Study of the Role of Plasmin-mediated Cleavage of Erythropoietin-Producing Hepatocellular A4 Receptor and its Molecular Binding Partners in Anxiogenesis

Submitted by Alberto Labrador Ramos to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Medical Studies

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Signature: .....

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ABSTRACT

Stressful experiences can produce a variety of emotional states, including elevated and prolonged levels of anxiety that can lead to anxiety disorders. Anxiety disorders are the most prevalent class of mental disorders worldwide; however, the neurobiological mechanisms that regulate anxiety and its disorders are still not well understood. In this context, the current project delves into downstream effects of the cleavage of EphA4 receptor by tissue plasminogen activator (tPA)/plasmin proteases; an event recently discovered by former members of our laboratory. The initial hypothesis of this project was, therefore, that tPA/plasmin-induced proteolysis of the murine EphA4 receptor is present in the mouse brain and can regulate stress-related phenomena.

An initial necessary first step was to confirm the presence of the proteolytic cascade in areas relevant for the study of anxiety. In agreement with this hypothesis, I demonstrated that tPA and plasminogen co-localise with EphA4 in the GABAergic neuronal synapses of the central amygdala (CeA) through immunhistochemical techniques. In line with this discovery, the relevant literature sufficiently proves that inhibitory interneurons in the central amygdala of the mouse brain regulate anxiety-related processes by controlling the activity of downstream output cells. Specifically, those of the lateral subdivision of the central amygdala (CeL) expressing protein kinase C delta ($PKC\delta^{+}$) are important for aversive stimuli processing and memory. In the present work, I show that all tPA-expressing cells in CeA are also PKCδ+, which establishes a strong link between PKCδ+ cell-types and the location of an assumptive tPA/ plasmin/EphA4 cascade in areas relevant for stress-related events and anxiety-like behaviours.

Conceivably, $PKC\delta^{+}$ (tPA) cells can regulate the properties of their downstream GABAergic synapses during stress through a cleavage of EphA4 associated with the tPA/plasmin proteolytic cascade. It is known that stressful stimuli produce the tPA-mediated conversion of plasminogen into the active enzyme, plasmin; and, as demonstrated here, plasmin would subsequently be able to cleave the tyrosine kinase receptor, EphA4.

Cleavage of EphA4 has multiple neurobiological consequences. At the molecular level, I examined how shedding of EphA4 affects postsynaptic GABAergic protein-protein interactions. Here, I show that cleavage induces the

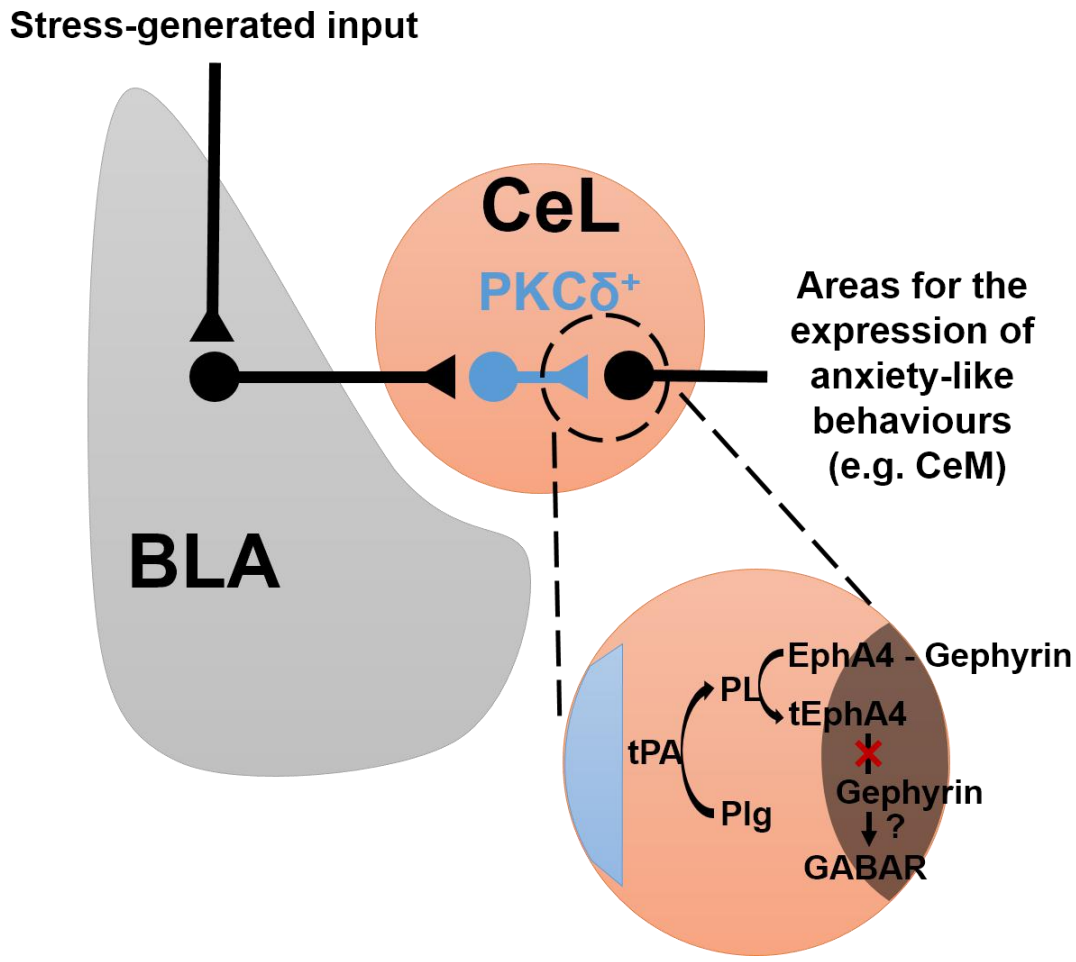
dissociation of EphA4 from the GABA-receptor anchoring protein, gephyrin. The repercussions of this event are still unknown. Furthermore, this shedding can regulate the dendritic spine shape as evidenced by spine morphology experiments. Spine morphology is thought to reflect the strength and activity of a synapse whereby the excitatory or inhibitory tone of a neuron can be tuned.

Moreover, consistent with a crucial role of the tPA/plasmin/EphA4 signalling cascade in anxiogenesis, EphA4 main cleaved form of EphA4 is increased after restraint stress. Accordingly, increased protein levels in the central amygdala of a plasmin-resistant variant of EphA4 (crEphA4) prevents the expression of stress-induced anxiety-like behaviours in mice, whereas the expression of a truncated EphA4 variant that mimics the cleavage by plasmin (tEphA4) increases this expression. This indicates that the cleavage of EphA4 potentially helps to modulate the expression of anxiety-like behaviours.

Therefore, the present work identified a central molecular cascade that potentially controls the structure and function of GABAergic synapses downstream of CeL-PKCδ^{+} interneurons in the CeA and has the ability to modify the expression of anxiety-like behaviours.

Additional pieces of data presented in this work indicate that the cleavage of EphA4 is affected in other brain conditions in which tPA/plasmin cascade is involved, such as rodent models of stroke or epilepsy. Therefore, this work opens future possibilities for the study of other mechanisms regulated by tPA/plasmin/EphA4 cascade.

VISUAL ABSTRACT



BLA = basolateral amygdala; CeL = centrolateral amygdala; CeM = centromedial amygdala; PKCδ+ = interneuron containing protein kinase C delta; tPA = tissue type of plasminogen activator; Plg = plasminogen; PL = plasmin; EphA4 = erythropoietin-producing hepatocellular A4 receptor; tEphA4 = truncated EphA4; GABAR = gamma aminobutyric acid receptor.

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LIST OF ABBREVIATIONS

15 m S	15 min restraint stress
18 h R	18 Hours Recovery
5-HT	Serotonin
5 m S	5 min restraint stress
6 h R	6 Hours recovery
6 h S	6 Hours restraint stress
A	Adenine
AC	Associational commissural fibres
ACN	Acetonitrile
ADAM10	A disintegrin and metalloproteinase domain-containing protein 10
AF	Alexa Fluor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
Arg	Arginine
ATD	Amino-terminal domain
A.u.	Arbitrary units
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
C	Cytosine
CA1-4	*Cornu Ammonis* areas 1 to 4
Ca^{2+}	Calcium ions
CAMKII	Calcium/calmodulin-dependent protein kinase II
cDNA	Complementary DNA
CeA	Central amygdala
CeL	Centrolateral amygdala
CeM	Centromedial amygdala
Cl^-	Chloride ion
CMV	Cytomegalovirus
CNS	Central nervous system
CoA	Cortical Amygdala
CORT	Corticosterone
COS	CV-1 in origin carrying the SV40 genetic material
Cre +/-	mice carrying cyclic adenosine monophosphate response element insertion
crEphA4	Cleavage-resistant EphA4 mutant
CREB	Cyclic adenosine monophosphate response element-binding
CRF	Corticotropin-releasing factor
CRFR2	Corticotropin-releasing factor receptor 2
CRH	Corticotropin-releasing hormone
CS	Conditioned stimulus
CUMS	Chronic unpredictable mild stress
Cy-5	Cyanine 5

DAPI	4 ',6-diamidino-2- phenylindole
ΔG	Increment in Gibbs free energy
DG	Dentate gyrus
DIG	Digoxigenin
DNA	Deoxyribonucleic-acid
DTT	Dithiothreitol
E-LTP	Early-LTP
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EGTA	Ethyleneglycoltetraacetic acid
EM	Electron microscope
Eph proteins	Denotes both Eph receptors and ephrin ligands
EPM	Elevated plus maze
ERK	Extracellular signal-regulated kinase
FISH	Fluorescent *in situ* hybridisation
FKBP5	FK506 binding protein 5 gene
FKBP51	Fk506 binding protein 51
FN3	Fibronectin type III
G	Guanine
GABA	Gamma-aminobutyric acid
GAL2	Galanin receptor 2
GC	Glucocorticoid
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GluNA	Metabotropic glutamate receptor A
Gly	Glycine
GPI	Glycosylphosphoinositol
Grb4	Growth factor receptor-bound protein 4
GRIP	Glutamate receptor interacting protein 1
GSK3β	Glycogen synthase kinase 3β
GTP	Guanosine triphosphate
GuHCl	Guanidinium chloride
HCl	Hydrochloric acid
HEK293	Human embryonic kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HO	(buffer)
HPA	Hypothalamic pituitary adrenal
HRP	Horseradish peroxidase
IEG	Immediate early gene
IgG	Immunoglobulin G
Ile	Isoleucine
JM	Juxtamembrane

JNK	c-Jun NH2 terminal kinase
KCl	Potassium chloride
L1CAM	L1 Cell adhesion molecule
LA	Lateral amygdala
LBD	Ligand binding domain
LPS	Lipopolysaccharide
LRP1	Low-density lipoprotein receptor-related protein 1
LSM5	Laser Scanning Microscope 5
LTD	Long term depression
LTP	Long term potentiation
LVDCC	L-type voltage dependent calcium channel
Lys	Lysine
MABT	Maleic acid buffer with tween
MAP	Mitogen-activated protein
MeA	Medial Amygdala
MEM	Minimum essential medium
MF	Mossy fibres
Mg^{2+}	Magnesium ion
MMP	Matrix metalloprotease
mPFC	Medial prefrontal cortex
N2A	Neuro-2A
Na_3VO_4	Sodium orthovanadate
NaBH3CN	Sodium cyanoborohydride
NAc	Nucleous accumbens
NaCl	Sodium chloride
NaF	Sodium fluoride
NCAM	Neural cell adhesion molecule
NeuN	Neuronal nuclei
NLS	Nuclear localisation sequence
NL	Neuroligin
NMDA	N-methyl-D-aspartate
$NP^{-/-}$	Neuropsin knockout mice
$NP^{+/+}$	Wild-type mice
NR1, 2	NMDA receptor subunit 1, 2
OF	Open field
P10	Post-natal day 10
p75NGFR	p75 nerve growth factor receptor
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor-1
PAR-1	Protease-activated receptor 1
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline – Tween® 20
PDZ	Post-synaptic density-95/Drosophila disc-large tumour suppressor/Zonula occludens 1
PFC	Prefrontal cortex

PKA	Protein kinase A
PKCδ	Protein kinase C delta
PL	Plasmin
PLC	Phospholipase C
Plg	Plasminogen
PP	Perforant pathway
PS1	Presenilin 1
PSD	Postsynaptic density
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
Q	Glutamine
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
R	Arginine
Rac1	Ras-related C3 botulinum toxin substrate 1
RAP	Ribosomal acidic P proteins
RGS3	Regulator of G-protein signalling 3
RHBDL2	Rhomboid like 2
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SAM	Sympathetic adrenal medullary
SAM	Sterile alpha motif
SOM	somatostatin
SB	Subiculum
SC	Shaffer collateral fibres
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SSC	Saline-sodium citrate
T	Thymine
tEphA4	Truncated EphA4
Tiam1	T-cell lymphoma invasion and metastasis 1
TM domain	Transmembrane domain
TNF	Tumour necrosis factor
tPA	Tissue plasminogen activator
Tris	Tris (hydroxymethyl) aminomethane
tRNA	Transfer RNA
uPA	Urokinase-type plasminogen activator
US	Unconditioned stimulus
UTR	Untranslated region
WT	Wild type
wtEphA4	Wild-type EphA4

LITERATURE REVIEW, SEARCH STRATEGY AND SELECTION CRITERIA

I searched in PubMed and the Cochrane Library for review articles published in English between Jan 1, 2000, and Nov 31, 2018 with the following terms (or a combination of them) depending on the particular topic of interest:

1. (“emotions” OR “feelings”) AND (“human” OR “mouse” OR “Mus musculus” OR “animals” OR “species” OR “Darwin” OR “evolution”).

2. (“stress” OR “allostasis”) AND (“Selye” OR “allostatic overload” OR “stress-related disorders” OR “chronic stress” OR “acute stress” OR “animal model” OR “psychiatric disorders” OR “medical condition” OR “anxiety disorders” OR “depression” OR “therapy” OR “treatment” OR “medication” OR “drug”).

3. (“anxiety”) AND (“anxiety-like behaviours” OR “definition” OR “anxiety disorder” OR “separation anxiety disorder” OR “selective mutism” OR “specific phobia” OR “social anxiety disorder” OR “panic disorder” OR “agoraphobia” OR “generalized anxiety disorder” OR “substance-induced anxiety disorder” OR “phobia” OR “PTSD” OR “pathophysiology” OR “obsessive-compulsive disorder” OR “symptoms” OR “epidemiology” OR “economy” OR “cost” OR “incidence” OR “prevalence” OR “animal model” OR “human” OR “mouse” OR “Mus musculus” OR “behaviour” OR “behavioural test” OR “test”).

4. (“amygdala” OR “hippocampus” OR “medial prefrontal cortex”) AND (“anxiety” OR “anxiety disorder” OR “anxiety-like behaviours” OR “stress” OR “stress disorder”) AND (“central amygdala” OR “circuit” OR “network” OR “connectivity” OR “anatomy” OR “central nervous system” OR “sympathetic system” OR “parasympathetic system” OR “hypothalamus” OR “lesion” OR “studies” OR “neurotransmitters” OR “electrical stimulation” OR “chemical stimulation” OR “human” OR “mouse” OR “Mus musculus” OR “brain” OR “central nervous system”).

5. (“protease” OR “serine protease” OR “tissue plasminogen activator” OR “urokinase plasminogen activator” OR “plasmin” OR “cascade” OR “serpin”) AND (“anxiety” OR “anxiety disorder” OR “anxiety-like behaviours” OR “amygdala” OR “stress” OR “stress disorder” OR “human” OR “mouse” OR “Mus musculus” OR “brain” OR “central nervous system”) AND (“protease receptor” OR “protease-activated receptor” OR “PAR” OR “function” OR “MMP” OR “function” OR “structure” OR “localisation” OR “location”).

6. (“eph” OR “ephrin”) AND (“epha” OR “ephb” OR “receptor” OR “RTK” OR “tyrosine kinase” OR “ligand” OR “system” OR “structure” OR “membrane receptor” OR “regulation” OR “function” OR “role” OR “evolution” OR “phylogenetic” OR “orthologues” OR “localisation” OR “expression” OR “proteases” OR “serine protease” OR “cleavage”)

7. (“eph” OR “ephrin”) AND (“synaptic plasticity” OR “synapse” OR “anxiety” OR “anxiety disorder” OR “anxiety-like behaviours” OR “amygdala” OR “stress” OR “stress disorder” OR “human” OR “mouse” OR “Mus musculus” OR “brain” OR “central nervous system”).

8. (“plasticity OR “synaptic plasticity” OR “neuronal plasticity” OR “synapse” OR “synaptic transmission” OR “long-term potentiation” OR long-term depression” OR “LTP” OR “LTD” OR “spine” OR “spine morphology” OR “spine density” OR “functional plasticity”) AND (“anxiety” OR “fear” OR “fear conditioning” OR “anxiety disorder” OR “anxiety-like behaviours” OR “amygdala” OR “stress” OR “stress disorder” OR “human” OR “mouse” OR “Mus musculus” OR “brain” OR “central nervous system”) AND (“eph” OR “ephrin” OR “protease” OR “serine protease” OR “tissue plasminogen activator” OR “urokinase plasminogen activator” OR “plasmin” OR “cascade” OR “serpin”).

9. (“GABA” OR “Gamma-Aminobutyric acid” OR “sub-units” OR “GABAA” OR “GABAAR” OR “gephyrin” OR “interaction” OR “interactome” OR “proteome” OR “signalling cascade” OR “downstream”) AND (“anxiety” OR “fear” OR “fear conditioning” OR “anxiety disorder” OR “anxiety-like

behaviours” OR “amygdala” OR “stress” OR “stress disorder” OR “human” OR “mouse” OR “Mus musculus” OR “brain” OR “central nervous system”) AND (“eph” OR “ephrin” OR “protease” OR “serine protease” OR “tissue plasminogen activator” OR “urokinase plasminogen activator” OR “plasmin” OR “cascade” OR “serpin”).

10. (“Alzheimer” OR “stroke” OR “epilepsy” OR “epileptic attack” OR “seizure” OR “pentylenetetrazol” OR “PTZ” OR “Medial Cerebral Artery Occlusion” OR “Photochemically Induced Thrombosis” OR “model” OR “MCAO” OR “MCA” OR “PIT”) AND (“pathology” OR “anxiety” OR “fear” OR “fear conditioning” OR “anxiety disorder” OR “anxiety-like behaviours” OR “amygdala” OR “stress” OR “stress disorder” OR “human” OR “mouse” OR “Mus musculus” OR “brain” OR “central nervous system”) AND (“eph” OR “ephrin” OR “protease” OR “serine protease” OR “tissue plasminogen activator” OR “urokinase plasminogen activator” OR “plasmin” OR “cascade” OR “serpin”).

I also reviewed the lists of references within the articles identified by this search. Given the numerous references extracted, the present thesis provides a representative rather than a complete review of the citations and representative articles are presented for each topic. Conclusions and arguments extracted from this search were included in the introduction chapter and introduction sections of each chapter.

PART 1: INTRODUCTION AND MATERIAL AND METHODS

CHAPTER 1. INTRODUCTION

EMOTIONS, STRESS-RELATED DISORDERS AND THEIR IMPORTANCE

Emotions are a major research area continuously growing in the fields of neuroscience and psychology. A PubMed search on “emotion” grows from around 400 hits in 1960 to more than 8000 publications only in 2016.

However, the content of these publications is diverse since there is not total consensus about what an emotion is and how it differentiates from other aspects of mind and behaviour (LeDoux 2012; Anderson and Adolphs 2014). Nevertheless, a broadly accepted aspect of emotions is that, although singular features of human emotions can be described, some aspects of emotional phenomena are conserved through evolution and are reflected in species close enough to the *Homo sapiens* (Anderson and Adolphs 2014; LeDoux 2012). This judgment is the foundation for neurobiological approaches to emotions, in which animal research is an essential tool to study many aspects of emotional phenomena.

To date, it has been impossible to agree on a single definition of emotion in objective scientific terms that can be used by different disciplines (e.g. neuroscience, psychology or philosophy). A lot of this disagreement arises from the internal brain states that the human beings subjectively experience and report as ‘‘feelings’’ (Dolan 2002; Damasio 2003). The existence of feelings (e.g. joy, sadness, disgust, envy) can currently only be assessed by verbal report, therefore their study is exclusive in humans; and, although they are considered emotions, emotions are not limited to feelings.

Since Darwin’s 1872 monograph, *The Expression of the Emotions in Man and Animals*, many have considered other types of emotional expression in beings other than humans, as well as ways of generalising the concept of emotion to diverse organisms (LeDoux, 2012; Anderson and Adolphs, 2014). A heuristic approach to this problem has been addressed by Anderson and Adolphs (Anderson and Adolphs 2014 for review), and it is the one that I adopted for the discussion of the present work. They proposed that model organisms exhibit primitive emotion states that are expressed by externally observable behaviours.

These states present a number of defined properties coined as ‘‘emotion primitives’’, disregarding they are internally or externally expressed. For these authors, a given group of emotion primitives can share combinations of fundamental features and they constitute the “*evolutionary building blocks of emotion*”.

Furthermore, these emotion primitives are shared by different emotions and across phylogeny, even if the behaviour observable in each species is different. This approach facilitates investigation of features of emotions in model organisms and their emotion primitives without the need to associate them with human feelings or behaviours and it makes it easier to methodically dissect the neural circuit related to these states. In their paper, Anderson and Adolphs (2014) provide a very illustrative example of the advantages of this point of view: *“By analogy, in the same way that we have learned a great deal about the neurobiology of vision by studying animal models without worrying about trying to solve the problem of how we have conscious visual experiences, we can learn much about the neural encoding of central emotion states in animals without concerning ourselves with the subjective, conscious perception of such states”.*

Two of the most important emotion primitives are valence (positive or negative) and scalability (intensity); and this is because they have been classically used to categorise emotions. Gradations in intensity are thought to reflect the level of arousal, and this can be evaluated using objective measurements, such as locomotion or sensitivity to noxious sensory stimuli in the case of *Drosophila melanogaster*. Emotions are also regarded as sets of antithetical pairs which are expressed by complementary and physically opposite behaviours. One of the most usual opposite pairing is locomotor activity, whereby the living system can approach or withdraw from the stimulus.

Another important characteristic of emotions is that they are pleiotropic, which is to say that they have multiple parallel consequences affecting other elements or responses in a system. As a contrasting example, reflexes typically produce a stereotyped outcome without affecting other elements or responses. Therefore, emotions can mediate many different biological processes apart from behaviours, such as somatic responses or cognition. For instance, responses caused by a

central nervous system state produced by a noxious stimulus range in many species from defensive behaviours (e.g. flight response), to endocrine changes (e.g. the release of stress hormones), to autonomic function (e.g. increased heart rate), to sleep patterns, attention or memory. Moreover, emotions can alter cognition and responses to a different stimulus and generalise that internal state to a different context. A good example of this characteristic is the Pavlovian fear conditioning paradigm in rodents (Davis, 1992), in which, after a training session, an animal learns to associate a negative stimulus (e.g. mild electric footshock) with a context that involves sensory information (e.g. sound, light, odours). Subsequently, when the animal is exposed to the learnt associated context (in the absence of the negative stimulus), it will elicit an aversive response similar to the one produced by the negative stimulus.

One of the most studied and intriguing sets of behavioural phenomena is that provoked by the proximity of a noxious stimulus. For example, a common defensive response of many species against that noxious stimulus is to avoid being detected by a threatening witness through freezing, which is transformed into a confrontation or an escaping response if the threatening stimulus is close enough. This reaction has been termed “freeze-fight-flight” response by many scientists (Bolles and Fanselow 1982; Blanchard et al. 1998; Brown and Fee 2002). In octopi, their freeze-fight-flight response turns from camouflage and freezing into flight and ink-jetting with the potential threat. Humans share these types of behaviours, but they can be extended to correlative subjective emotional states that can be described in a gradation from mild concern to anxiety, fear or panic.

In this line of research, “*stress*” is a word that has been commonly associated with experiences that are challenging emotionally and physiologically (McEwen, 2007), but the original term *stress* was coined by Hans Selye in 1936. He defined it as “*a syndrome produced by diverse nocuous agents*” (Selye 1998). He used it to describe the effect that various noxious stimuli had on laboratory rats. These included cold, surgical injury, excessive exercise or sublethal doses of drugs. He characterised stress as a stereotypical triphasic common pattern, divided into an initial alarm phase, followed by a stage of resistance and a final exhaustion phase if the exposure to the noxious agent was long enough. This concept stemmed

from an initial speculative idea which originated when he observed similar characteristic patterns in his patients, who showed non-specific adaptive physiological responses to injury not related to the diagnostic of their diseases. He named that combination of reactions: “general adaptation syndrome” (GAS), which he would later define as “*the sum of all non-specific, systemic reactions of the body which ensue upon long-continued exposure to stress*”.

Since Selye, several terms have been created to describe and study the biological response to stress. One of the most commonly used ones is “allostasis”, which refers to the active process of adapting to stressors via mediators (autonomic, metabolic, endocrine and immune system) to survive. Allostasis (“stability through change”) contrasts with (and has the aim to substitute) the previous concept of homeostasis (“stability through constancy”). In homeostasis, the fluctuations of biological internal parameters out of its normal constant numbers are understood as necessary inappropriate values and these deviations must be corrected to bring back the body’s physiological “normal” parameters. However, models based on homeostasis, although useful to describe certain biological responses are not free of criticism. Evidence shows that biological parameters are not constant throughout time and their variation seems to be a coping mechanism rather than an error. This is not to say that there are not parameters that are tightly regulated (e.g. oxygen, glucose, temperature or osmotic pressure in the brain). In allostasis, these tight regulations do not reflect constancy, but system functioning features that optimise performance to succeed in the survival goal. Therefore, allostasis describes a model where the goal is not constant parameters, but survival (or, as Stirling describes it, “fitness” under natural selection). Prediction is an essential component of survival that minimises errors and reduces metabolic costs. In the allostasis model this prediction (and not a system failure) would be the cause of unusual parameter values. Hence, parameters vary and variation anticipates demand. This anticipation, in turn, would help to coordinate fluctuations in different biological processes to optimise performance at the minimum metabolic cost (Sterling, 2004).

“Allostatic load” makes reference to the cumulative effect of multiple stressors (e.g. production of adrenalin or increment in heart rate in response to a

challenge). “Allostatic overload”, in turn, describes the response that exceeds body demands and can result in pathological forms of stress (McEwen 2003b, 2003a; McEwen, Gray, and Nasca 2015). Therefore, allostasis is a process with two sides of the same coin. It can result in positive protective changes that allow an organism to cope with stressful events. However, these same changes can become uncontrolled and generate detrimental effects that further develop morbidities and comorbidities with other disorders. The allostatic overload has no practical use or purpose against a stressor and predisposes the individual to disease (Carrasco and Van de Kar 2003; McEwen 2003a, 2003b, 2005; McEwen, Gray, and Nasca 2015). Therefore, an acute stress response is needed for many species to cope with challenging situations to increase the probability of survival (also known as “*the good stress*” in popular jargon). It is a completely natural response to a threat. In ancient times, humans would be stressed by dealing with predators or enemy tribes, whereas nowadays we face daily stressors ranging from caring for a child to unexpected events such as a work deadline, a traffic jam or starting an argument. However, at least in humans, such responses can be deleterious for health and survival when they are prolonged or dysregulated (McEwen 1998; Heim and Nemeroff 1999; McEwen 2003b; McEwen, Gray, and Nasca 2015; Davidson, 2000; Goldsmith and Lemery 2000). These dysregulated or lengthened responses are often produced by experiences in which a sense of control of the situation is missing. Moreover, negative situations that become recurrent, irritating, emotionally draining, physically exhausting or dangerous can be a source of harmful responses to stress. For instance, living in poverty, being underemployed or having intrusive thoughts can be the trigger for a stressful situation. Colloquially speaking, these responses have been termed as “bad stress” or “being stressed out” (McEwen 2005; McEwen, Gray, and Nasca 2015). Nonetheless, short but intense stress responses to traumatic events can also lead to long-term health problems, such as post-traumatic stress disorders (PTSD) (Mc Farlane, 2010). Therefore, one of the main reasons for studying stress is that it can eventually lead to the development of both neurological and psychosomatic illnesses.

The effects of stress on health can result in diseases that vary in their life span. The pathogenesis of acute stress-induced disorders can be attributed to various

causes, among which, increased stress mediators mixed with a vulnerable background are thought to be the main cause. The list of these pathologies is long. For example, exaggerated acute responses include allergic reactions mediated by immune corticotropin-releasing factor (CRF)-induced degranulation of mast cells. Immune dysregulation may have a deleterious effect in a vulnerable organ; such as asthma in the lungs, eczema in the skin or migraine´s local vasodilatation in meningeal blood vessels. This later effect is even able to cause panic or psychotic attacks if CRF is released in an area of the brain called central amygdala. But the repercussions are many; for example, stress can induce increased sympathetic or parasympathetic system activation that results in hypertensive or hypotensive attacks (Chrousos 2009).

Chronic stress-related disorders are related to continuous, excessive stress responses in magnitude and time. Such a response can result in the prolonged secretion of stress mediators, such as CRF, norepinephrine, cortisol and other hormones that activate the defence system. Such hormones eventually produce anxiety, anorexia or hyperphagia, as well as tachyphylaxis of the reward system. In turn, these conditions may produce depression, food disorders or substances abuse. The stress-related molecules also disrupt the sleep system patterns, which causes insomnia, loss of sleep and daytime somnolence. All these effects are concomitant with fatigue, nausea, headaches and other pains. Additionally, cognitive systems are affected, which produces bad intellectual performance and planning, as well as making bad decisions. As a result, all of these conditions can lead to psychosocial problems and prolong the cause of the stress system malfunctioning. Focusing on the somatic consequences, functioning of the growth hormone axis can be disrupted by stress, cause hypogonadism and the consequent loss of libido and hypo fertility, or hyperactivity of the sympathetic system that can result in hypertension. Moreover, chronic hypersecretion of stress hormones combined with a vulnerable phenotype may lead to visceral fat accumulation due to hypercortisolism or reactive insulin hypersecretion. These also provoke a sort of metabolic syndrome characterised by sarcopenia, osteopenia and osteoporosis, dyslipidaemias, hypercytokinemia, hypercoagulation, atherosclerosis or type 2 diabetes mellitus. Importantly, genetically or constitutionally vulnerable women of reproductive age may develop

polycystic ovary syndrome, hypothalamic oligomenorrhea and amenorrhea, reduced fertility or obligate athleticism. Also, the immune system seems to be impaired, which leads to a higher risk of infections and autoimmune disorders (such as Graves' disease, systemic lupus erythematosus and some allergic conditions). In the gastrointestinal tract, dysregulation of the autonomic nervous system leads to common gastrointestinal disorders, such as irritable bowel syndrome and peptic ulcer disease (Chrousos 2009).

Clinical reports indicate that in fact, stress-related disorders are present in up to 50% of patients with chronic disorders (e.g. pain, cancer, cardiovascular disease, obesity or diabetes), in a much higher rate than the general population (which only reaches 5 - 8%) (Duric et al. 2016). However, despite all the literature about stress, definitively establishing causal links between stress and disease in humans is difficult. One of the best-known examples is cardiovascular diseases (CD). A large body of clinical and correlational studies support the idea that stress affects CD (Steptoe and Kivimaki 2012) and there exist biological response components of stress that plausibly contribute to CD, including raised blood pressure, reduced insulin sensitivity, increased haemostasis and endothelial dysfunction. However, the key pathological mechanisms are still missing (Steptoe and Kivimaki 2012). Animal models provide an important tool to help us to study the specific causality between stressors and diseases. In this line, one well-known animal model relating stress to atherosclerosis was developed by Kaplan et al. (1982). Their study was performed on male cynomolgus monkeys, who normally live in social groups. The investigators socially stressed half of the group by isolating them and observed significantly more atherosclerosis when these monkeys were compared with the non-stressed group. This effect was reversed with a sympathetic nervous system-blocking agent, namely propranolol. However, this kind of studies is not common and their results are not always definitive.

In spite of the mentioned stress-related deleterious effects, the main adverse consequence of severe or prolonged stress is that it frequently promotes the development of psychiatric disorders (e.g. Johnstone, 2010; Heim and Nemeroff, 2001; McEwen, 2007). These disorders affect a large percentage of the population in the United Kingdom ("UK Data Service Discover » Adult Psychiatric

Morbidity Survey, 2007", 2007) and generate an enormous social and economic burden (Fineberg et al. 2013). Among them, stress-related and anxiety disorders (e.g. generalised anxiety disorder (GAD) and phobias) are the most prevalent and currently-available therapies are often ineffective and associated with numerous side effects (Bystritsky 2006; Bystritsky et al. 2013; Kozaric-Kovacic 2008). Details for this type of stress-generated conditions can be found in the next section.

ANXIETY

This section will address the main aspects of the pathophysiology of anxiety due to the implications of the current work on this condition. More details about animal models and brain connections related to anxiety will be mentioned in specific sections and chapters.

The word "anxiety" can allude to an array of related phenomena. These include the human "normal-range" trait-like negative affect and the animal model correlates that show particular patterns of behaviour. Also, anxiety can refer to a class of psychiatric disorders derived from the mentioned phenomena (Grupe and Nitschke, 2013). Subsequently, the definitions of anxiety vary. On the one hand, in clinical literature, anxiety can be defined as an emotional state that is characterised by persistent hyperarousal that result from the potential for threat in uncertain situations. It is defined by cognition, behaviour and physiological reactions among which avoidance and hypervigilance are very common; however, these characteristics vary depending on the particular type of anxiety studied (described later on this chapter). On the other hand, in the animal literature and our work, anxiety is defined as a temporary emotional state induced by diffuse threatening stimuli (Sylvers, Lilienfeld, and LaPrairie 2011), such as open spaces (Pellow et al. 1985) and bright lights (Crawley 1985). Since not all the features of human anxiety can be assessed or modelled in an animal model, these behaviours are often referenced as "anxiety-like behaviours". Especially the ones related to psychological changes, cognition and all kind of features that

can be only assessed by self-report.

Anxiety is an emotional state of negative valence and, as such, it might be confused with fear, but there are certain differences between them in which the majority of researchers agree. Whereas fear is characterised by an acute response to a defined and imminent threat (e.g. the presence of a predator [cat] for a prey [mouse]), anxiety is characterised by a more sustained response that might result from a diffuse, uncertain or anticipated threatening stimulus where threat is not clearly imminent (e.g. open spaces, insects, social interaction) (Grupe and Nitschke 2013; Sylvers, Lilienfeld, and LaPrairie 2011; Adhikari 2014).

Anxiety is considered a non-pathological and primitive state generally thought to be a method for many species to respond to a potentially threatening stimulus and provide adaptive survival to protect the individual from danger (Porges 1995). However, in humans, excessive levels of anxiety produce a pathological state that generates distress and suffering and falls under the umbrella term of “anxiety disorders” (Wu, Kim, and Zhuo 2008).

Non-pathological anxiety can be divided into two categories. First, state anxiety is an acute level of anxiety that is produced as an adaptation to specific situations or stressors. And second, trait anxiety, which is used to evaluate the long-term tendency of an individual to show an increased anxiety response across time and situations (Bystritsky, 2006).

The pathological form of anxiety has been classified in different ways throughout history, which highlights the complexity and variety of these disorders. The Diagnostic and Statistical Manual of Mental Disorders (DSM) (American Psychiatric Association 2013) criteria have been used the most in epidemiological studies. This widely accepted categorisation of anxiety disorders is based on behavioural and subjective reports. However, whether neural mechanisms underlying anxiety symptomatology are similar in differentiated disorders is still a matter of research. The recent DSM-5 includes twelve typified anxiety disorders. The symptomatology is different depending on the particular type of condition. The International Classification of Diseases 10th edition (ICD-10) is another

broadly used categorisation (World Health Organisation 2018). These two classification systems present some differences that were reviewed elsewhere (Barton et al. 2014).

However, despite the fact that anxiety-related disorders are categorised as separate conditions, they probably share common characteristics. This hypothesis lays its foundations on the idea that most of the anxiety disorders respond to a similar array of pharmacological treatments.

The most common described forms of these conditions are:

1. Generalised anxiety disorder (GAD): Patients experience excessive and long-lasting worry, motor tension, restlessness, irritability, difficulty sleeping and hypervigilance without identifiable stimuli triggering it.

2. Panic disorder (PD): This type of anxiety disorder is characterised by acute, unexpected, intense fearful reaction (also known as panic attacks) without any apparent reason. Patients might experience sympathetic crises, dyspnoea, fear of dying and losing control.

3. Phobias: They are a group of anxiety disorders in which the person experiences persistent fear reactions resulting in the avoidance of a particular stimulus (situation, object, place, person, etc.). If the person cannot avoid the stimulus, this can trigger intense distress.

4. Social phobia: This particular type of phobia is triggered by unfamiliar social settings and it has been classified as a separate category.

5. Post-traumatic stress disorder (PTSD): This anxiety disorder is developed after a highly traumatic experience. The condition is defined by intrusive recurrent distress provoked by the traumatic memory.

6. Obsessive-compulsive disorder (OCD): People can experience repeated obsessive thoughts and the need to perform compulsive actions aiming to relieve the distress they produce.

Numerous studies have been developed to address the importance and influence

of these conditions in human populations. However, the variability of the scope, the different methodological factors and the location of the populations studied make it difficult to extract clear conclusions. Together, anxiety-related conditions present a twelve-month prevalence of about 20% and are estimated to affect about one-third of the population at least once in their life (Stein and Steckler 2010). A meta-analysis study by Baxter et al. (2013), extracted from 87 community-sampled studies of 44 countries, indicates that the current global 12-month prevalence of anxiety disorders adjusted for methodological differences is 7.3% (4.8–10.9%) and ranged from 5.3% (3.5–8.1%) in African cultures to 10.4% (7.0–15.5%) in Euro/Anglo cultures. In addition, these studies would need to consider the age of the population studied, since, in general terms, the age-of-onset (AOO) of anxiety disorders is normally the childhood or adolescence and the course of the disease tends to be chronic-recurrent producing a higher prevalence in the third age (Stein and Steckler 2010). In terms of economic burden, a reputed and ambitious study by Chisholm et al. (2016) estimates that, across the 36 largest countries in the world, these diseases produce a cost of 4.7 billion extra days of work in productivity, and an extra cost of USD 592 billion.

Currently, available therapies are often ineffective and associated with numerous side effects (Bystritsky, 2006; Kozaric-Kovacic 2008; Bystritsky et al. 2013; Baldwin et al. 2014, 2017). Treatment of pathological anxiety has been addressed pharmacologically with the use of drugs that have calming properties (e.g. alcohol (often consumed as self-treatment), barbiturates, opiates, beta-blockers and benzodiazepines). More recently, cognitive behaviour therapy and other psychological treatments have been added to the therapeutic toolbox.

Current pharmacological treatments show a positive high placebo response incidence; however, only around one-half of the medicated patients will show improvement symptoms at the end of acute treatment. Therefore, there is still ambiguity about the real efficacy and tolerability of the diverse pharmacological treatments. During the last 30 years, selective serotonin reuptake inhibitors (SSRIs) have become the first-choice pharmacological treatment in evidence-based treatment guidelines due to their efficacy and tolerability in randomised controlled trials (RCTs). Due to the relapse phenomenon of anxiety conditions, long-term treatment is often required. Selective treatments for these periods

include some SSRIs, such as escitalopram and paroxetine; the serotonin-noradrenaline reuptake inhibitors (SNRIs), duloxetine and venlafaxine; the anticonvulsant drug, pregabalin; the antipsychotic drug, quetiapine and the novel antidepressant, agomelatine (Baldwin et al. 2014). Additionally, despite the big controversy around the use of benzodiazepines due to their various problems (i.e. risks of tolerance and dependence or long-term risk of dementia), they are still routinely used because of their robust relative efficacy (Craske and Stein 2016). They are used in short-term treatments (up to four weeks), while a co-administered SSRI becomes effective. In addition, long-term treatments also include them when refractory patients do not respond to an SSRI, SNRI, pregabalin, buspirone or psychological interventions (Baldwin et al. 2017).

Among psychological treatments, cognitive behavioural therapy (CBT) is the most used evidence-based one for youths and adults. CBT is a short-term treatment, based on patients' skills, that helps to empower patients and to interpret ambiguous threatening stimuli and replaces aversive behaviours with coping behaviours (Craske and Stein 2016). Other psychological treatments include exposure therapies (the patient is gradually exposed to the feared stimulus), relaxation training or self-help (they involve new forms of technology that offer all the elements found in live therapy).

Together, the overall limited effectiveness of available therapies and the associated tolerability and dependency effects provide a solid justification for the discovery of novel pharmacological targets and a better understanding of these types of conditions (Baldwin et al. 2017).

NEURAL CIRCUITRY OF ANXIETY: CENTRAL ROLE OF THE AMYGDALA

Extensive evidence from human and animal studies suggests that the amygdala is an important area involved in anxiety-related response (for reviews: Grupe and Nitschke 2013; Etkin and Wager 2007; Tovote, Fadok, and Lüthi 2015). This

section will summarise general aspects of the neuronal connections associated with anxiety, especially those affecting amygdalar structures. Details about particularly interesting areas will be mentioned in specific sections.

Some of the brain areas most important for stress and anxiety-like behaviours belong to a cluster of structures that form the limbic system, which is an old term (and arguably obsolete) that describes a very well-known region that mediates the processing of emotions in vertebrates (e.g. fear, anxiety, addiction reward, appetitive, sexual or attention-cognition behaviours) (Heimer and Van Hoesen 2006). The amygdala is a bilateral group of nuclei within the medial temporal lobe that belongs to the limbic system and is key in processing anxiety-like behaviours (Sah et al. 2003; Wu, Kim, and Zhuo 2008; Roozendaal, McEwen, and Chattarji 2009). Anxiety-like behaviours are closely related to other aversive states, such as conditioned-fear behaviours. Hence, increasing evidence suggests that the neural mechanisms underlying fear and anxiety states share overlapping brain substrates, among which, the amygdala is one of the most important (Tovote, Fadok, and Lüthi 2015).

The seminal studies proposing the amygdala as an important region for emotional reactions involved lesion studies in rhesus monkeys (*Macaca mulatta).* In this early work, Klüver and Bucy (1939) described a behavioural syndrome of monkeys with bilateral removal of the temporal lobes (including the amygdala, hippocampus and surrounding cortical areas). These animals would exhibit “psychic blindness”, i.e. no hesitation on approaching new objects (food, faeces, a snake or a light bulb) and a tendency to use the mouth to examine them. They seemed to pay more attention to visual cues. They also had a marked absence of emotional, motor and vocal reactions normally associated with negative-valence situations.

Subsequent studies showed that the ablation of the amygdala and surrounding perirhinal and entorhinal cortex could replicate the components of the Klüver–Bucy syndrome (Davis and Whalen 2001). These results were later observed also in rodents (LeDoux et al. 1990; Blanchard and Blanchard 1972) and humans (Grupe and Nitschke 2013; Etkin and Wager 2007 for review) indicating conservation of function across species. Therefore, it is inferable that a big part

of the complex response pattern observable during emotional states related to this area (i.e. anxiety and fear conditioning) has been "hard-wired" during evolution in the mammalian brain.

In support of this idea, recent studies about the activation of the central nucleus of the amygdala show that it produces a state similar to fear in the absence of previous explicit fear conditioning. Additionally, experiments electrically stimulating different sub-areas of the amygdala result in a subset of different signs of fear and anxiety (for reviews Davis 1992; LeDoux 2000; Tovote, Fadok, and Lüthi 2015). Moreover, in the rodent amygdala, there are a wide variety of monoaminergic, amino acid and peptidergic neurotransmitters and receptors (Sah et al. 2003; Gilpin 2012), and the activation or inhibition of each of them yields diverse results depending on the nuclei injected and the behavioural test used (Engin and Treit 2008). Numerous intra-cerebral microinfusions of selective receptor agonist or antagonist have also been performed to address the function of the amygdala in anxiety-related behaviours (Engin and Treit, 2008 for a detailed review).

As mentioned before, although fear and anxiety seem to produce similar symptoms and activate shared brain regions, clinically speaking, fear is considered more stimulus-driven than anxiety (Davis 1992). A large number of studies have evidenced the importance of the amygdala in Pavlovian fear conditioning (in seminal papers: Blnchar and Blanchard, 1972; Davis, Hitchcock and Rosen, 1987; Kapp, Pascoe and Bixler, 1984; Kapp et al., 1990; LeDoux, 1987, 1990; and in more modern ones: Ciocchi et al. 2010; Haubensak et al. 2010. Tovote, Fadok and Lüthi 2015 for a comprehensive review). However, the role of the amygdala in the aversive reactions of models of unconditioned anxiety seems to be more complex than a fear reaction. Furthermore, the relevant literature suggests that the response produced depends on specific amygdalar nuclei and sub-nuclei as well as on the behavioural tests or parameters used to study it (Tovote, Fadok, and Lüthi 2015). The study of all these circuits can help to understand the normal functioning of the brain that leads to physiological anxiety and the features that can cause a defective circuit which would trigger pathological forms of anxiety.

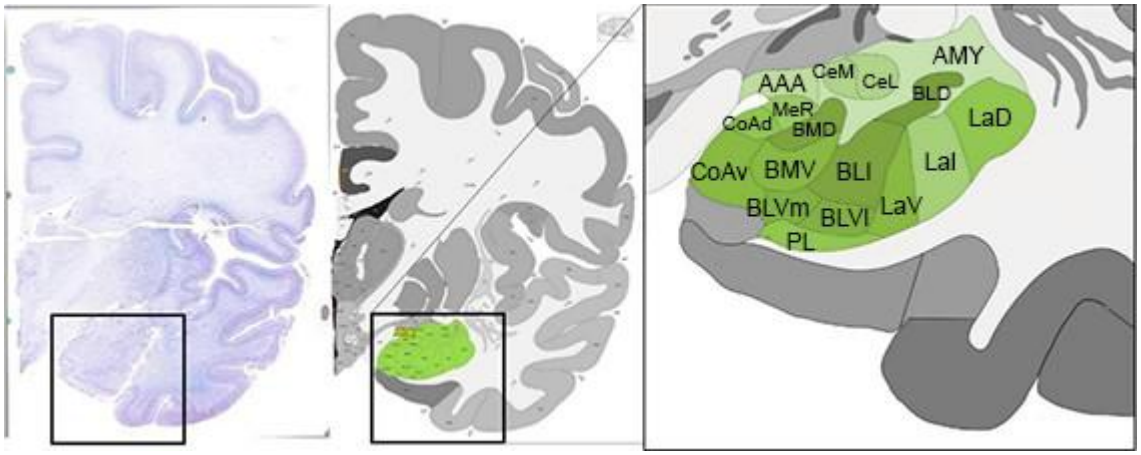


Image 1. Coronal section of the human brain showing the subdivisions of the amygdala. AAA anterior amygdaloid area; Amy amygdala; BLA basolateral nucleus; BLD basolateral nucleus, dorsal; BLI basolateral nucleus intermediate; BLVm basolateral nucleus, ventromedial; BMD basomedial, dorsal; BMV basomedial, ventral; CeA central nucleus; CeM centromedial amygdala; CeL centrolateral amygdala; CoAd cortical amygdala, dorsal; CoAv cortical amygdala, ventral; LA lateral nucleus; LaD lateral nucleus, dorsal division; Lal lateral nucleus, intermediate division; LaV lateral nucleus, left, ventral division; MeR medial amygdala; PL paralaminar nucleus. Modified from: © 2019 Allen Institute for Brain Science. Allen Human Brain Atlas.

Anatomically, the amygdala is an almond-shaped structure located in the medial temporal lobe. It is formed by functionally and morphologically heterogeneous sub-nuclei with intricate interconnectivity, also known as the amygdaloid complex. The amygdala is divided in about 13 nuclei. Although this is a common anatomic separation, other groups distinguish smaller subdivisions within those nuclei based on histology, histochemistry, and the connections with other regions (Sah et al., 2003). All these nuclei and sub-nuclei have been classically classified in four major groups: 1) Basolateral complex of the amygdala (BLA), which comprises the lateral amygdala (LA), the basal amygdala (BA) and the accessory basal nucleus (AB). 2) Central amygdala (CeA), which includes a lateral subdivision (CeL) and a medial subdivision (CeM). 3) Medial amygdala (MeA). 4) Cortical amygdala (CoA) (Sah et al., 2003; Roozendaal, McEwen, and Chattarji, 2009; Janak and Tye, 2015).

A layer of GABAergic neurons between the BLA and the CeA forms what is called intercalated cells, which also modulates the neurotransmission between these two areas of the amygdala (Ehrlich et al. 2009; Marowsky et al. 2005).

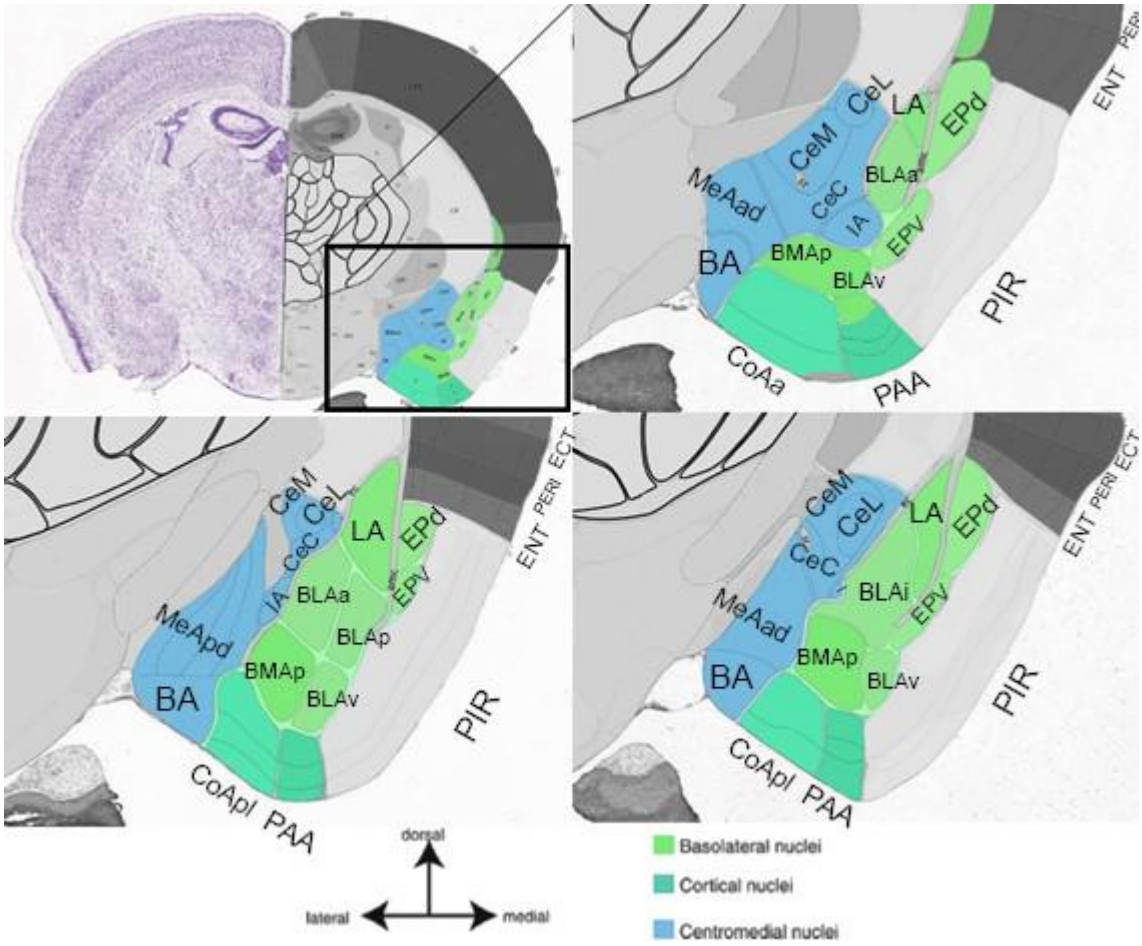


Image 2. Coronal section of the mouse brain showing the subdivisions of the amygdala. AAA Anterior amygdalar area; BA Bed nucleus of the accessory olfactory tract; BLA Basolateral amygdalar nucleus; BLAa Basolateral amygdalar nucleus, anterior part; BLAi Basolateral amygdalar nucleus, intermediate part; BLAp Basolateral amygdalar nucleus, posterior part; BLAv Basolateral amygdalar nucleus, ventral part; BMA Basomedial amygdalar nucleus; CeC Central amygdalar nucleus, capsular part; CeL Central amygdalar nucleus, lateral part; CeM Central amygdalar nucleus, medial part; CoA Cortical amygdalar area; CoAa Cortical amygdalar area, anterior part; CoApl Cortical amygdalar area, posterior lateral part; ECT Ectorhinal cortex; ENT Entorhinal cortex; EPd Endopiriform nucleus, dorsal part; EPv Endopiriform nucleus, ventral part; IA Intercalated amygdalar nucleus; LA Lateral amygdalar nucleus; MeAad Medial amygdalar nucleus, anterodorsal part; MeApd Medial amygdalar nucleus, posterodorsal part; PAA Piriform-amygdalar area; PERI Perirhinal cortex; PIR Piriform cortex. Modified from: © 2019 Allen Institute for Brain Science. Allen Mouse Brain Atlas.

In addition, researchers often consider other connected parts of the forebrain as a continuum with the classical areas of the amygdala, which are referred to as “extended amygdala” (e.g. bed nucleus of the *stria terminalis* or BNST) (LeDoux 2000; Davis and Whalen 2001 for review).

The complex interconnectivity of the amygdala is known to process input signals from other parts of the brain (e.g. hypothalamus, septal area, orbital cortex) and to induce output responses to other target areas, which are translated into autonomic, endocrine and behavioural responses related to anxiety (Davis 1992; Gross and Hen 2004). A simplified view of the signalling flow through the amygdala would start with input information being sent from sensory association areas that project profusely to the LA, via projections from the thalamus, cortex or hippocampus. The information then is transferred to other areas within the BLA (BA and BM) and the adjacent CeA. The BLA also projects back to cortical regions (such as the midline and orbital prefrontal cortices [PFCs]), the hippocampus and sensory association areas (McDonald 1998). Other unidirectional outputs from the BLA include striatal structures (such as the nucleus accumbens [NAc] or the BNST) and the CeA. This simplified model does not fit perfectly with reality since the BA and CeA also receive other inputs and the CeA can also function independently from the BLA. In addition, the BA also projects to the medial amygdala (MeA) and MeA, in turn, sends efferents to the BNST and hypothalamus (Corbit and Balleine 2005; Holland and Gallagher 2003). As mentioned, CeA receives information from BLA, but it also projects to autonomic, behavioural and hormonal regulatory centres in the hypothalamus, the midbrain, the pons periaqueductal grey (PAG) and the BNST. The main output region of the amygdalar complex is the CeA, which mediates autonomic and behavioural responses associated with fear and anxiety when it is chemically or electrically stimulated (Kapp et al., 1979; Applegate et al., 1982; Pascoe et al., 1985; Iwata et al., 1987; LeDoux et al., 1988; Wilensky et al., 2000, 2001; Goosens et al., 2003; Samosn et al., 2005; Tye et al. 2011).

The amygdala and, in general, the anxiety circuitry is closely related to the stress-response system. In mammals, the two main physiological components of anxiety related to the stress response are the autonomic nervous system (ANS) and the endocrine hypothalamic-pituitary-adrenal (HPA) axis. The activation of the ANS produces other physiological responses and the fast release of catecholamines; i.e. noradrenaline (a.k.a. norepinephrine) from sympathetic nerve terminals and adrenaline (a.k.a. epinephrine) from the adrenal medulla, which locates on top of each kidney. At the same time, the induction of the HPA axis produces the

activation of parvocellular neurons in the paraventricular nucleus of the hypothalamus (PVN) and the release of a number of hormones (corticotropin-releasing factor (CRF), arginine vasopressin (AVP)) and other secretagogues into the small portal circulatory system in the median eminence of the brain. These released hormones activate the corticotropic cells of the pituitary gland to produce the secretion of adrenocorticotropic hormone (ACTH). In turn, ACTH is released into the peripheral circulation and induces the synthesis and release of glucocorticoids in the adrenal cortex (mainly corticosterone in rats and mice and cortisol in humans and other mammals) within a range of less than 15 min. Negative feedback mechanisms control this system. Receptors for the mentioned steroid hormones (i.e. glucocorticoids) are expressed throughout the brain. They can act as transcription factors and therefore regulate gene and protein expression and potentially have long-lasting effects on the functioning of the brain regions affected (e.g. mPFC, hippocampal formation, the PVN and the anterior pituitary). In fact, high glucocorticoid levels are associated with chronic or recurrent cases of stress and can produce hyperactivation of the HPA axis via reduction of regulatory negative feedback mechanisms, especially in the limbic brain (Ulrich-Lai and Herman 2009; Lupien et al. 2009).

Moreover, chronic stress is also accompanied by complex consequences in the immune system, in both innate and acquired immunity. Glucocorticoids and catecholamines affect different types of trafficking immune cells and abolish the secretion of proinflammatory cytokines (PICs), such as tumour necrosis factor (TNF), IL1, IL6, IL8 and IL12. Conversely, PICs activate the stress system in both the CNS and peripheral nervous system (hypothalamus, central noradrenergic system, pituitary and adrenal glands). This activation increases glucocorticoid levels and thus suppresses the inflammatory reaction (Gadek-Michalska et al. 2013 for review).

These findings demonstrate that anxiety is regulated by various pathways within and outside the amygdala, and they give examples of the importance of differentiating specific projections and neuronal populations in the study of neural circuit function relevant to anxiety disorders.

SERINE PROTEASES IN THE BRAIN: THE TISSUE PLASMINOGEN ACTIVATOR / PLASMIN CASCADE

This section will comprehensively explore aspects of proteases and, more specifically, of serine proteases and the tissue plasminogen activator/plasmin cascade in the brain. Further facets of these proteases will be mentioned in specific sections.

Proteases are enzymes that produce the hydrolysis of peptide bonds belonging to either proteins or peptides. A typical human genome contains approximately 4% of genes encoding proteolytic enzymes and, among them, serine proteases have been shown to be the most abundant and functionally diverse group (more than one-third of all known proteolytic enzymes belong to this group). Serine proteases are named after one of the three critical amino acids that form their catalytic site: serine, histidine and aspartic acid (Davies et al. 1998; Di Cera 2009). Serine proteases, serine protease inhibitors (serpins) and protease-activated receptors (PARs) have been studied in the circulatory system for a long time due to their roles in coagulation, haemostasis and haemodynamics, inflammation and wound healing (Coughlin 2000; Macfarlane et al. 2001; Molinari et al. 2003; Wang, Luo, and Reiser 2008; Di Cera 2009 for reviews).

However, some of these proteases and related molecules have been observed to be endogenously expressed in the central nervous system, in different cell types and locations. Indeed, many projects have demonstrated that serine proteases, their zymogen precursors and endogenous inhibitors, as well as their protease-activated receptors (PARs), can affect synaptic function, behaviour and a number of neurological pathologies (e.g. Alzheimer's disease, Parkinson's disease, traumatic brain injury or stroke) (Almonte and Sweatt 2011). Importantly, it has been demonstrated that extracellular proteases and their target molecules are critical for the development of stress-induced anxiety and fear in mice (Almonte and Sweatt 2011; Brown et al. 2009; Horii et al. 2008; Meins et al. 2010; Melchor and Strickland 2005; Pawlak et al. 2003). Among these stress-related enzymes, one of the most well-known groups of serine proteases is the thrombin-like class, which includes tissue plasminogen activator (tPA) and plasmin (Coughlin 2000 for review).

Both tissue plasminogen activator (tPA) and plasmin, belong to one of the better-known proteolytic systems in the human body: the plasminogen activator (PA)/ plasmin proteolytic cascade. The PA/plasmin system consists of an inactive zymogen (a.k.a. proenzyme), called plasminogen, which is cleaved by plasminogen activators (PAs) to produce the broader spectrum active enzyme, plasmin. Plasmin, in turn, has protease properties such as degradation of fibrin clots in the circulatory system or the activation (via cleavage) of matrix metalloproteases (MMPs) than can process extracellular matrix (ECM) components (Baricos et al., 1995; Collen 2001). There are two mammalian PAs, namely, tissue-type (tPA) and urokinase-type (uPA). PAs are selective serine proteases that cleave a specific peptide bond within the plasminogen structure just after arginine codon 561 to release the active protease, plasmin (Carmeliet et al. 1994). In the brain, these activators participate in cell migration, neuronal plasticity, neuronal survival, the maintenance of the blood-brain barrier integrity and inflammatory processes (Hébert et al., 2016). This system is tightly regulated by serine protease inhibitors, also known as serpins. Serpins present a carboxy terminus (C-terminus or C-term) region with a specific reactive site peptide bond (Arg-X or Lys-X) that is cleaved by its respective protease, subsequently producing an inactive enzyme-inhibitor complex. Among them, plasminogen activator inhibitor-1 (PAI-1) and neuroserpin are more specific towards tPA, while α_2-antiplasmin shows high affinity for plasmin (Melchor and Strickland 2005). However, plasmin also has some other inhibitors such as α2-macroglobulin (Castellino and Ploplis 2005; Irigoyen et al. 1999; Lijnen 1996). Furthermore, low-density lipoprotein receptor-related protein (LRP)-mediated endocytosis can clear extracellular tPA and this process is increased by binding of annexin-II or fibrin (Melchor and Strickland 2005).

TPA/plasmin cascade has been classically associated with thrombolysis because of its mediation in the cleavage of fibrin in blood clots (Collen 2001). Plasmin efficiently cleaves fibrin, thus helping to break down fibrin clots, a key factor in the regulation of haemostasis and vascular patency. In fact, tPA and other derivative products are used as treatments after myocardial infarction or thrombotic stroke to treat it and restore blood flow. tPA itself can be used in a short time frame after stroke in suitable populations (Melchor and Strickland

2005).

Interestingly, all components of the tPA/plasmin system, including serpins, have been shown to be present in the CNS (Qian et al. 1993; Seeds, Williams, and Bickford 1995; Seeds, Basham, and Haffke 1999; Salles and Strickland 2002; Pawlak et al. 2003; Seeds, Basham, and Ferguson 2003; Rodrigues, Schafe, and LeDoux 2004; Teesalu et al. 2004; Masos and Miskin 1997; Yepes and Lawrence 2004b). In the adult mouse brain, tPA is highly expressed and released by neurons, glial cells, and endothelial cells, especially in highly plastic structures involved in learning and memory (e.g. hippocampus and amygdala), fear and anxiety (e.g. amygdala) and motor learning (e.g. cerebellum); but also in areas related to autonomic and endocrine functions (e.g. hypothalamus) (Lochner et al. 2006; Melchor and Strickland 2005; Qian et al. 1993; Salles and Strickland 2002; Shin, Kundel, and Wells 2004; Tsirka et al. 1995; Yepes and Lawrence 2004b, 2004a).

TPA is subject to a highly regulated proteolytic function. This protease is induced as an immediate early gene (IEG) depending on neuronal activity, which can be induced by epileptic seizures, kindling processes and long-term potentiation (LTP) paradigms (these paradigms will be explained in further chapters). IEGs are rapidly and temporarily activated as a reaction to a variety of cellular stimuli. In fact, it has been reported that some of these genes are upregulated upon stressful events in rodents (Butler et al., 2012). The basal levels of tPA mRNA are very low or undetectable, but they can be quickly increased when tPA synthesis is activated. Moreover, mRNA still accumulates when protein synthesis is inhibited, which means that the levels of mRNA are not dependent on the final protein synthesis (Qian et al. 1993). In neurons, translational control has been demonstrated to be dependent on the cytoplasmic polyadenylation element binding (CPEB) protein, which allows the polyadenylation of tPA mRNA and a subsequent increase in tPA protein synthesis (Shin, Kundel, and Wells 2004). These tight regulations allow a prompt increase in active tPA upon specific stimuli whereas the existence of cognate serpins of tPA allows a specific inactivation. The idea of a high regulation of this system is reinforced by the selective localisation of tPA in axon terminals. In these structures, tPA is contained in vesicles that can be released as secretory vesicles after membrane

depolarisation or stimulation (Gualandris et al., 1996; Parmer et al., 1997; Echeverry et al., 2010; Wu et al., 2015). Therefore, this mechanism of regulated tPA secretion provides a fast and localised increase of this enzyme at the synaptic cleft. Of note, tPA has been detected in dendrites, but the release of this protein from these structures has not been demonstrated (Melchor and Strickland 2005).

The second component of the system, plasminogen (plg), was thought for a long time to be exclusively produced in the liver (Bohmfalk et al., 1980). Sappino and colleagues acknowledged the presence of its mRNA in the mouse hippocampus (in 1993), which was confirmed later on by Tsirka et al. (1997). The presence of plasminogen mRNA and its encoded protein has been demonstrated in the cerebellum, the cortex, and the hippocampus of the neonatal and adult mouse, in a particularly important manner at times when experience and activity can induce tPA expression (Basham and Seeds 2001; Salles and Strickland 2002; Melchor and Strickland, 2005). It is considered that, as it happens in the periphery, tPA can locally convert plasminogen to plasmin in the brain. Then, plasmin has the ability to degrade extracellular matrix or modify synapses by proteolysis of synaptic proteins, including receptors or adhesion molecules. This localised and tightly controlled process can provide broad-based proteolysis involved in neural plasticity. However, since some tPA-dependent biological responses cannot be attributed to plasmin, it is still discussed whether tPA and plasmin have independent or coordinated functions in the brain (Melchor and Strickland 2005).

As mentioned in this introduction, stress is the trigger of many biological processes in the body. There is a plethora of possible mechanisms for the body to cope with stress. Of particular importance is the protease-mediated cleavage of extracellular matrix proteins, adhesion molecules, transmembrane receptors or ion channels (Overall and Blobel 2007; Lopez-Otin and Overall 2002), and indeed, tPA/plasmin proteolytic cascade has been suggested to be involved in stress-related disorders (Melchor and Strickland 2005).

tPA itself has been proposed to mediate a number of responses to stress. In the acute restraint stress model of anxiety, tPA activity is increased in the medial and

central amygdala preceding the onset of the stress-induced anxiety-like behaviour measured by elevated plus maze behavioural test (EPM) in wild-type (WT) mice. These behavioural changes are accompanied by an elevation of circulating levels of the stress-related hormone, corticosterone, which can be detected 90 min after stress (Pawlak et al. 2003). In the hippocampus of WT mice, acute restraint stress paradigm produces molecular changes, such as the post-synaptic phosphorylation of extracellular signal-regulated kinase (ERK1/2) and increased expression of the NMDA glutamatergic receptor subunit, GluN2B. These events were not found in $tPA^{-/-}$ mice. Furthermore, a different chronic stress model has also shown differences between wild-type and $tPA^{-/-}$ or $Plg^{-/-}$ mice. The reduced expression of the NR2A and NR2B glutamatergic NMDA receptor and the neuronal axonal growth-associated protein (GAP-43) in the hippocampus of WT and $tPA^{-/-}$ stressed mice is prevented in $Plg^{-/-}$ animals. Also, stressed WT mice undergo a decrease in the glutamatergic NR1 subunit, but this effect was reduced in $tPA^{-/-}$ mice and abolished in $Plg^{-/-}$. Additionally, tPA can affect morphological plasticity of neurons. For example, the dendritic spine counting in CA1 neurons decreased after stress, but this effect is prevented in $tPA^{-/-}$ animals (Pawlak et al. 2005). Furthermore, changes produced by tPA seem to influence behaviour, since $tPA^{-/-}$ mice showed less avoidance in the elevated plus maze (EPM) and less freezing behaviour than stressed wild-type mice in contextual fear conditioning (Norris and Strickland 2007).

TPA is also influenced by the hormonal stress response, as it is demonstrated by the elevated tPA activity in the amygdala after the infusion of CRF, a principal component of the stress-related pathways of the HPA axis. Accordingly, c-Fos (a marker of neuronal activation) immunoreactivity increases in central and medial amygdala upon intraventricular CRF infusion (Matys and Strickland 2003). However, all these effects produced by tPA seem to be plasmin-independent, since plasminogen-deficient mice do not present lower levels of anxiety in the elevated plus maze after restraint stress (Pawlak et al. 2003) or increased c-Fos expression after intraventricular infusion of CRF (Matys et al. 2004). This indicates that tPA could exert a plasmin-independent mechanism that may affect stress and anxiety-like behaviours. However, as it will be explained and summarised in Chapter 4, plasmin may have important roles in neuronal plasticity

and other cell processes.

As it can be deduced from this section, the activity of tPA/plasmin cascade has an instrumental role in anxiety. In the present thesis, I focus on the study of these proteases to try to understand the underpinning mechanisms of these conditions and tackle them from the molecular point of view. Particularly, the cleavage of the Eph receptor, EphB2, has been observed to modulate anxiety-like behaviours in rodents in the past (Attwood et al., 2011), and more recently, EphA4 was observed to be processed by tPA/plasmin cascade but its presence in the brain or its involvement in anxiety processes has not been assessed. Therefore, the main aim of this study was to observe whether this cascade would be happening in the murine brain and whether this cleavage would produce any modulation in stress-induced anxiety-like behaviours.

EPH RECEPTORS AND EPHRINS IN THE ADULT BRAIN

tPA and plasmin target a small and specific subset of known proteins and peptides in the brain (e.g. pro-brain-derived neurotrophic factor [proBDNF], low density lipoprotein receptor-related protein [LRP], GluN1, GluN2B, laminin, amyloid-β, DSD-1-proteoglycan/phosphacan and neurocan) (Wu et al., 2000; Melchor and Strickland, 2005). A recent discovery in our laboratory has posed a member of the Eph family of receptors, called EphA4, as a candidate target for these two proteases. This section will explain the particular characteristics of this family of proteins and general aspects of their relationship with the current project.

Description of the family

Erythropoietin-producing hepatocellular receptors (Eph receptors) is the largest family of receptor tyrosine kinases in humans. These multifaceted family of tyrosine kinases account for fourteen receptors at the last count (http://cbweb.med.harvard.edu/eph-nomenclature) (Murai and Pasquale, 2002).

They are divided into two classes, EphA receptors (A1–8, and A10) and EphB receptors (B1–4, and B6), depending on the homology of their sequences and their binding affinities for their innate ligands, called ephrins. In terms of binding capacity, there is a high degree of promiscuity within the family members, meaning that individual ligands are able to bind different receptors and vice versa; but in general terms, EphA receptors interact with ephrin-A ligands (A1–A5), and EphB receptors with ephrin-B ligands (B1–B3) (Gale et al., 1996; Himanen and Nikolov, 2003). There are two notable exceptions to this rule: EphA4, which is also activated by ephrin-B2 and ephrin-B3 (Gale et al., 1996), and EphB2, which is activated by ephrin-A5 (Himanen et al., 2004) (Image 3).

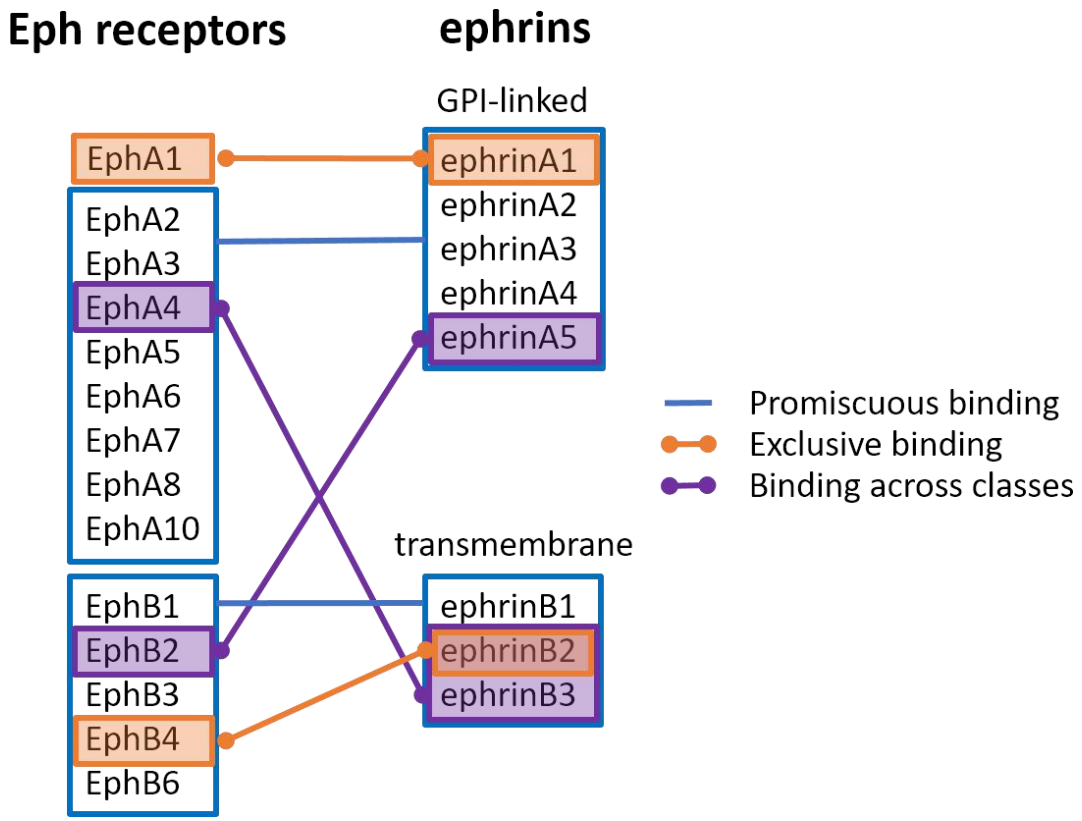


Image 3. Structural classification and binding affinities of Eph receptors and ephrin ligands.

Interactions

The valence of these interactions (activating or deactivating) is not clear. Data collected so far suggests that, in most of the cases, high-affinity binding seems

to involve a subsequent autophosphorylation of Eph receptors (Brambilla et al., 1995; Davis et al., 1994; Gale et al., 1996; Shao et al., 1995; Winslow et al., 1995). Interestingly, some receptors or their ligand ephrins (e.g. EphB3 and ephrin-B1) can be autophosphorylated when cells are transfected with only the intracellular tropomyosin receptor kinase B (TrkB) domain (Brambilla et al., 1995). Furthermore, protein structure research shows that Eph receptors and ephrins expressed on adjacent cell surfaces can crosslink their amino-terminal domains to form multimers at the cell-cell interface (Himanen et al., 2001). In most cases, the interaction leads to the clustering of receptors and induces bidirectional signalling (Klein, 2001; Pasquale, 2008 for more on this topic), which propagates both towards the cell containing the receptor and the cell presenting the ligand.

Importantly, unlike other families of RTKs, in Eph family, only the membrane-bound forms of the ligands can activate the receptors. However, the ligands' soluble forms (non-membrane bound monomer) can be artificially activated via clustering (using linking tags or antibodies) (Davis et al., 1994). Dose-response studies demonstrated that clustered ligands have at least 100 times greater potency than unclustered ligands, which also would explain why ligands need to be bound to the membrane to be active. Membrane attachment facilitates ligand clustering, which in turn promotes dimerisation, multimerisation and hence activation (understood as phosphorylation / auto-phosphorylation) of the corresponding receptors. Nonetheless, in their physiological state, affinities can potentially change and may involve various receptors/ligands (Davis et al., 1994; Flanagan and Vanderhaeghen, 1998; Stein et al., 1998). This transmembrane arrangement of ligands is not exclusive of ephrins. Other ligands of tyrosine kinase receptors also exhibit this location (e.g. kit ligand, colony-stimulating factor-1, and various members of the epidermal growth factor family) (Massague and Pandiella, 1993). All these ligands can be cleaved to produce active soluble molecules, but in the case of the ephrins, artificial truncated soluble forms of ephrins (presented outside the membrane) have not been demonstrated to be able to activate their respective Eph receptors.

The functional consequences of this tight regulation of location and activation are not known, but in the case of other ligands (e.g. kit ligand), evidence suggests that the membrane anchorage helps to spatially restrict signalling activity

(Brannan et al., 1991; Flanagan, Chan and Leder, 1991). The need for nerve cells to precisely navigate during development and modifications in synaptic plasticity entails a necessity of an equally precise cell-cell communication. This tight regulation also may explain why, as in the case of many growth factors, ligands for tyrosine kinases receptor (such as Eph receptors) can be found anchored either to cell surfaces or to extracellular matrix in a non-freely diffusible way (Massague and Pandiella, 1993; Taipale and Keski-Oja, 1997).

Structure

The Eph receptors comprise an extracellular region, which contains an amino-terminal (N-terminal or N-term) part with cysteine-rich motifs followed by two fibronectin type III (FN3) motifs implicated in the assembly of multimeric signalling clusters (Lackmann et al., 1998; Wimmer-Kleikamp et al., 2004). Three exons link these three motifs (Connor and Pasquale, 1995). As other tyrosine kinases receptors, they exhibit a single transmembrane domain and an intracellular domain, which involved in signal transduction. The cytoplasmic fragment of Eph receptors incorporates four domains which differ in their function. These include a juxtamembrane region containing an Src-homology-2 (SH2)-docking site, an uninterrupted dual-lobe tyrosine kinase domain (Wybenga-Groot et al., 2001), a sterile-A-motif (SAM) domain and a postsynaptic density protein/disc large/zona occludens (PDZ) binding domain (Himanen and Nikolov, 2003). In general, Eph receptors are closely related to each other, with sequence identities of approx. 30–70% in the extracellular domain and 65–90% in the kinase domain (Flanagan and Vanderhaeghen, 1998) (Image 4).

Interestingly, they are the only RTKs that need oligomerisation to trigger the activation of the receptor (Davis et al., 1994; Stein et al., 1998), and the only

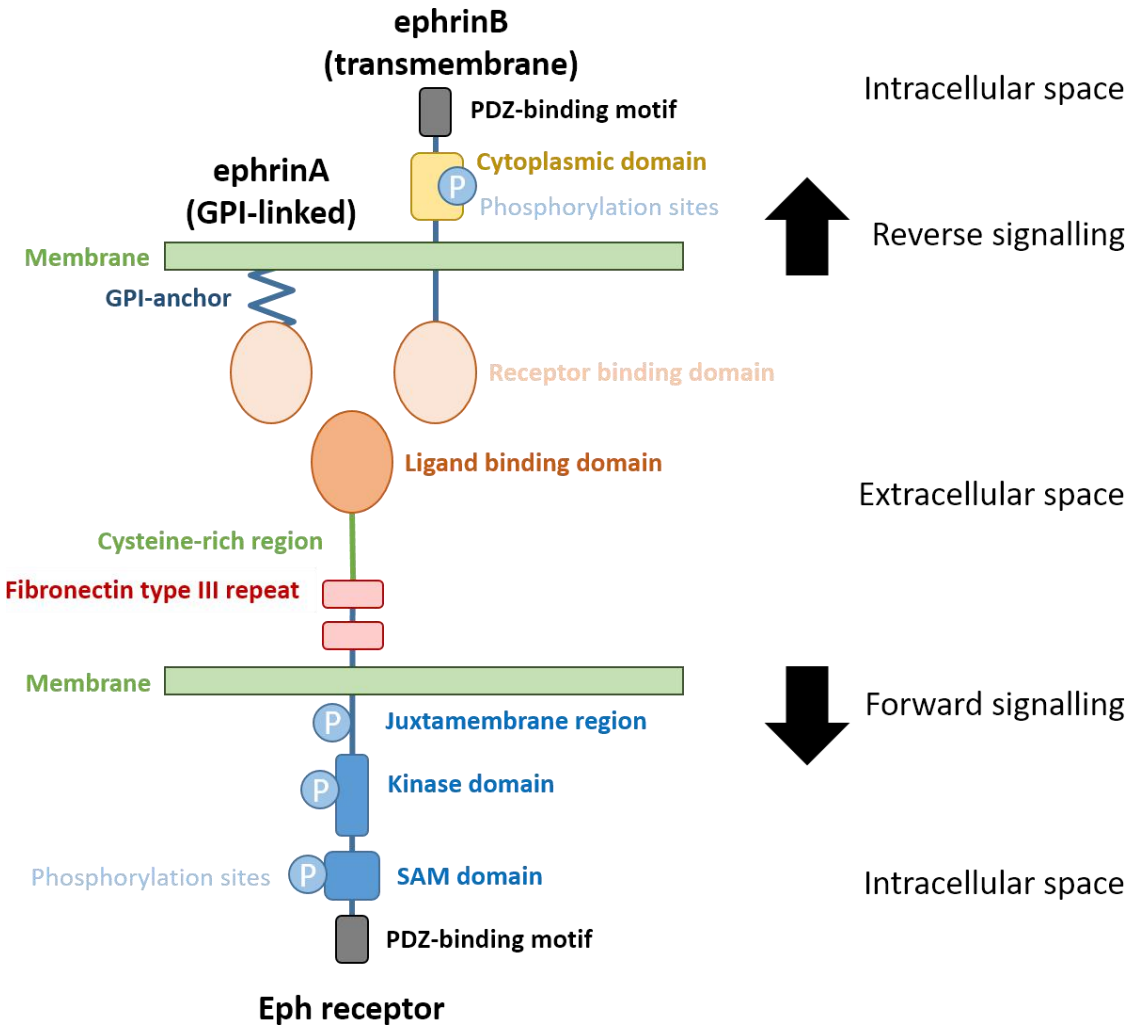


Image 4. Structure of Eph receptors and ephrin ligands. The image represents a scheme of the tertiary structure of Eph receptors and the two ephrin ligands (a full description of these structures can be found in the section "Structure" [page 61]). Forward signalling refers to the signalling produced by Eph receptors (A or B) upon the respective ligand binding. Conversely, reverse signalling alludes to the signalling generated by ephrin molecules that is triggered by Eph receptors.

RTKs containing a SAM domain (Hubbard and Till, 2000). SAM domain has been implicated in regulating protein/protein interactions and homodimerisation (Behlke, Labudde and Ristau, 2001), which might indicate a link of this domain within the clustering of Eph receptors to form active complexes (Stapleton et al., 1999). However, the real functional relevance of the SAM or the PDZ domains is not well understood, because the ablation of the SAM and PDZ domains does not seem to influence various receptor's functions *in vivo* or *in vitro* (Kullander et al., 2001; Park et al., 2004). Cytosolic ligands for PDZ domain of Eph receptors

have been reported (Buchert et al., 1999; Hsueh and Sheng, 1998; Torres et al., 1998), which may indicate a different function for this domain.

Observation of the crystal structure of isolated EphB2 and ephrin-B2 revealed a heterotetrameric complex comprised of two Eph-ephrin heterodimers (Himanen et al., 2001; Kullander and Klein, 2002). Further research on EphA4–ephrin-B2 complex revealed new structures that enable the characteristic binding promiscuity of this family. These molecules, apart from the ligand/receptor binding domain, present an additional low-affinity interface between the ephrin and the so-called H–I loop of the Eph globular structure, which, along with four residues in the A-type of Ephs is thought to provide subclass-specificity to ephrin binding (Himanen, Henkemeyer and Nikolov, 1998; Himanen et al., 2001). The D–E and J–K loops of Eph receptors experience the largest conformational change when the ligand is bound, forming the so-called “ligand binding channel”. This structural view of specificity is reinforced by the fact that the described interaction is missing when comparing to a non-putative EphB–ephrin-A complex, such as the EphB2–ephrin-A5 complex (Himanen et al., 2004). Further analysis in EphA3–ephrin-A5 complex confirmed the position and functionality of these high- and low-affinity binding sites, but it also identified a third binding site (Smith et al., 2004). This third site is important for receptor phosphorylation and downstream recruitment of proteins and responses (Smith et al., 2004), but it contributes only moderately to ligand binding, supporting the notion that the tetrameric Eph–ephrin complex observed in the crystal structure is necessary but not sufficient for signalling.

Phylogenetic Comparisons

Eph receptors and ephrins have been cloned from various invertebrate and vertebrate species (Scully, McKeown and Thomas, 1999; Bossing and Brand, 2002; Kaneko and Nighorn, 2003), with a more intensive analysis in human (*Homo sapiens*), mouse (*Mus musculus*) and chicken (*Gallus gallus*) (Flanagan and Vanderhaeghen, 1998). Overall numbers of ligands and receptors seem to be conserved in mammals and birds. Orthologs for the human receptors and

ligands have been assigned unequivocally in mouse and chicken showing a diverse structure related to “speciation rather than gene duplication” (Flanagan and Vanderhaeghen, 1998).

Only one Eph gene has been found in sponges (Suga et al., 1999), *C. elegans* (George et al., 1998) and *Drosophila* (Dearborn Jr. et al., 2002) compared to fourteen different Ephs in vertebrates. This fact responds, for some authors, to a diversification during the evolution of a complex vertebrate body, and, in particular, of the more complex vertebrate’s vascular and nervous systems (Boyd and Lackmann, 2001; Drescher, 2002).

Localisation

Details about the different receptors can be found in projects aiming to describe the expression of the large number of genes in the mouse brain (Heintz, 2004; Magdaleno et al., 2006; Lein et al., 2007), as well as specific research focusing on this type of receptors in the mouse and the non-human primates (Liebl et al., 2003; Xiao et al., 2006).

Eph receptors have been shown to be located in areas related to plasticity and memory formation such as the hippocampus (e.g. mouse [Grunwald et al., 2001]; primates [Xiao et al., 2006]; human [Rosenberger et al., 2014]), amygdala (e.g. mouse: [Grunwald et al., 2001]; primates: [Xiao et al., 2006]) and cortex (e.g. rat: [Martone et al., 1997]; primates: [Xiao et al., 2006]). Given that specific location, it is plausible that Ephs are involved in synaptic transmission, plasticity and synaptogenesis, which are cellular events intimately involved in memory formation and anxiety.

The Eph receptors have been demonstrated to play an important role in the development and segmentation of the mouse nervous system (Flanagan and Vanderhaeghen, 1998; Cramer and Miko, 2016); however, the gene expression in embryonic stages is not exclusive to the CNS (Murai et al., 2003). When non-neuronal cells ectopically express the Eph family receptors, they do not present the usual effect in proliferation and differentiation, although they provoke changes

in cytoskeletal structures (Lhoták and Pawson, 1993; Davis et al., 1994; Brambilla et al., 1995; Vearing and Lackmann, 2005). Within the brain, the expression of these receptors is widespread and varies with the different receptors and ligands (Murai et al., 2003). In the same manner, the profile of expression changes over time during the lifespan, which sets the foundation for the study of the different roles of these molecules in developmental processes and adult experience-driven plasticity. Expression of these proteins can be either extended until adulthood or reduced, to the point that they can be lost, like in the case of a big part of the midbrain and preoptic area (Liebl et al., 2003). Interestingly, in highly plastic regions, including the amygdala and hippocampus, their expression is higher in the adulthood (Liebl et al., 2003; Magdaleno et al., 2006; Lein et al., 2007).

A vast amount of scientific evidence shows that many Eph receptors are localised presynaptically in the growth cone (the leading portion of the extending axons) in the developing nervous system. This location is in line with a very well-known function of these receptors in establishing connections between different areas of the nervous system and axon navigation. Nonetheless, this location is not exclusive. Immunoelectron microscopy experiments show that Eph receptors are also located postsynaptically in dendrites of the adult brain (Martone et al., 1997; Buchert et al., 1999). This alternative location in dendrites is in agreement with the notable relation of Eph receptors with synaptic function. Some neuronal dendrites extend filopodia-like protrusions to examine the environment (Luscher et al., 2000). That extension, in some cases, may lead to the finding of a new axonal target and develop a more mature synaptic connection. Accordingly, establishing contact with other neurons and electrical activity appears to be important in regulating Eph receptor expression (Moreno-Flores and Wandosell, 1999; Henderson et al., 2001; Murai and Pasquale, 2002).

Functions of the family

Tyrosine kinase receptors are disseminated throughout the whole nervous system and it is well-established that they have important roles in the construction

of the neuronal circuits during the embryo development (Murai and Pasquale, 2002; Kania and Klein, 2016). In 1995, Winslow et al. set the foundations of these observations by reporting that adding rat EphA5-IgG to the culture medium completely prevented the formation of axon bundles, while having little or no effect on dendritic processes (Winslow et al., 1995). Over the last two decades, numerous projects have unveiled the important role of Eph/ephrin activity in the development of the nervous system. I will only briefly summarise it here, but a number of reviews describe this topic in more detail (Boyd and Lackmann, 2001; Klein, 2001, 2012; Kullander and Klein, 2002; Poliakov, Cotrina and Wilkinson, 2004; Klein and Kania, 2014; Kania and Klein, 2016; Ventrella, Kaplan and Getsios, 2017).

However, more recent work about the adult brain is creating increasing attention among researchers and it is connecting Eph family to changes in the strength of existing neuronal connections, particularly the synaptic connections. More about this topic will be referenced in further chapters but, as an example, they have been observed to modify these connections in close association with at least one family of ion channels, the NMDA receptors (Murai and Pasquale, 2002). Specifically, direct interaction between EphB receptors and the NMDA receptors' subunits has been reported (Dalva et al., 2000; Attwood et al., 2011). This interaction seems to be promoted by ephrin-B ligands and intriguingly, ephrin-B-induced clustering of EphB receptors and NMDAR is a mechanism that reminds the formation of postsynaptic specialisations. Adding up to the possible functionality of this interaction, various papers showed that EphB receptors may play a role in the regulation of functional synaptic plasticity through NMDA currents (Ali and Salter, 2001; Henderson et al., 2001; Grunwald et al., 2001; Takasu et al., 2002).

Eph receptors as a target for stress-related phenomena

Members of our laboratory first identified a member of the Eph family (EphB2) as a target for proteases in amygdala linked to stress-related plasticity and to anxiety-like behaviours (Attwood et al., 2011). In this study, it is shown that

cleavage of EphB2 by neuropsin (a kallikrein-like serine protease) in the amygdala results in the dissociation of EphB2 from the GluN1 subunit of the NMDAR. At the functional level, that interaction enhances NMDAR current and induces Fkbp5 gene expression, which is a co-chaperone of glucocorticoid receptors that has been linked with anxiety disorders. In addition, that interaction increases anxiety-like behaviours in mice as measured by different behavioural paradigms, which was not observed in neuropsin knockout mice. This anxiety-like behaviour was abolished by blocking EphB2 in the amygdala of wild-type mice.

Long-term potentiation (LTP) refers to the remodelling of neuronal connections leading to increased synaptic strength after a repeated excitatory stimulation and it is considered to be a correlate of learning processes. Recent findings highlight the importance of EphA4 in the amygdala, and thus the control of emotions. Deletion of EphA4 reduces BLA's LTP, which implicates this receptor in the normal expression of synaptic plasticity in that area. The same work shows that EphA4 interaction with ephrin-A3 induces the increase of endocytosis of EphA4 through a mechanism that involves Ras and Rab interactor 1 (Rin1). Accordingly, mice deficient in Rin1 show a higher level of LTP, suggesting that the internalisation of EphA4 associated with Rin1 may control LTP in the amygdala (Deininger et al., 2008). However, these defects of EphA4$^{-/-}$ during synaptic plasticity do not seem to be clearly mirrored in fear and anxiety-related behavioural paradigms (Willi et al., 2012), although other groups claim that impeding EphA4 signalling could achieve so (Dines and Lamprecht, 2014).

Altogether, these findings pose Eph receptors, such as EphB2 and EphA4, as important targets of stress-induced mechanisms in the brain. The fact that a serine protease (neuropsin) is able to modify stress-related events through interaction with EphB2 indicates that similar mechanisms may be occurring through the interaction of proteases and other membrane molecules.

OBJECTIVES OF THE STUDY

As mentioned in this introduction, the main objectives of this study are to observe whether an tPA/plasmin/EphA4 cascade is present and relevant in the murine brain and (given the instrumental role of tPA/plasmin cascade in anxiety) whether a hypothetical cleavage of EphA4 would produce any modulation in stress-induced anxiety-like behaviours, as it has been observed to happen with the cleavage of EphB2 receptor (Attwood et al., 2012). With the goal of characterising tPA/plasmin/EphA4 cascade in the mouse brain, Chapter 3 aims to localise the cascade in two brain areas relevant for the development of anxiety, i.e. hippocampus and amygdala by using immunofluorescence techniques. In addition, the same chapter includes the characterisation of the cleavage sites in the structure of EphA4 produced by tPA and plasmin using mass spectrometry and Western blot techniques; plus, the identification of the cascade in vitro and in vivo. This necessary characterisation was of help to develop new tools and hypothesis about the cascade. In light of the characterisation in Chapter 3, further chapters try to address downstream interactions of EphA4 in the GABAergic synapse that may enable synaptic plasticity changes. In line with the role of the receptor and its presence in the synaptic spines, I tried to stablish whether there is any difference in dendritic and spine morphology of neuronal primary cultures, as a representative form of synaptic plasticity. Moreover, due to the role to tPA/ plasmin cascade in stress-induced anxiety-like behaviours, Chapter 6 tries to explore changes in these behaviours by overexpressing different variants of the receptor in the mouse amygdala, the area where tPA is most active. Finally, we analysed other animal models for different conditions in which the tPA/ plasmin cascade and where EphA4 could be relevant for the development of their processes.

CHAPTER 2: MATERIALS AND METHODS

Reagents were purchased from Sigma-Aldrich unless otherwise specified.

All experiments were performed by myself unless otherwise specified.

SPECIFICITY OF ANTIBODIES

The specificity of all the primary antibodies referenced in the respective experiments of this chapter has been sufficiently proved and tested against negative controls in similar conditions in previous experiments of either former members of the laboratory or the broad scientific literature (normally specified in the manufacturer's datasheet). Therefore, particular experiments about it were not explicitly included in this thesis. The specificity of the secondary antibodies was also checked by incubating the corresponding samples with the secondary antibodies used in absence of primary antibodies. The images of this controls have been also omitted in the current manuscript.

ANIMALS

Experiments were performed on 8- to 12-week adult male C57BL/6J mice of three strains: wild-type, $tPA^{-/-}$ or $plasminogen^{-/-}$ backcrossed to C57BL/6J for 12 generations. All animals were housed in groups of three to five male mice per cage in a colony room with a 12-hour light/dark cycle in standard group cages with *ad libitum* access to commercial food pellets and water. All experiments were conducted during the light half of the cycle. The experiments were performed under the Project License Number 80/3502 and approved by the Animal Welfare Ethical Review Body (AWERB) at the University of Exeter.

EXTRACTION OF THE MOUSE BRAIN AND AMYGDALA DISSECTION

Mice (stress-naive or subjected to the restraint stress as described below) were euthanised using intraperitoneal sodium pentobarbital (50 mg/kg). Then, the animals were fixed in a stereotaxic frame and perfused transcardially with ice-cold phosphate buffer saline (PBS). The brains were removed and amygdalae dissected from slices at -0.58 to -2.3 mm relative to Bregmal, where amygdala sits, using a mouse brain matrix (Stoelting Co, USA), frozen immediately on dry ice and stored at -80ºC until use.

If the animals were used for immunohistochemistry (IHC), PBS + protease inhibitor cocktail (cOmplete®, Roche, Germany) was used for perfusion. For IHC, the brains were extracted and fixed in 4% paraformaldehyde (PFA) dissolved in PBS at 4ºC, overnight, with constant agitation. Next day, brains were washed for approximately 6 h in PBS. Then, brains were cut with a vibrating microtome (Campden Instruments, UK) in coronal sections with a thickness of 70 µm.

MICROARRAY STUDY

This microarray study was performed by former members of our laboratory. Amygdalae were isolated from $tPA^{+/+}$ (n = 30) and $tPA^{-/-}$ (n = 30) mice using a dissecting microscope in ice-cold ACSF (glucose 25 mM, NaCl 115 mM, $NaH_2PO_4 \cdot H_2O$ 1.2 mM, KCl 3.3 mM, $CaCl_2$, 2 mM, $MgSO_4$, 1 mM, $NaHCO_3$ 25.5 mM, pH 7.4 and stored at −20 °C in RNAlater solution [Qiagen, UK]). RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen, UK), the ribosomal fraction of RNA reduced with RiboMinus Kit (Invitrogen, USA) and the RNA integrity verified by electrophoresis using Agilent Bioanalyser 2100 (Agilent Technologies, USA). RNA pulled from three mice was reverse-transcribed and hybridised with GeneChip Mouse Exon 1.0 ST Array (Affymetrix, UK; 10 arrays per genotype).

The Bioconductor bundle of R packages and The Partek Genomics Suite (PGS) (Partek Incorporated, USA) were employed to analyse differential gene expression and perform pathway analyses. The results were verified using Enrichr and g:Profiler servers using Kegg Pathway Database (Kanehisa Laboratories, Japan) (Jüri et al., 2007; Kuleshov et al., 2016) as the basis for performing Statistical Overrepresentation Test.

IMMUNOSTAINING OF BRAIN SLICES

Brain sections were incubated for 1 h at RT in 1× PBS with gentle rotation for normal immunostainings, or 1× PBS + 3% H_2O_2 if TSA amplification was added to the process. Then, sections were washed three times for 10 min with 1× PBS. After blocking (0.1% Triton X-100, 10% foetal bovine serum [FBS] in 1× PBS for 1 h at RT), slices were probed with the primary antibodies at 4°C overnight with gentle shaking. Antibodies used (host; brand; concentration; catalogue number): EphA4 (mouse; Invitrogen, USA; 1:500; #37-1600); plasminogen (rabbit; Innovative Research, USA; 1:500; #IRBAMSPLGAP100UG); tPA (rabbit; Molecular Innovations, UK; 1:500; #ASHTPA-GF-HT); gephyrin (rabbit; Abcam, UK; 1:1000; #ab136343); GFAP (chicken; Abcam, UK; 1:500; ab4673); NeuN (mouse; Chemicon [Millipore]; 1:500; MAB377); NeuN-GFP (mouse; Chemicon [Millipore]; 1:500; MAB377X); CRF SOM; EAAT3 (goat; Santa Cruz; 1:500; sc-7761). Next day, sections were washed (0.1% Triton X-100 in 1× PBS, for 15 min, three times at RT) before applying the corresponding secondary fluorescent antibodies (i.e. Alexa Fluor 647, Alexa Fluor 555, FITC, Cy5 and Cy3) (Invitrogen, USA; 1:500) (0.1% Triton X-100, 10% FBS in 1× PBS, for 1 h at RT in darkness). DAPI Vectashield® for counterstaining (Vector Laboratories, #H-1200).

In the case of immunostaining with amplified fluorescence for tPA and plasmin(ogen) antigens (Image 5), secondary fluorescent antibodies were substituted for biotin-conjugated antibodies (horse; Vector Labs, UK; 1:500; #BA-1100-1.5). After that, slices were rinsed once with 0.1% Triton X-100 in 1× PBS and then washed for 15 min, three times with 0.1% Triton X-100 in 1× PBS. After

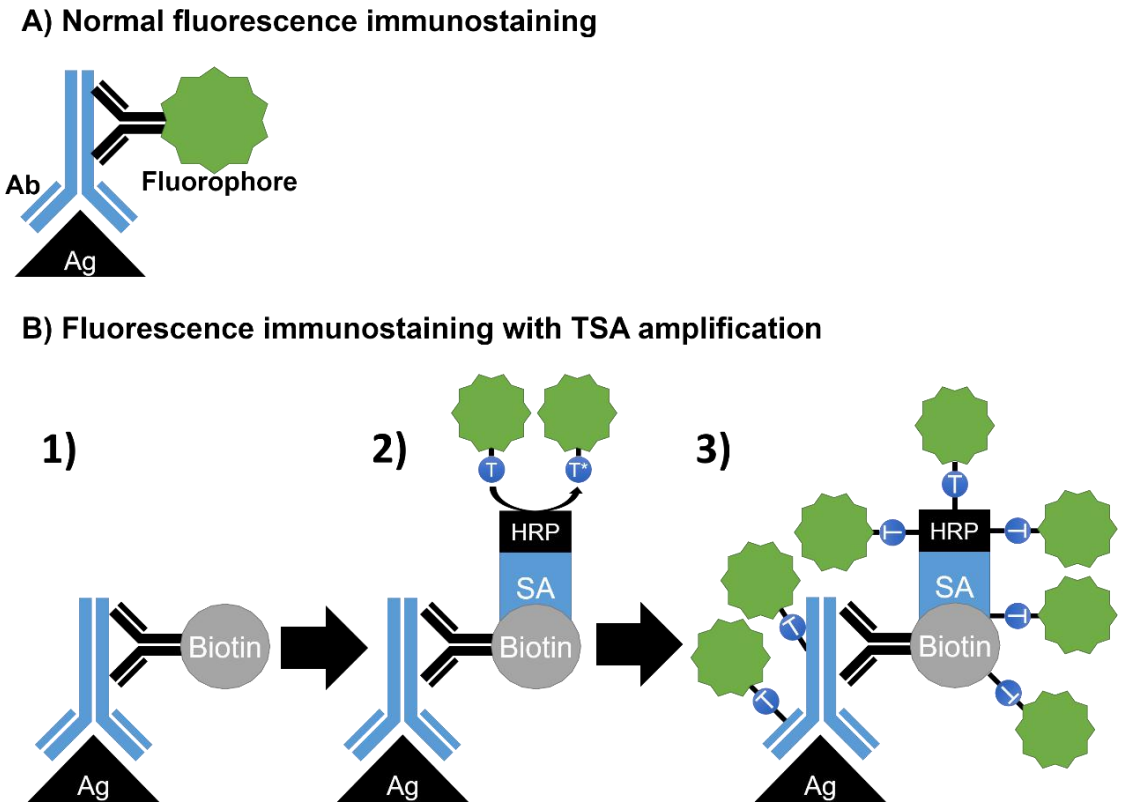


Image 5. Comparison between normal fluorescence immunostaining and TSA-amplified immunostaining. The TSA amplification procedure starts as a standard immunohistochemistry or immunocytochemistry experiment by using a primary antibody (Ab) against the antigen (Ag) of interest. In the TSA amplification method, instead of a secondary antibody labelled with a fluorophore (A), a secondary antibody labelled with biotin is directed against the primary antibody (B1). Then, horseradish peroxidase (HRP) is introduced into the system in conjunction with the biotin-affinity label, streptavidin (SA) (B2). HRP catalyses the reaction in which TSA substrate (T) is converted into a highly reactive free-radical species (T*) (B2). TSA is covalently bound to a fluorophore that will produce the fluorescent signal. Finally, the reactive TSA forms covalent bonds with tyrosine residues immediately proximal to the enzyme, so many fluorescent molecules are deposited near the primary antibody, hence amplifying the fluorescent signal (B3).

that, avidin-biotin HRP solution was applied according to manufacturer's instructions (VECTASTAIN® Elite® ABC HRP Kit; PK-6100). Then, Tyramide Signal Amplification (TSA) Systems kit (Perkin Elmer, USA) was used according to manufacturer's instructions. After all the steps, slices were washed at least three times for 15 min each with 0.1% Triton X-100 in 1× PBS, before mounting the sections.

Negative controls for secondary antibodies were performed following the same steps except for the addition of the primary antibody.

FLUORESCENT *IN SITU* HYBRIDISATION

682bp long fragment of tissue plasminogen activator mRNA 3'UTR was amplified from mouse amygdala cDNA by Taq PCR with 5'-GTGCCTGGGGTCTACACAAA and 5'-AAATCATACAGTTCTCCCAACCA primers. Next, the PCR product was cloned into a dual-promoter pCRII vector and the insert orientation and integrity were confirmed by restriction analysis and DNA sequencing. The plasmid was linearised with SacI or XhoI (for transcription from T7 or SP6 promoter respectively), and 3'UTR fragment was transcribed in vitro using SP6 or T7 polymerases (New England Biolabs, UK) and the DIG RNA labelling Mix (Roche, UK), generating sense and antisense DIG-labelled probes.

The extracted mouse brains (see "extraction of the mouse brain and amygdala dissection") were fixed overnight in 4% PFA in PBS at 4ºC. The next day, brains were washed in PBS treated with diethylpyrocarbonate (DEPC), to inactivate RNase enzymes, and sectioned at 50 µm. The sections were transferred onto polylysine slides (VWR, UK), left to dry and stored at -80 ºC until use. Before *in situ* hybridisation commenced, slides were submerged in PBS/DEPC until the PBS precipitate dissolved. In the meantime, the RNA probe was incubated with hybridisation buffer (50% v/v deionized formamide, 0.2 M NaCl, 50 mM EDTA, 10 mM Tris-HCl pH 7.5, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 0.05 mg/mL tRNA from baker's yeast) for 5 min at 70°C and placed onto slides. The dilution of the probe

in the hybridisation buffer that generates the best signal to noise ratio was empirically established at 1:1000. Sections were covered with cover glasses and the hybridisation reaction was initiated. Hybridisation with the DIG-labelled RNA probes was carried out overnight at 65°C in a chamber humidified with 50% v/v formamide containing 1× saline-sodium cytrate (SSC) buffer. The next day sections were washed three times at 65°C for 30 min each in wash solution (50% v/v formamide, 0.1% Tween® 20, 1× SSC), followed by two washes for 30 min each in 1× MABT (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.1% Tween® 20) at RT. The sections were blocked with blocking buffer (0.5% blocking reagent [Roche, UK] dissolved in TNT buffer [0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween® 20]) for 1 h at RT. Anti-digoxigenin-HRP antibody (sheep; Merck, UK; # 11207733910) at 1:200 dilution in blocking buffer was applied onto the sections and incubated for 1 h at RT, followed by three washes with PBS-T (0.1% Triton X-100 in 1× PBS). The biotin deposition was performed using TSA Plus Biotin Fluorescein Kit (Perkin Elmer, UK), followed by three subsequent washes with PBS-T and a 45-min incubation with ABC reagent (Vector Laboratories, UK). Then, fluorescein was deposited using the TSA Plus Fluorescein Kit (Perkin Elmer).

To immunohistochemically visualise corticotropin-releasing factor (CRF), somatostatin and protein kinase C (PKC)δ proteins on the same sections, the slides were blocked with 10% FBS in PBS-T for 1 hour at RT followed by overnight incubation at 4°C with either anti-CRF (Sigma, UK), anti-SST (Merck Millipore, UK) or anti-PKCδ (BD Biosciences, UK) primary antibodies diluted 1:200. The primary antibodies were visualised by corresponding Alexa Fluor-conjugated secondary antibodies (Invitrogen, USA) as described in the Immunohistochemistry section.

A first negative control was performed following the same steps except for the addition of the DIG-labelled RNA probes. A second negative control was carried out without the addition of the anti-digoxigenin-HRP antibody. A third negative control was performed following the same steps except for the primary antibodies targeting the interneuronal markers (CRF, somatostatin or PKCδ).

These experiments were carried out with the help of Dr Mariusz Mucha.

GENERATION OF INSERTS FOR EPHA4 CONSTRUCTS

Three inserts were generated corresponding to the three different EphA4 variants used in this work. This is, an insert for the wild-type mouse EphA4, an insert for the cleavage-resistant form of EphA4 and an insert for the truncated form of EphA4 that mimics tPA's or PL's main cleavage activity.

For the generation of the cleavage-resistant EphA4 mutant (crEphA4) insert, single-amino acid substitution was performed using a site-directed mutagenesis assay. In this cleavage-resistant form, the arginine residue located at the cleavage site named P5 (R516) was substituted by glutamine residue (R516Q), which is the amino acid adding less steric tension to the structure. To this aim, a PCR reaction was performed using cDNA of wild-type mouse EphA4 (wtEphA4) as a template and the pair of self-reverse-complement primers introducing the required mutation (forward primer: 5'-GTTTTTCACGTGCGAGCCCAGACCGC TGCTGGCTACGG-3'; reverse primer: 5'-CCGTAGCCAGCAGCGGTCTGGG CTCGCACGTGAAAAAC-3'). Then, PCR DNA products were treated with DpnI restrictase to eliminate the initial methylated DNA sequence and the amplified crEphA4 plasmid was subsequently electroporated into 5-alpha electrocompetent E. coli cells (New England BioLabs, UK). The introduction of the desired mutation, insert orientation and integrity were confirmed by restriction analysis and DNA sequencing.

In order to generate constructs for the expression of the different EphA4 variants, cDNAs corresponding to the wtEphA4, the crEphA4 mutant or the truncated EphA4 variant at the residue R516 (tEphA4), were amplified by PCR from wild-type mouse EphA4 cDNA or the crEphA4-containing plasmid. Restriction sites were added to the PCR DNA-primers to allow further cloning into the appropriate vectors. XbaI on the forward primers and SalI on the reverse one. wtEphA4 forward primer: 5'-AAATCTAGAATGGCTGG GATTTTCTATTTC-3'; crEphA4 forward primer: 5'-AAAGTCGACTCAGACAGG AACCATCCTGCC-3', Reverse primer for both wt and crEphA4: 5'-AAAGTCGACTTACCTGGCTCGCACGTG AAAAACATAGGAAGTCAGAGGG-3'. The truncated form of EphA4 (tEphA4) was amplified using a forward primer containing a NheI restriction site and wtEphA4's signal peptide, which allows tEphA4 to localise on the cell membrane:

5'-AAAGCTAGCATGGCCGGCATCTTCTACTTCATCCTGTTCTCCTTCCTGT TCGGCATCTGCGACGCCACCGCTGCTGGCTACGGAGACTTCAGC-3'. The reverse primer containing SalI site is the same one used for wtEphA4 and crEphA4 amplification. Sequence orientation and integrity were confirmed by restriction analysis and DNA sequencing.

For the overexpression of EphA4 receptor and its variants, these inserts were subcloned into an empty plasmid backbone as described in further sections.

CELL LINE CULTURES EXPERIMENTS

For the transient overexpression of EphA4 variants in mammalian cell line cultures, four constructs were generated. The backbone plasmid used to synthesise these constructs was Foxd3-pIRES2-eGFP (Addgene plasmid catalogue #37269) and the inserts described in the previous section were introduced using XbaI and SalI or NheI and SalI restriction sites respectively. All these constructs co-express enhanced green fluorescence protein (eGFP) after the corresponding sequence of interest taking advantage of an internal ribosome entry site (IRES). The baseline control for the expression of these inserts consists of the expression of eGFP by the empty backbone plasmid. All these constructs use a CMV promoter.

For enzymatic proteolytic cleavage experiments, Neuro-2A (N2A) cell line (passage number <10) was incubated (37°C, 5% CO_2) in cell culture media (DMEM, 1% penicillin-streptomycin, 1% non-essential amino acids) + 5% v/v foetal bovine serum (FBS), until 70-80% confluence. Once appropriate confluence is reached, cells were transfected with the control vector (containing only eGFP), wild-type EphA4 or any of the variants of EphA4 (crEphA4 or tEphA4) using polyethyleneimine (PEI) MW25000 (Polysciences, USA) as transfection agent at a 3:1 ratio of PEI:DNA. Cells were then left to overexpress these constructs for 24 to 48 h. After that, cells were washed twice with culture medium without FBS before being incubated with culture media without FBS for

3 h. Then, the medium was removed and cells were rinsed once with medium without FBS. Subsequently, cells were treated with medium + tPA (Abcam, UK, #ab92715; 2.5 µg/ml [39.73 nmol]) or medium + tPA (2.5 µg/ml [39.73 nmol]; Abcam, UK, #ab92715) + plasminogen (10 µg/mL [110.41 nM] / 50 µg/mL [552.07 nM] / 100 µg/mL [1104.14 nM]; R&D Systems, UK, #1939-SE) for 15 min. After that, culture dishes were placed on ice, the medium was removed, the dishes were rinsed twice with ice-cold phosphate buffer saline, pH 7.4 (PBS) and finally, RIPA buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate [SDS], 1 mM ethylenediaminetetraacetic acid [EDTA] solution, 10 mM NaF, 1 mM Orthovanadate, 1x Inhibitor Cocktail [cOmplete®, Roche, UK]) + 1× protease inhibitors (Halt®, Thermo, UK) was incorporated. The cells from the dishes were collected using a cell scraper and homogenised with a 25 gauge, 5/8th needle and a syringe at 0°C. The resulting protein sample was analysed by Western blot as described in further sections.

For imaging experiments in cell lines, coverslips were flame-sterilised and put on culture plates. Neuro-2A (passage number <10) cells were then cultured on them using culture medium + 5% FBS and incubated (37°C, 5% CO_2) until 70-80% confluence. Once appropriate confluence is reached, cells were transfected with the appropriate plasmid using polyethyleneimine (PEI) MW25000 (Polysciences, USA) as transfection agent at a 3:1 ratio PEI:DNA. After 1-3 days of expression, coverslips were fixed with 4% paraformaldehyde (PFA) in 1× PBS for 5 min. Coverslips were next washed three times for 15 min with 1× PBS. Then unspecific interactions of the antibodies were blocked with blocking buffer: 0.1% Triton X-100, 10% FBS in 1× PBS for 1 h at RT. Samples were then probed with the primary antibodies in blocking buffer at 4°C overnight with gentle agitation. Antibodies: EphA4 C-term (mouse, Invitrogen, USA; 1:500; #37-1600); GFP-AF488 (rabbit, Invitrogen, USA; 1:500; #A-21311). Next day, coverslips were washed (0.1% Triton X-100 in 1× PBS for 15 min, 3 times at RT) before applying the corresponding secondary fluorescent antibodies (Alexa Fluor 555 or FITC) (Invitrogen, USA; 1:1000) diluted in blocking buffer for 1 h at RT in darkness. Images were taken with Zeiss LSM5 Exciter and processed with Zen 2009 software (Zeiss Ltd., Germany). Negative controls for secondary antibodies were

performed following the same steps except for the addition of the primary antibody.

LENTIVIRAL DELIVERY SYSTEM

A lentiviral delivery system was used to achieve the overexpression of EphA4 or its variants (detailed in “generation of inserts for Epha4 constructs” section) in the mouse brain. Plasmids aiming the production of lentiviruses were constructed by subcloning the sequence of EphA4 (or its mutated and truncated variants) upstream of a sequence coding eGFP and separated from it by a self-cleaving peptide (P2A), which allows the subsequent and independent expression of both proteins. EphA4 receptor and its variants for overexpression were inserted into an LV-pUltra plasmid empty backbone (Addgene plasmid catalogue #24129) using NheI (restriction site at the 5′ terminus) and the SacI (restriction site at the 3′ terminus) as restriction enzymes. LV-pUltra plasmid uses an UbC (ubiquitin C) promoter.

For the lentivirus production, HEK293T cells were grown in DMEM media (Sigma-Aldrich, USA D6046) supplemented with 1% penicillin-streptomycin and 10% v/v FBS until reaching a confluence of 70-80%. Then, cells were transfected with the addressing and packaging plasmids (pCMV delta R8.2, Addgene plasmid #12263; pCMV-VSV-G Addgene plasmid #8454). After 48 hours of expression, virus extraction was performed. After shaking the cell culture plates to detach the viruses, the supernatant was collected and spun down (7 min, 1.500 rpm) to pellet the detached cells. Then supernatant is filtered with a wide pore and low protein affinity filter (Roche Millipore Millex®-HV syringe driven filter unit, low protein binding Durapore [PVDF] 0.45µm pore; Roche, UK). Then $MgCl_2$ 10 mM of final concentration was added as a DNase cofactor. RNase-free DNase I solution (Thermo, USA) was added at a 0.3 unit/mL concentration to the media. Then, the mix was incubated at 37°C for 15 min and ultracentrifuged at 26.000 rpm (~115.000 × 1 g) for 90 min at 4°C with slow deceleration (Beckman coulter Optima LE-80K Ultracentrifuge with SW40 T_I rotor). Then, media leftovers were

discarded and the pellet was resuspended in ice-cold PBS.

To assess viral functional infectivity, a focus forming assay (FFA) was used. Different volumes of viral particles solution for each of the plasmids were diluted in 2 mL of cell culture media and then this media was applied to Neuro-2A cell line monolayer in a 6 well plate at 100% confluence. After 48 h of overexpression, foci of infection were counted and infective units were calculated according to their respective dilution. As our lentiviral particles are designed to co-express eGFP after a P2A motif, infective units were characterised as fluorescent-green single cells or groups of adjacent co-infected cell focal points. Finally, the viral particle stocks were diluted accordingly to achieve equal functional infectivity.

These viruses were created with the help of Dr Mariusz Mucha.

STEREOTAXIC SURGERY FOR INTRACRANIAL LENTIVIRAL PARTICLE INJECTION UNDER ISOFLURANE ANAESTHESIA

Adult mice of 8 to 9 weeks of age were firstly anaesthetised with inhaled isoflurane (5%) and oxygen (4 L/min) in an induction chamber. Then, mice were positioned in a mouse stereotaxic frame (Kopf Instruments, Germany) and anaesthesia was changed to a constant lower rate of isoflurane (2.5%) and oxygen (1 L/min) of through a facemask. After that, a midline incision on the skin was made and burr holes were carefully drilled in the desired Cartesian coordinates. The central amygdala (CeA) was targeted bilaterally at two injection sites in each hemisphere (from Bregma: −1.5 mm anteroposterior [AP], ±3.0 mm mediolateral [ML] and two virus delivery sites at −4.45 and -4.3 mm dorsoventral [DV]). Injection volumes were 500 nL in each injection delivery site. Viruses were injected at 10 nL/min using a metal gauge needle attached to a NanoFil© 100 µL syringe (World Precision Instruments, USA). The needle was positioned into the first target site and remained there for 10 min before the beginning of the injection. After the injection, the needle stayed in the same position for 10 min more before it was moved to the second delivery position. After the second injection was

performed, the needle remained in the same position for another 10 min before it was completely withdrawn. The midline incision was closed with Vetbond® surgical glue (3M, USA). Mice were given an initial dose of buprenorphine (0.15 mg/Kg) straight after the incision was closed and carprofen (5 mg/Kg) on the first and second day after the surgery as analgesic and anti-inflammatory drug respectively. Body weights and pain signs were assessed during, and after surgeries. Then, mice recovered and the viral particles were allowed to express for four weeks before behavioural experiments took place. All injection sites were verified immunohistochemically by assessing the protein levels of the co-expressed eGFP with an antibody against GFP protein: GFP-AF488 (rabbit, Invitrogen, USA; 1:500; #A-21311).

These surgeries were carried out with the help of Jaison Kolenchery.

RESTRAINT STRESS

Upon arrival, C57BL/6J mice were kept undisturbed for at least one week in their home cages to become familiar with the environment. Restraint stress was performed during the light period of the circadian cycle. Mice were held in falcon tube restrainers, secured at the tail end of the restrainer with a cap, within their home cage for the required period of stress. Control animals were left undisturbed, and stressed animals were subjected to single restraint stress of 15 min, 1 h or 6 h in a well-lit area of a separate room, depending on the experiment of interest. Another time, namely 2 h, was also assayed and yielded similar results as 1 h.

BEHAVIOURAL EXPERIMENTS

All mice were allowed to habituate for at least 1 h before undergoing any of the

behavioural tests. A battery of behavioural tests was performed in the following order: first, elevated plus (EPM) maze, then light/dark box (LDB) and finally open field test (OFT) (Schematic representations on Image 6), allowing the animals to rest for at least 1 h in between tests. All experiments were performed during the light cycle between 09:00 and 14:00 hours maintaining the hour of the day consistently for each of the tests. Behavioural testing was not performed on days when, as a part of routine animal husbandry, home cages are scheduled for change.

The EPM, LDB and OFT were used to measure anxiety-like behaviours in the experimental mice. Following each test, faecal boli and urine were removed from the equipment, and the equipment was wiped clean with Virkon® solution followed by 70% industrial methylated spirit (IMS) solution to eliminate olfactory cueing influencing behaviours. A camera connected to a video recorder was positioned above the arena. Each test was analysed in a blinded and automated manner using the computer software ANY-maze (Stoelting Co., Dublin; http://www.anymaze.co.uk).

Elevated plus maze

The elevated plus maze was made out of non-toxic acrylic plastic. It consisted of an elevated platform with four 10 × 50 cm arms in a cross-shape configuration. Two of them are opaquely-walled closed arms located opposite to each other and the other two are non-walled open arms. An open square centre of 10 cm on each side connected the four arms. The apparatus was elevated 50 cm above the floor and was lit by two white-light lamps (60 W each) placed above the end of the open arm. Experimental animals were placed on the centre area between the plus maze arms and were recorded when exploring the plus maze for 5 min. The amount of time spent in the closed and open arm was taken as a measure of anxiety-like behaviours. Other parameters, such as the number of entries in the open arm, total number of entries or the latency to the first entry were also measured. Immobility was detected with ANY-maze detection software with min. freeze duration of 200 ms and thresholds for on and off of 30 and 40 respectively.

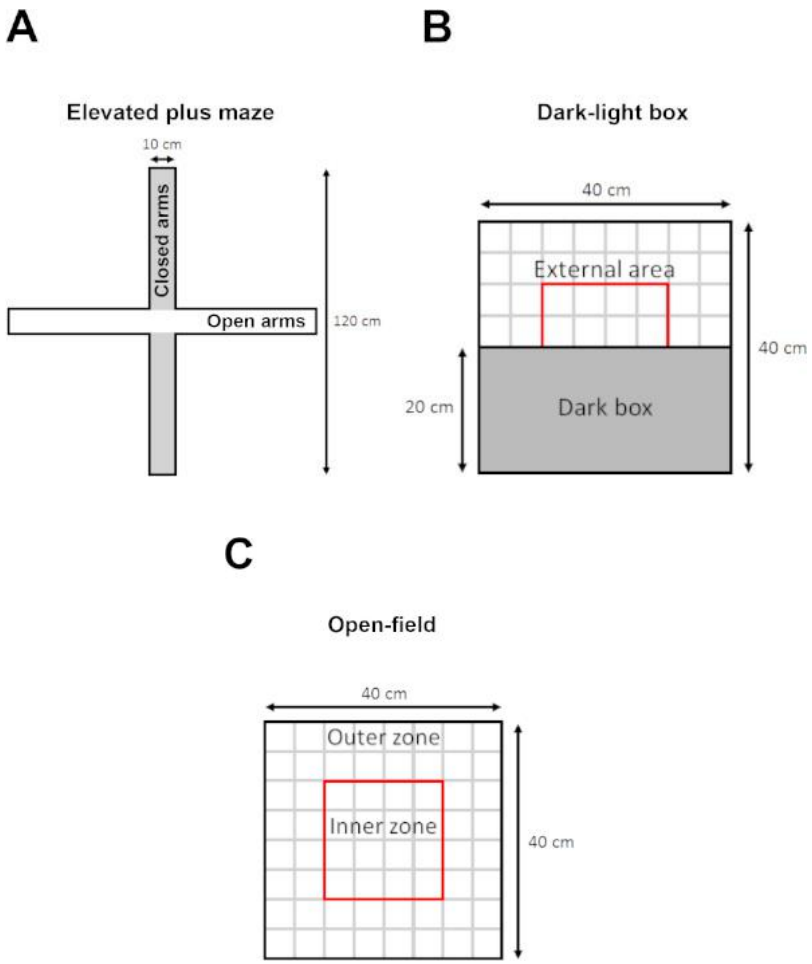


Image 6. Top-view scheme of the apparatus used for the elevated plus maze test (A), the dark-light box test (B) and the open-field test (C).

Light/dark box

The box was made of transparent acrylic with internal measurements 40×40×40cm. The box was divided into two equal-measurement compartments; a dark chamber of 20×40 cm and a light chamber of the same size that was lit with a bright white light. A small opening (semicircle of 7 cm diameter) was made on the centre of the separator wall between the chambers at the ground level which allowed the mice to move between the two compartments freely. The test started after the mouse was placed onto the light area facing the hole that separates both compartments. The animal was allowed to freely move between chambers for 5 min. Following the completion of the task, the mouse was returned to its home cage. Activity measures were evaluated (e.g. mean speed [cm/s] and

distance travelled [cm] in the light compartment). The number of entries into the light compartment and time spent [s] in the light compartment were taken as anxiety parameters.

Open field

The open field was made of non-toxic transparent acrylic, with internal measurements 40×40×40 cm. Squares of 5×5 cm were drawn on the bottom surface of the arena. White light was evenly distributed across the arena during the test. Mice were placed onto the centre of the arena, and the testing was recorded with a camera placed above the open field. Each animal was allowed to freely explore the open field for 10 min. After that, the mouse was returned to its home cage. For the analysis of the data, a central 10×10 cm square area was defined and called the “central zone”. The rest of the open field outside the central zone was defined as the “outer zone”. Anxiety parameters measured included the number of entries, and time spent [s] in the central zone of the arena. Activity parameters measured were mean speed [cm/s] and distance travelled [cm] in the whole area of the arena.

EPHA4 CLEAVAGE IN BRAIN HOMOGENATES

After hippocampi and amygdalae were extracted as described in the “Western blot” section, samples were homogenised in a cleavage buffer (0.1 M Tris, 0.1% Triton X-100, pH 7.4), containing phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_4). The homogenate (100 µL) was incubated with active tPA protein alone (2.5 µg/mL [39.73 nM]; Abcam, UK; #ab92715); tPA (2.5 µg/mL [39.73 nM]) + plasminogen (10 µg/mL [110.41 nM] / 50 µg/mL [552.07 nM] / 100 µg/mL [1104.14 nM]; R&D Systems, UK) or without any of these proteases for 15 min. After that the homogenate was placed on ice and proteases inhibitors (cOmplete®, Roche; Halt®, Thermo Fisher) were added to stop the reaction. The

samples were then analysed by SDS-PAGE as described in the “Western blotting” section (below).

EPHA4-FC CLEAVAGE IN VITRO

Recombinant Mouse EphA4-Fc Chimeric Protein (R&D Systems, UK; #641-A4; 1 mg/ml) was incubated with tPA (1 mg/ml; Abcam; #ab92715), tPA (1 mg/ml) + plasminogen (1.5 / 10 / 20 mg/mL; R&D, UK, #1939-SE) or without proteases in a HEPES-Tween® buffer (0.1 M HEPES, 0.01% Tween®, pH 7.4) as previously described (Quagraine et al., 2005) for 15 min. After the reaction was completed, the samples were placed on ice and protease inhibitors (cOmplete®; Roche, UK) were added to stop the enzymatic reaction. The samples were then analysed by SDS-PAGE as described in the “Western blot” section.

WESTERN BLOT

Samples were normally homogenised in, 0.1% Triton X-100, 0.1 M Tris pH 7.4, containing phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_4) and protease inhibitors (cOmplete®, Roche) and the protein concentration was adjusted to 2 mg/mL using the Bradford method (Pierce™, ThermoFisher Scientific, USA). Samples were then reduced using DTT, denatured (100°C for 5 min) and subjected to SDS-PAGE electrophoresis and transferred onto a nitrocellulose membrane (Thermo Scientific, USA). After blocking (5% skim milk in TBS-T [0.01 % Tween®20 in 1× Tris-buffered saline]) for 1 h at RT, the membranes were probed with the following primary antibodies at 4°C overnight (host; brand; concentration; catalogue number): EphA4 C-terminus (mouse; Invitrogen, USA; 1:1000; #37-1600) (this was also used in blots to recognise the C-terminus of EphA4); EphA4 N-terminus (goat; R&D Systems, UK; 1:500; #AF-641); EphA4 polyclonal (rabbit; Proteintech, UK; 1:1000; #21875); gephyrin (rabbit; Abcam,

UK; 1:1000; #ab136343); gephyrin-biotin (mouse; Synaptic Systems, Germany; 1:1000; #147-111BT); GFP (mouse; Roche, UK; 1:1000; #11814460001); human IgG-Fc (donkey; Jackson Immunoresearch; 1:10000; #009-000-008); thrombin (rabbit; Bioss, USA; 1:1000; #bs-0828R); tPA (rabbit; Molecular Innovations, USA; 1:1000; #ASHTPA-GF-HT). The membranes were then washed in PBS-T (3 × 5 min) before incubation with the HRP-conjugated secondary antibody (Vector Labs, UK; 1:1000) relevant to the species used in the primary antibody for 1 h at RT. After washing with PBS-T (3 × 15 min), the membrane was developed using Amersham ECL detection reagent (GE Healthcare, USA). To normalise the results, all membranes were stripped using a Restore PLUS buffer (Thermofisher, UK), blocked, washed as above described and re-blotted using mouse anti-β-actin antibody (mouse; Sigma-Aldrich, USA; 1:2500; #A5441) for 1 h at RT. Again, the membranes were washed, incubated and developed as described before. To quantify the results, the optical density of the bands was analysed using Image Lab software (Bio-Rad, UK) and normalised to their respective β-actin controls or total EphA4 protein in the case of quantifying cleaved forms of the receptor.

CO-IMMUNOPRECIPITATION

Unless otherwise mentioned the processes were carried out at low temperature (0 - 4°C).

For EphA4 immunoprecipitation, frozen tissue (-80°C) was thawed and homogenised with the following buffer (modified from Buchert et al., 1999; Calò et al., 2005): 50 mM Hepes-NaOH, 1% v/v Triton X-100, 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 10% glycerol, pH 7.4, containing phosphatase inhibitors (10 mM NaF, 1mM Na_3VO_4) and protease inhibitors (cOmplete®, Roche) and 5 mg (10 mg/mL) of total protein were used for each immunoprecipitation as quantified by the Bradford method (Pierce™, ThermoFisher Scientific, USA). Homogenates were incubated with 2 µg (1:250) of either an irrelevant IgG (Cell Signalling Technology, USA; 1:1000; #2729) or EphA4 polyclonal antibody

(rabbit; Proteintech, UK; 1:1000; #21875). Then, 50 µL of beads (Protein G Sepharose 4 Fast Flow, GE Healthcare, USA) were pre-washed and equilibrated (3x for 5 min with homogenising buffer 50:50; 12,000 × g, 30 s to pellet down the beads) and added to the sample mix. This mix was incubated overnight at 4ºC with gentle agitation. Next day, the sample was centrifuged at 12,000 g, for 30 s to pellet down the beads and the supernatant was stored for further analysis. After that, pelleted beads were gently resuspended in homogenising buffer 50:50 and left for 5 min with gentle agitation at 4ºC to wash. The washing process was repeated seven times. The final pellet was resuspended in Laemlli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris-HCl, pH 6.8) 50:50 and boiled for 5 min to elute proteins from the beads. Beads were pelleted down and the supernatant was analysed with SDS-PAGE as described in the “Western blot” section.

For Gephyrin immunoprecipitation, Neuro-2A cell line overexpressing gephyrin (Addgene, USA; catalogue #68816) and/or EphA4 variants (described in “Lentiviral delivery system” section) were homogenised in: TBS pH 7.4 and 1% NP-40 containing phosphatase inhibitors (10mM NaF, 1mM Na_3VO_4) and protease inhibitors (cOmplete®, Roche). An amount of 10 mg (10 mg/mL) of total protein was used for each immunoprecipitation quantified by the Bradford method (Pierce™, ThermoFisher Scientific, USA). Streptavidin beads (Pierce™, ThermoFisher Scientific, USA) were pre-cleared with 5% BSA in homogenising buffer. Homogenates were incubated with 5% BSA and 1 µg (1:400) of either an irrelevant IgG-biotin as isotype control (mouse; Abcam, UK; 1:1000; #ab131367) or biotinylated anti-gephyrin antibody (mouse; Synaptic Systems, Germany; 1:1000; #147-111BT). Then, 50 µL of streptavidin-coated beads (Pierce™, ThermoFisher Scientific, USA) previously pre-washed and equilibrated (with 3x for 5 min with homogenising buffer 50:50; at 12,000 × g, for 30 s to pellet down the beads) were added to the samples and the sample mix was incubated overnight at 4ºC with gentle agitation. Next day, the sample was centrifuged at 12,000 g, for 30 s to pellet down the beads and the supernatant was stored for further analysis. After that, pelleted beads were gently resuspended in washing buffer: 4× TBS, 2-5% NP-40 containing phosphatase inhibitors (10mM NaF, 1mM Na_3VO_4) and protease inhibitors (cOmplete®, Roche). Then they were left for 5

min with gentle agitation at 4ºC to wash. The washing process was repeated 10 times. The final pellet was resuspended in Laemlli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris-HCl, pH 6.8 approx.) 50:50 and boiled for 5 min to elute proteins from the beads. Beads were pelleted down and the supernatant was analysed with SDS-PAGE as described in the “Western blot” section.

DENDRITIC SPINE MORPHOLOGY EXPERIMENTS

These experiments were performed with the help of Marta Pyskaty our collaborators in Prof Jakub Wlodarczyk’s laboratory (Nencki institute, Poland).

Transfection of primary amygdala cultures

For morphological analysis of dendritic spines, cells were transfected with Lipofectamine 2000 Reagent (Invitrogen, USA) according to the manufacturer’s protocol at 7-9 day in vitro (DIV) with plasmid carrying RFP under β-actin promoter together with the lentiviral plasmids carrying the receptor variants (empty vector as a control, wtEphA4, crEphA4 or tEphA4). Imaging experiments were performed at 14-16 DIV. For studies of dendrite morphogenesis, amygdala neurons were transfected on DIV 2 and dendritic arbours were analysed at DIV 7.

Imaging

Images were acquired using the ZEISS LSM 780 confocal microscope with a PL Apo 40x/1.4 NA oil immersion objective using a 488 nm and 561 nm diode-pumped solid-state lasers at 10% transmission with 1024×1024 pixels of resolution. A series of z-stacks were acquired for each cell at 0.4 µm steps, with additional digital zoom that results in a lateral resolution of 0.07 µm per pixel.

Analysis of dendritic morphology

Morphometric analyses of dendrites were performed using ImageJ with NeuronJ software (Meijering et al., 2004) and the Sholl plugin (Perycz et al., 2011). The axons were excluded during marking tracings for Sholl analysis. For each experimental condition, 20 cells were analysed.

Analysis of dendritic spines morphology

The images of dendrites were semi-automatically analysed using the custom-written software, SpineMagick (patent no. WO/2013/021001). The dendritic spine shape parameters were determined, i.e. length, head width and the length-to-width ratio (the length divided by the head width), which reflects the spine shape. The head width was defined as the diameter of the largest spine section while the bottom part of the spine (1/3 of the spine length adjacent to the dendrite) was excluded. Only the spines protruding in the transverse direction (contained in the single image plane) that could be clearly distinguished were selected. Only the spines belonging to the secondary dendrite were chosen. The motivation for this restriction is to eliminate possible systematic differences in spine morphologies that are due to the location of spines on dendrites with different ranks. All objects (protrusions) with an area smaller than 0.2 μm were discarded owing to the limitation in the resolution of typical confocal setup. For each experimental condition, the total number of spines analysed was found 1131 (13 cells) for wtEphA4, 1088 (14 cells) for crEphA4, 1173 (15 cells) for tEphA4 and 1241 (9 cells) for the control vector.

AMINO TERMINAL ORIENTED MASS SPECTROMETRY OF THE SUBSTRATE

These experiments were carried out by Dr Małgorzata Bajor.

The cleavage reaction of EphA4-Fc was performed with EphA4-Fc (R&D, #641-A4) or plasminogen (R&D #1939-SE) or rt-tPA (alteplase) lyophilized proteins. They were reconstituted in 50 mM HEPES buffer, pH 7.4. To remove the interfering compounds contained in the protein solution, 50 mM HEPES buffer, pH 7.4 was exchanged three times using Millipore Amicon Ultra devices with cut off 3000 Da (#UFC500324) at 10,000 x g for 10 minutes at 4°C. The filtrates from the three steps were pooled and the total amount of protein in all samples was determined by UV absorption A_{280}. EphA4-Fc (10 µg/ml) was incubated with either tPA (10 µg/ml) or tPA (10 µg/ml) + plasminogen (1.5/10/20 µg/ml) or without proteases in a 50 mM HEPES buffer, pH 7.4 for 15 min at 37°C. tPA + plasminogen mix was preincubated for 15 min at 37°C before mixing with EphA4-Fc. After cleavage reaction samples were subjected to the ATOMS analysis.

To this aim, each sample was added one volume of 8.0 M GuHCl was added to denature all proteins. the pH of the samples was adjusted to 7.0 and DTT was added (to final concentration 5 mM) to reduce disulphide bridges. Samples were incubated at 65^{o}C for 1 h. Then, iodoacetamide was added (to final concentration of 15 mM) followed by DTT (to final concentration 30 mM) and samples were incubated at room temperature in the dark for 30 min and at room temperature for 30 min, respectively. Then samples were labelled with either heavy formaldehyde (formaldehyde containing the isotope 13C and deuterium ($13C_2D_2O$ from Cambridge Isotope Laboratories, Inc.) to a final concentration of 60 mM or light formaldehyde (regular formaldehyde ($12C_1H_2O$ from Sigma) to a final concentration of 60 mM. Next, $NaBH_3CN$ to a final concentration of 30 mM was added to all samples for the reduction of imines. Samples were vortexed and the pH was adjusted to 6-7 followed by overnight incubation at 37^{o}C. To quench the excess formaldehyde ammonium bicarbonate (final concentration, 100 mM) was added and pH was adjusted once again to 6-7. Samples were incubated at 37^{o}C for 4 h. Then, samples were combined and precipitated with cold acetone/methanol. After precipitation dried protein pellets were resuspended with 60 µL of 50 mM HEPES, pH 8.0 and 1 µg of mass spectrometry-grade trypsin was added to each sample followed by overnight incubation at 37^{o}C. Then samples were subjected to mass spectrometry analysis.

For the mass spectrometry analysis, the resulting peptide mixtures were applied to an RP-18 pre-column (Waters, Milford, MA, USA) using water that contained 0.1% formic acid as a mobile phase and then transferred to the RP-18 column (75 μM internal diameter; Waters) of the nanoACQUITY UPLC system (Waters) using an ACN gradient (0–30% ACN in 45 min) in the presence of 0.1% formic acid at a flow rate of 250 nL/min. The column outlet was coupled directly to the ion source of an LTQ Orbitrap Velos mass spectrometer (Thermo Electron, San Jose, CA, USA) working in the regime of data-dependent MS to MS/MS switch. A blank run that ensured the absence of cross-contamination from previous samples preceded each analysis. The obtained mass spectra were preprocessed with Mascot Distiller software (v.2.2.1, Matrix Science) and searched against the EphA4-Fc protein sequence using on-site-licensed-processor-engine MASCOT software (Mascot Server v. 2.2.03, Mascot Daemon v. 2.2.2, Matrix Science). Fixed modification for carboxymethylation of cysteines and variable modification of methionine oxidation were applied to all searches. Enzyme specificity was semi-Arg-C, precursor and fragment ion mass tolerance was 0.8 Da, peptide mass tolerance was 40 ppm, and a maximum of three miscleavages were allowed. The variable modifications lysine and N-terminal dimethylation with heavy formaldehyde (34.0631 Da) and with light formaldehyde (28.0311 Da) was conducted for all samples. Protein MASCOT scores above expectation values of 0.05 were required for a hit. All liquid chromatography-MS-MS/MS measurements were performed in the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics PAS at the Polish Academy of Sciences, Warsaw (Poland).

STATISTICAL ANALYSIS

The statistical values are expressed as mean ± standard error of the mean (SEM) unless otherwise noted. P values for the tests used are typically noted on the corresponding graphs. The statistical analyses were performed using GraphPad Prism 7 Software (GraphPad Software, USA).

Datasets were firstly analysed with Shapiro-Wilk test as an initial test to compare means (confidence interval of 95%). Levene test was used as an initial test to compare variances (confidence interval of 95%). Student's t-test or ANOVA were used to compare the differences between two or more groups (respectively) with equal variances and means (confidence interval of 95%). Bonferroni's Multiple Comparison Test was performed as a post-hoc test for ANOVA significant results to compare all sets of data.

When means and variances did not allow for parametric analysis of the data (assessed by Shapiro-Wilk test), the Mann Whitney test or Kruskall-Wallis test were performed to compare differences between two or more groups respectively (confidence interval of 95%). Dunn's Multiple Comparison Test was used as a post-hoc test (confidence interval of 95%).

Brown-Forsythe test was used to compare differences between groups when equal means with different variances were compared. Games-Howell was used as a post-hoc test for significant results (confidence interval of 95%).

PART 2: TISSUE PLASMINOGEN ACTIVATOR / PLASMIN CASCADE IN THE MOUSE BRAIN: LOCATION AND FUNCTIONAL RELEVANCE OF TISSUE PLASMINOGEN ACTIVATOR / PLASMIN CLEAVAGE OF EPHA4 IN THE MOUSE BRAIN

CHAPTER 3: LOCATION OF THE COMPONENTS OF THE CASCADE AND CLEAVAGE OF EPHA4

INTRODUCTION

In this chapter, I investigate the existence of a tPA / plasmin(ogen) / EphA4 system in the mouse brain based on the expression pattern of these enzymes. Former members of our lab found that Eph-receptor tyrosine kinases are subject to cleavage by extracellular serine proteases and, specifically, they found that tPA/plasmin system can cleave EphA4 in the mouse brain. Therefore, in this chapter, I will also explore the presence of such event in specific areas of the brain related to stress and anxiety.

Eph and ephrins as a target for proteases

Eph receptors and ephrins belong to the family of receptor tyrosine kinases (RTKs). Many of these RTKs are subjects of cleavage by a variety of proteases (subsequently producing or facilitating autocrine or paracrine molecular signalling) and Eph and ephrins are not an exception (Nievergall, Lackmann and Janes, 2012). There are various groups of proteases able to cleave and interact with Eph/ephrin family. These include A disintegrin and metalloproteases (ADAMs), matrix-metalloproteases (MMPs) and intramembranous cleaving proteases (iCLiPs), such as γ-secretase (Atapattu, Lackmann and Janes, 2014).

As far as serine proteases are concerned, there are some examples of interactions with this family of receptors as well. Perhaps neuropsin is the clearest example of a serine protease that is able to modify stress behaviours as a consequence of Eph receptor cleavage. Neuropsin is a secreted serine protease that belongs to the family of kallikrein endopeptidase 8-like (KLK8) enzymes and it is highly expressed in the limbic system (Chen et al., 1995). This protease's enzymatic activity has been shown to be important for synaptic plasticity, by enabling the early phase of LTP. The effects of neuropsin on synapses are speculated to be related to mammalian working memory and consequently integration of learning and memory (Attwood et al., 2011; Shiosaka and Ishikawa, 2011; Attwood, 2016). The work of Attwood et al. is particularly relevant because

it puts forward the idea that interactions between serine proteases and Eph receptors might be necessary for the regulation of anxiety and fear development (Attwood et al., 2011).

Apart from neuropsin, there are other serine proteases reported to cleave Eph/ephrin family of receptors. For instance, rhomboids are intramembranous serine proteases that have been reported to be present in virtually all eukaryotic species. A research group tried to find potential substrates for rhomboid-related protein 2 (RHBDL2). Based on a sequence motif susceptible to cleavage, this group developed a structural analysis of several Type-I membrane proteins. Among all ephrin-Bs detected by this analysis, only ephrin-B3 was efficiently cleaved by RHBDL2 as evidenced by the overexpression of mammalian Rhomboid-1 or Rhomboid-2 along with ephrin-B3 (Atapattu, Lackmann and Janes, 2014).

Apart from these proteases, specific interactions between EphA4 and different proteases in the nervous system have been reported and they will be described in the following section.

EphA4 as a target for proteases

ADAM19 interacts with EphA4 in the peripheral nervous system, in particular, in the neuromuscular junction (NMJ). The interaction blocks the internalisation of the EphA4-ephrin-A5 complex in EphA4-expressing motor neurons in a way that is not dependant on the proteolytic activity (Yumoto et al., 2008). This process prevents repulsion of the axon terminal at the NMJ.

In a broader spectrum type of experiment, another group created a cleavage-resistant isoform of EphA4 (Gatto et al., 2014). They showed that cleavage of EphA4 promotes cell-cell and growth cone-cell detachment in vitro. Moreover, mice in which EphA4 cleavage is genetically abolished (knock-in mice bearing the cleavage resistant isoform) have motor axon guidance defects during development. However, when EphA4 cleavage resistant isoform is expressed

locally, it has the same function as the wild-type EphA4 in redirecting motor axons in limbs. This suggests a valuable role of EphA4 cleavage in axon guidance outside of the brain. This group also found that blocking EphA4 cleavage increases expression of full-length EphA4 in limb mesenchyme.

The only study to date identifying EphA4 as a direct protease target was developed by Inoue et al. (2009). They identified EphA4 as a substrate of gamma-secretase, a multimeric intramembranous protease complex that includes presenilin 1 (PS1) and is markedly dysfunctional in cases of early-onset familial Alzheimer's disease (AD). This research group describes the interaction of EphA4 and γ-secretase in synaptic lipid raft membranes, a region of the cell surface enriched in γ-secretase. In this area, there is an apparent increase in the formation of an intracellular EphA4-C-terminal fragment (CTF). Additionally, the inhibition of γ-secretase, or its downregulation through siRNA, reduces the production of soluble EphA4-CTF. Additional data shows that EphA4-CTF cleavage by γ-secretase activates the Rac signalling pathway, which subsequently modifies dendritic spines formation and maturation. Notably, a typical PS1 mutation of familial AD presents a reduction in EphA4- CTF cleavage and inhibits dendritic spine formation (Inoue et al., 2009). In addition, AD patients' occipital lobe express lower soluble EphA4-CTF levels and Rac1 activity (Matsui et al., 2012), which suggests that γ-secretase might be playing a role in EphA4 cleavage. Interestingly, in line with this discovery, another study in post-mortem hippocampal tissue from patients with incipient AD showed a reduction in EphB2 and EphA4 receptor levels (Simón et al., 2009).

Altogether, these data show that EphA4 can be cleaved by serine proteases and that the cleavage of different Eph receptors by proteases may result in different outcomes depending on the parts of the CNS, the protease involved and the cleavage site targeted by them.

Location of the components of the cascade: tPA and plasmin location in the adult mouse brain

As mentioned in Chapter 1, all elements of the tPA/plasmin system are expressed in the CNS. Highly plastic regions in the adult brain that show activity-dependent structural plasticity (e.g. amygdala, hippocampus and cerebellum) express tPA (Sappino et al., 1993; Davies et al., 1998; Salles and Strickland, 2002; Pawlak et al., 2003). And, although tPA can be found throughout the brain, its enzymatic activity is restricted to discrete brain regions, including the above mentioned highly plastic structures (i.e. hippocampal mossy fibres, MeA and CeA, BNST, hypothalamus, and cerebellum). It has been hypothesised that the lack of activity in other areas might be due to the presence of tPA or plasmin inhibitors (Sappino et al., 1993; Salles and Strickland, 2002; Pawlak et al., 2003; Matys et al., 2004).

With regard to the amygdala, the expression pattern of tPA measured by immunoreactivity is marked in the CeA and MeA amygdala, but it is almost absent in the basolateral amygdala. Acute restraint stress (RS) paradigm produces an increase in extracellular tPA activity, in the MeA and CeA, 30 min after the commencement of a restraint stress protocol. This effect continues until 18 hours after the end of the protocol. Interestingly, tPA activity seems to be elevated in the amygdala but not at the same level of that in the hippocampus. An additional piece of data indicates that stress does not modify the urokinase-type plasminogen activator (uPA); therefore the effects of these experiments are attributed to tPA (Pawlak et al., 2003). Thus, attenuation of tPA activity in the amygdala after restraint stress might be due to inhibition or clearance of the enzyme. Plasminogen activator inhibitor-1 (PAI-1) protein, but not neuroserpin, is upregulated in the areas showing reduced tPA activity, which suggests a possible mechanism for that activity inhibition. The upregulation of PAI-1 was absent in $tPA^{-/-}$ mice showing that tPA (either directly or indirectly) induces PAI-1 expression.

In the mouse hippocampus, tPA is more abundant in the projections of the mossy fibre pathway (DG granule neuron's unmyelinated axons that project to CA3 pyramidal layer), whereas in the fibres of the perforant path, in the Schaffer collaterals or in the neuronal cell bodies there is little or no expression (Davies et

al., 1998; Salles and Strickland, 2002). Another work reports changes in protein expression after excitotoxic injury. Enzymatically-active tPA protein levels are transiently induced in CA1 pyramidal neurons that survive KA excitotoxic injuries Within the mossy fibre pathway, KA injuries resulted in decreased tPA protein. However, mossy fibre tPA's activity also increases at 8 h and then decreases 24 h after injury, a time frame similar to the one observed in the amygdala. In a like manner, PAI-1 expression is upregulated after 24 h and it may be involved in the decrease of tPA activity in the HPC and amygdala (Salles and Strickland, 2002).

The cerebellum is another area where tPA is present and active during development and adulthood (Krystosek and Seeds, 1981; Soreq and Miskin, 1983; Sappino et al., 1993). In this area, tPA mRNA is expressed in the granule cell layer, but its presence does not seem to be complemented with high proteolytic activity. However, some overall tPA activity has been observed in the cerebellum with a zymographic technique (Sappino et al., 1993).

Plasminogen mRNA staining of the hippocampus produces a detectable signal in the developing mouse CA1 and the DG regions. Protein immunolabelling in the adult brain correlates with these findings. With this method, plasminogen can be detected in the cortex, the hippocampal pyramidal cells, the cerebellar granule cell layer and Purkinje cells (Basham and Seeds, 2001). Sappino et al. (Sappino et al., 1993) observed that, after excitotoxic injury, the mossy fibre pathway and the hilus of the dentate gyrus present a detectable increase in mRNA immunofluorescence. This pattern matched the one achieved using zymography. Interestingly, immunohistochemistry also indicates an increase of plasminogen protein levels in the mossy fibre pathway after excitotoxic injury (Salles and Strickland, 2002).

When looking at the cellular and sub-cellular level, in the adult brain, tPA is present in neurons and glial cells but it is also expressed in vascular endothelial cells throughout the brain parenchyma (Qian et al., 1993; Tsirka et al., 1995; Salles and Strickland, 2002; Shin, Kundel and Wells, 2004; Yepes and Lawrence, 2004a, 2004b; Melchor and Strickland, 2005). Upon depolarisation, tPA is released into the extracellular space (Gualandris et al., 1996; Parmer et al., 1997) and tPA mRNA is upregulated (Qian et al., 1993; Carroll et al., 1994). TPA has

been reported to be secreted from neuronal growth cones and to promote the outgrowth of neurites (Krystosek and Seeds, 1981; Wu et al., 2012). More precisely, fluorescent tPA chimaeras were found to be localised in dense-core granules (DCGs) in neuroendocrine cells (Lochner et al., 1998; Taraska et al., 2003) and in developing hippocampal neurons (Silverman et al., 2005). Also, they were localised in Golgi apparatus (DCGs site of formation), which points out the secretory nature of this enzyme (Lochner et al., 2006; Wu et al., 2012; An et al., 2014). In mature hippocampal neurons, tPA-eGFP produces punctate fluorescence throughout the neuronal processes in both axons and dendrites, but according to the authors, DCGs containing tPA chimaera are located preferentially in dendritic spines (Lochner et al., 2006).

Plasmin(ogen), in turn, is a secreted protein, but it can be also localised on the cell surface through the binding to lysine residues of the N-terminal part of various membrane proteins, such as annexin II. In most cases, this type of cell surface-bound plasmin is more protected from circulating inhibitors and therefore, it presents an increased activity (Myöhänen and Vaheri, 2004).

Plasminogen mRNA can be found in the CNS (Sappino et al., 1993). Using a more accurate method of mRNA measurement, Tsirka et al. (1997) found plasminogen mRNA in neurons of the hippocampal cell body layers. Moreover, plasminogen mRNA was found in the dendrites, indicating a local post-synaptic production and release. Immunohistochemical staining analysis has also detected the existence of plasminogen protein in the hippocampus (Basham and Seeds, 2001).

Location of the components of the cascade: EphA4 location in the mouse brain

EphA4 is largely expressed in the embryonic and adult nervous system in rats (Martone et al., 1997), mice (Canty et al., 2006; Yun et al., 2003; Tremblay et al., 2007; Bouvier et al., 2008) and humans (Hafner et al., 2004; Yamaguchi and

Pasquale, 2004).

In the developing mouse brain, EphA4 expression has been assessed by *in situ* hybridisation technique. mRNA is mostly expressed in rhombomeres 3 and 5 and the neural crest adjacent to rhombomere 5, although expression occurs in several other developing tissues and later in development until adulthood (Nieto et al., 1992; Mori et al., 1995).

More importantly, the EphA4 receptor has been studied in the adult mouse CNS. Research groups have performed *in situ* hybridisation and immunohistochemistry techniques to show the localisation of EphA4 in various of its regions and cell types. Adult expression includes highly-plastic areas, such as the subventricular zone (SVZ) of the lateral ventricles (which is the largest remaining germinal area of the mammalian brain after development) (Conover et al., 2000), cerebellum (Karam et al., 2002) and the amygdala (Deininger et al., 2008). However, its expression seems to be enriched in the neocortex and hippocampus (Moreno-Flores and Wandosell, 1999; Vanderhaeghen et al., 2000; Murai et al., 2003; Liebl et al., 2003; Fu et al., 2007; Tremblay et al., 2007; Deininger et al., 2008). Similarly to mice, EphA4 is also present in highly plastic areas of the macaque brain (Xiao et al., 2006).

Although a systematic assessment of the distribution of all Eph receptors at the subcellular level has not been performed, data about their localisation has been gathered. Literature describes these receptors to be mainly associated with dendritic spines and their post-synaptic densities (PSDs) (Martone et al., 1997; Torres, Firestein, et al., 1998; Buchert et al., 1999; Grunwald et al., 2001; Murai et al., 2003) and they have been linked with dendritic spines morphology and LTP (Henderson et al., 2001; Grunwald et al., 2001; Contractor et al., 2002; Murai et al., 2003; Grunwald et al., 2004; Armstrong et al., 2006; Inoue et al., 2009).

At the subcellular level, EphA4 immunoreactivity has been studied in rat and mouse hippocampus in great detail by Tremblay et al. and Bouvier et al. (Bouvier et al., 2008; Tremblay et al., 2007). In Tremblay's study, a precise pre-embedding immunogold protocol, combined with silver intensification, revealed a subcellular localisation on the plasma membrane of small unmyelinated axons, dendritic

spines, axon terminals and astrocytic processes (Tremblay et al., 2007). Conversely, neuronal cell bodies and dendritic branches were found to be immunonegative in those studies. Dendritic spines are only stained in the PSD and generally extended to the dendritic spine apparatus. In axon terminals, the identified regions only included a subset of the varicosities. The axonal presynaptic active zone was also labelled in some instances. Labelled neurites were mostly contacting neurites and in rare occasions, pre and postsynaptic ends were immunopositive (Tremblay et al., 2007). Likewise, a small number of astrocytic processes in the hippocampus (but not cell bodies or proximal processes) showed EphA4 immunoreactivity (Tremblay et al., 2007) and the expression of EphA4 in astrocytes has been also reported in spinal cord and retina (Goldsmith and Lemery, 2000; Fabes et al., 2006).

Notably, biochemical work is in agreement with the immunohistochemical one. The subcellular fractionation of mouse brain homogenates shows that EphA4 is related to synaptic vesicles, as well as to the synaptosomal membranes, which contain both pre and postsynaptic membranes. To be more precise, authors relate EphA4 with PSDs and presynaptic active zone (Bouvier et al., 2008). In this in-depth study of localisation of EphB2 and EphA4 in discrete compartments, Bouvier et al. (2008) used synaptosomes (an isolation of synaptic membranes), fractionation and pre-embedding immunohistochemistry (immunoperoxidase and immunogold with silver intensification) in conjunction with electron microscopy to show that EphA4 and EphB2 are enriched in microsomes (vesicle derived from endoplasmic reticulum) and synaptosomes (cell fraction isolated from synapses). In synaptosomes, they were more related to the membrane and the synaptic vesicle fractions. Both receptors were found presynaptically in the active zone fraction, but only EphA4 was associated with postsynaptic density fraction. Electron microscopy experiments in the cortex and the hippocampus showed EphA4 detection in axon terminals, and EphB2 in large dendritic shafts.

Central amygdala functional roles in anxiety

Observing the literature mentioned in the introductory chapter, it is plausible that the circuitry within the amygdala sub-nuclei and its long-range projections might have different functions in anxiety-like behaviours. Complex relations between the brain regions connected by these projections may underlie various features related to anxiety, such as memory, aversion or motivation (Tovote, Fadok and Lüthi, 2015). The high presence of tPA's activity in the CeA made this nucleus a central point on which to focus our research.

Recent real-time manipulations combining viral vector expression and optogenetic techniques have shed some light on the functions of these different neuronal sub-populations in anxiety-like behaviours. Optogenetics used in murine models of anxiety has aimed to unveil specific intra-amygdala circuits (Tye et al., 2011) and long-range projections (Felix-Ortiz et al., 2013). Interestingly, in these studies, it is shown that activation via soma of BLA neurons projecting to CeA resulted in increased anxiety-related behaviours, whereas more selective activation of axons from BLA excitatory neurons projecting into the CeL had anxiolytic effects. Thus, these results suggest a more complex connectivity of these areas than a single projection connection between BLA and CeA.

A further study suggested that this anxiolytic neuron population in the CeA is mainly composed of CeL amygdala protein kinase C delta positive neurons (CeL:PKCδ^{+}), a known group of cells that has also been implicated in conditioned fear response (Cai et al., 2014). This study also showed that this CeL:PKCδ^{+} population have effects opposite to CeL:PKCδ^{-}. A later study by Botta et al. (2015) showed that optical stimulation of PKCδ^{+} cells provoked increased anxiety-like behaviours (measured by elevated plus maze) and fear generalisation (measured by an increase in the ratio of freezing in reaction to CS−/CS+ stimuli). Furthermore, the increased tonic activity and behavioural changes in the EPM related to PKCδ^{+} neurons were correlated with decreased α_5-GABA$_A$R-mediated conductance (Wolff et al., 2014; Botta et al., 2015), which is in line with previous studies showing that modifications in GABAergic signalling in amygdala can affect anxiety-like behaviours (Tasan et al., 2011; Gilpin, Herman and Roberto, 2015). Moreover, α_5-GABA$_A$R mRNA reduction via shRNA

tools was sufficient to increase anxiety-like behaviour and fear generalisation (Wolff et al., 2014; Botta et al., 2015). Altogether, these results suggest different roles of discrete neuron sub-populations, probably GABAergic, within the central amygdala in anxiety-like behaviours.

Other specific cell-types determined by biomarkers have been described in the amygdala for conditioned fear behaviours (e.g. McCullough et al., 2016). Cell-type-specific examination of these kinds of pathologies will allow the identification of selective targets that can modulate distinct circuitries involved in different aspects of anxiety-related behaviours and associated disorders. In this project, I provide information about different new actors in the above-described complex circuitry of the amygdala that may offer unique approaches to fear- and anxiety-related disorders' therapies.

RESULTS

Tissue plasminogen activator, plasmin and EphA4 expression in the adult brain

The current study is based on previous works referenced in this chapter's introduction, in which, the cleavage of EphB2 and EphA4 receptors by serine proteases (neuropsin and tPA/plasmin system respectively) was demonstrated (Attwood et al., 2011; Attwood, 2016). The cleavage of EphB2 by neuropsin in the amygdala was shown to regulate levels of anxiety (Attwood et al., 2011), although the role of tPA/plasmin interaction with EphA4 remained unknown.

Preliminary studies showed that EphA4 undergoes proteolytic cleavage by a combination of tPA and plasmin in SHSY-5 neuroblastoma cell line (Attwood, 2016). Therefore, the appropriate immunohistochemistry staining was performed, as described in *Materials and Methods,* to examine whether all elements required for tPA/plasmin cleavage are present in the mouse brain.

Localisation of EphA4, tPA and plasminogen in the mouse hippocampus

EphA4

Hippocampus structure follows a pattern of laminar organisation and EphA4 shows a different expression throughout these anatomical layers. This pattern has been described in previous works from other labs and our lab (Attwood, 2016; Figure 1).

The strongest signal for EphA4 was found in the stratum oriens (SO), the stratum moleculare (SM) and the stratum radiatum (SR). These layers are mainly formed by processes from basal dendrites (dendrite close to the soma). Depending on the stratum, these basal dendrites have a different origin. Pyramidal neurons and septal/commissural fibres from the contralateral hippocampus send afferents and

form the SO, whereas axonal projections from the contralateral hippocampus, Schaffer collateral fibres projecting from the CA3 and apical dendrites of pyramidal cells of the CA1 form the SR (Figure 1). An example of these basal dendrites at higher magnifications can be found in Figure 3, which shows the stratum radiatum of CA3 region of the hippocampus (Figure 3 B). In contrast, EphA4 is less abundant in the stratum lacunosum-moleculare (SLM) and in the stratum moleculare (SM) of the dentate gyrus (Figure 1). SLM contains processes from Schaffer collaterals and perforant path fibres projecting onto distal, apical dendrites of pyramidal cells. SM is the stratum where commissural fibres from the contralateral dentate gyrus, perforant path processes and axonal inputs from the medial septum form synapses with the dendrites of the granule cells.

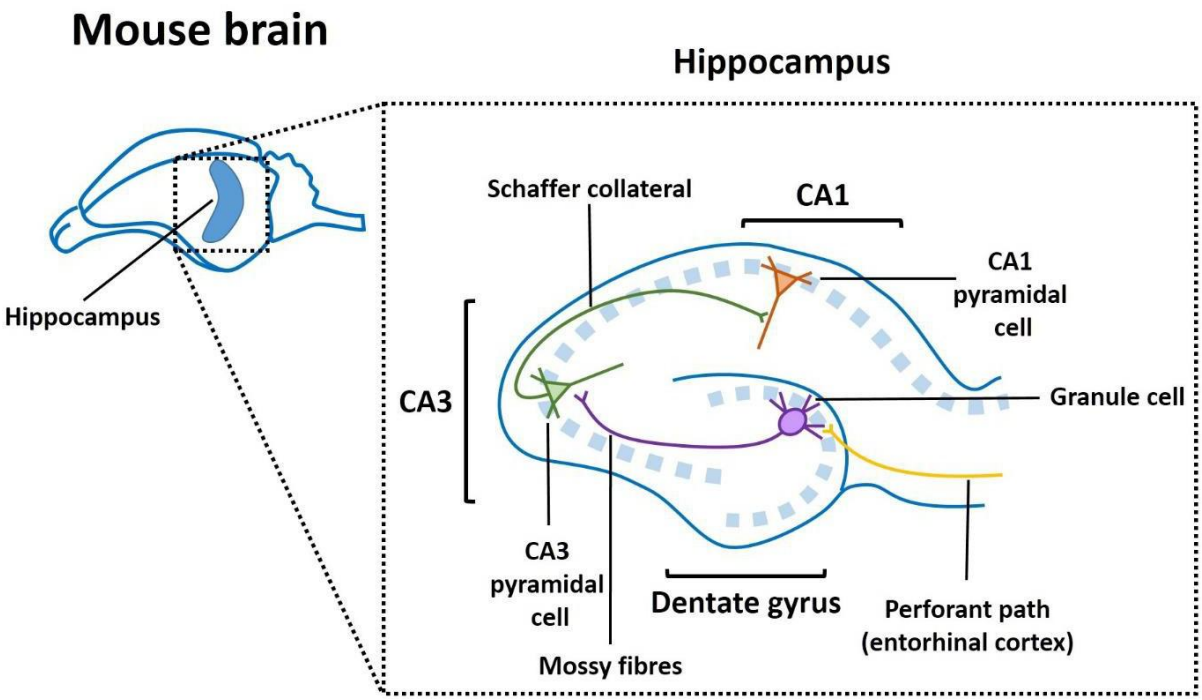


Image 7. Scheme of the main hippocampal pathways.

Higher magnification microscopy revealed high levels of EphA4 in cellular processes, which is in agreement with its higher amounts in Shaffer collaterals and the perforant path. EphA4 signal can be detected in areas distant from the soma in the form of puncta (Figure 3 B).

The layers in which cell bodies are more abundant (stratum pyramidale [SP] and stratum glanulosum [SG]) were also labelled but the intensity of the staining was considerably weaker (Figure 1).

tPA

Low magnification microscopy of tPA's TSA-amplified immunostainings revealed that this enzyme's distribution is restricted to the brain areas populated with cell bodies and their vicinities (Figure 2 A). Markedly intense immunofluorescence was detected in the CA2-CA4 regions corresponding to the stratum radiatum, which derive from the proximal areas of the stratum pyramidale. Also, some areas of the stratum granulosum in the dentate gyrus are moderately immunoreactive for tPA. The signal intensity is comparable in these two regions of the hippocampus; however, the protein levels in other parts of the hippocampus are almost undetectable. Of note, there is a group of sparsely distributed single cells labelled for tPA in this region. The identity of these cells is still to be investigated but their distribution may correspond to some sparse neuronal sub-type, such as interneurons, dopaminergic neurons or nascent cells (Figure 2).

Higher magnification microscopy shows strong tPA staining in clusters localised in the extracellular space and in the surrounding (perineuronal) areas of cell bodies of CA2-CA4/dentate gyrus (Figure 2 B). The perineuronal location would be consistent with the previously-demonstrated presence of tPA in neuronal growth cones. These structures end at the vicinities of the cell bodies and dendritic spines and make synaptic connection there with primary dendrites. Axons originate in neurons projecting into CA2-CA4/dentate gyrus, as described in previous works (Lochner et al., 1998, 2006; Silverman et al., 2005). Furthermore, the extracellular space location would be also consistent with the secretory nature of the enzyme, which is released to the extracellular space to exert some of its functions (Krystosek and Seeds, 1981; Pawlak et al., 2003; Wu et al., 2012).

tPA and EphA4 co-localisation

The expression patterns of EphA4 and tPA co-localise to a high degree in the areas in which tPA is most abundant, i.e. dentate gyrus and CA2-CA4 (Figure 2 A and B). Puncta corresponding to EphA4 overlap with tPA signal or are located in the close vicinities of the enzyme.

Plasmin(ogen)

Lower magnification of plasmin(ogen) immunostainings show a distribution that differs from those of EphA4 and tPA. Plasmin(ogen) is confined to regions populated with cell bodies and their proximities; i.e. the stratum pyramidale and granulosum, mainly at the CA1-CA4 areas and the dentate gyrus (Figure 3 A, Figure 7 C). The signal intensity is uniform in all of these areas. Similar to tPA, there is a sparse number of stained single cells whose identity has not been determined yet (Figure 3 A). in any case, the described localisation of the enzyme coincides with the previous works, where the enzyme's mRNA and protein expression is present in plasticity-related areas (Basham and Seeds, 2001).

High magnification microscopy reveals that plasmin(ogen) is localised around the cell perimeter. The protein is present in subcellular size cumuli that could be the consequence of confined anatomo-physiological structures in which this enzyme may develop its activity and whose identity is still unknown (Figure 3 B). This restricted superficial distribution is in line with its characterisation as a cell surface protein (Myöhänen and Vaheri, 2004); but this location is also in agreement with its presence in vesicular structures, and accordingly, with its secretory nature. This mechanism has been recognised in previous works involving tPA/plasmin cascade in the mouse hippocampus (Lochner et al., 2008).

Just as tPA, plasmin(ogen) also co-localises in high magnifications with EphA4 (Figure 3 B). Puncta corresponding to EphA4 overlap with plasmin(ogen) signal or are located in the close vicinities surrounding the enzyme (Figure 3 B).

tPA location is restricted to neurons

To assess whether the location of tPA was neuronal or glial, triple immunostainings were performed. Apart from the tPA antibody, I used an antibody against the specific neuronal marker protein (NeuN) along with an antibody against a specific glial marker, namely glial fibrillary acidic protein, or GFAP (Figure 4). This showed that the immunopositive areas for tPA correspond to NeuN-positive cells and that there was a complete lack of tPA-positive and GFAP-positive co-localisation (Figure 4). This means that the structures surrounded by tPA are mainly neurons, probably pyramidal and granular cells (Figure 9).

Plasmin(ogen) location is restricted to neurons

Triple immunostainings were performed for plasmin(ogen) plus NeuN and GFAP antibodies to mimic the experiments carried out to localise tPA. These experiments show evident plasmin(ogen) staining of the space surrounding the neuronal cell bodies of NeuN-positive cells (neurons) of all areas of the hippocampus, but no apparent co-localisation is observed with the glial marker GFAP. This indicates that the location of plasmin(ogen) is mainly, if not totally, neuronal (Figure 5).

Localisation of EphA4, tPA and plasminogen in the mouse amygdala

Unlike the hippocampus, the amygdala is lacking a laminar structure and boundaries of the structures are not as clear. The amygdala has been divided into up to 13 nuclei and sub-nuclei. However, they are commonly arranged into four groups: basolateral, central, medial and cortical. These groups will be used to describe the localisation of the components of the cascade.

EphA4

Immunostaining with anti-EphA4 antibody revealed that the protein was expressed in all groups of nuclei in the amygdala (Figure 6 A) i.e. BLA, CeA, MeA and CoA. However, the levels of this protein in this area are significantly lower compared to the ones found in the hippocampus (data not shown). This observation supports the previous reports related to the receptor's localisation (Liebl et al., 2003; Magdaleno et al., 2006; Lein et al., 2007). Different sub-structures show that levels of EphA4 slightly vary within amygdalar nuclei. A marginally more intense signal is present in the CeA, when compared to the BLA, MeA and CoA (Figure 6 A). As with the hippocampus, EphA4 was distributed in scattered puncta or groups of puncta surrounding cell bodies (Figure 6 B). The presence of puncta suggests that this protein is present in defined areas. Specifically, the localisation close to the cell bodies would suggest a likely presence in neuropil structures (an area around neurons rich in synaptic contacts) or its close vicinities.

tPA

tPA immunostainings indicate that the enzyme is mainly located in the central amygdala, with particular abundance in the extracellular space of the centrolateral amygdala (Figure 6 A). In the rest of the amygdalar areas, tPA's presence is limited or inexistent (Figure 6 A). This distribution is in agreement with previous reports of tPA location of mRNA, protein levels and activity (Melchor and Strickland, 2005; Pawlak et al., 2005; Skrzypiec, Buczko and Pawlak, 2008). Like in the hippocampus, there are some isolated cells which are highly immunostained for tPA. These cells have not been identified yet. High magnification microscopy shows a strong tPA staining in the extrasomatic space (Figure 6 B), which coincides with the secretory nature of the enzyme described in previous works.

Co-localisation of tPA and EphA4

EphA4 and tPA co-localise to a high degree in the area in which both proteins are most expressed, i.e. the central amygdala but it is negligible in basolateral, medial and cortical groups (Figure 6 A and B). However, although the overlap is significant, it is not complete, meaning that there are tPA puncta that do not coincide in space with EphA4 puncta and vice versa. Importantly, there is a large number of adjacent puncta corresponding to each of these two proteins (Figure 10). This fact indicates that EphA4 and tPA likely accumulate in spatially-related structures, such as the pre and postsynaptic components of the synapse and indeed, much of this tPA-EphA4 co-localisation is occurring in the context of the GABAergic synapse, as demonstrated by their co-localisation with the postsynaptic GABAergic synapse marker, gephyrin (Figure 10).

Co-localisation of tPA and EphA4 with Gephyrin and EAAT3

Approximately, 95% of the neurons in the central amygdala are GABAergic (McDonald, 1982) and, as it will be mentioned in the following sections, the GABAergic synapse is in close relation with tPA-expressing neurons. Therefore, it was interesting to observe whether the association between EphA4 and tPA/plasmin(ogen) interaction could take place in GABAergic synapses of CeA. To that aim, a broadly-used postsynaptic marker of GABAergic synapses, gephyrin, was used. The results of triple immunostainings for tPA, EphA4 and gephyrin in CeA showed that there is high co-localisation of tPA and EphA4 in GABAergic synapses (Figure 10), which indicates that proteolysis of EphA4 by the tPA/plasmin system is likely to be taking place in the GABAergic synapse of the CeA. However, the co-localisation tPA/EphA4/gephyrin is not absolute, indicating the presence of synapses lacking gephyrin which are tPA/EphA4+ and synapses lacking tPA which are gephyrin/EphA4+ (Figure 10).

Complementarily, immunostainings for the EAAT3 glutamate transporter were carried out. This transporter is located in the peri- and pre-synaptic areas of glutamatergic synapse. As Figure 12 shows, tPA population (46.59 ± 3.96%) and EAAT3 population (13.06 ± 2.89%) barely colocalise (2.25 ± 0.82%) meaning that

they form two separate populations. In practical terms, these data also mean that the type of neurons that tPA co-localises with in the central amygdala are non-glutamatergic. All EAAT3^{+} cells seem to be also plasmin(ogen)$^{+}$, therefore, all glutamatergic synapses present plasmin(ogen) in the CeA (Figure 13).

Localisation of tPA is restricted to neurons

Immunostainings in the amygdala reflected those performed in the hippocampus. Antibodies against NeuN, GFAP and tPA/plasmin(ogen) confirmed that the main body of cells where tPA is located in the amygdala are neurons (Figure 4 B).

Plasmin(ogen)

Low magnification analysis of plasmin(ogen) immunostainings shows high levels of this protein in the central amygdala (Figure 11 A and B). Similar to the hippocampus, cells display evenly distributed puncta constrained to the cell body boundaries and the proximities of the cell body.

Co-localisation of plasmin(ogen) and EphA4

Importantly, plasmin(ogen) and EphA4 microscopic co-localise to a high degree (Figure 11 B) in the GABAergic synapse. This overlap enables a system in which EphA4 would be a target protein receptor downstream of plasmin. Further characterisation to unveil the function of this cleavage will be discussed in the following chapters.

Characterisation of tPA-positive neurons in the mouse amygdala

The specific interest of our laboratory in the (highly tPA-stained) CeL area of the amygdala led me to perform further experiments aiming to confirm the identity of tPA-mRNA-expressing cells in the CeL. To this aim, fluorescent in situ

hybridisation for the tPA gene (Plat) was used, followed by immunohistochemistry for cellular markers of major interneuron subclasses that populate CeL (Figure 9). Experiments performed by Dr Mariusz Mucha, from the University of Exeter, showed that all PKCδ+ neurons co-expressed tPA-mRNA (100±0%, n = 1094 cells, N = 11 sections from 3 mice), while a significantly smaller proportion expressed corticotrophin-releasing factor (CRF, 13.4±0.5%, n = 832 cells, N = 11 sections from 3 mice, $p<0.0001$) or somatostatin (SOM, 3.3±0.6%, n = 1526 cells, N = 18 sections from 3 mice, $p<0.0001$), indicating that PKCδ+ interneurons are the main cell type expressing tPA in CeA.

Cleavage of EphA4

EphA4 is cleaved by tissue plasminogen activator and plasmin in the mouse amygdala

After evaluating the presence of the tPA-plasmin-EphA4 proteolytic system in the mouse brain, I examined the feasibility of the cleavage in the brain milieu. For this purpose, brain tissue homogenates from wild-type C57BL/6J mouse hippocampus and amygdala were extracted and incubated with either tPA or tPA plus increasing concentrations of plasminogen as described in *Materials and Methods*.

Similar to the initial studies done in our lab with SH-SY5Y cell line, after incubation of brain homogenates with tPA plus increasing doses of plasminogen, the levels of native EphA4 protein decreased with increasing concentration of plasmin, even at low concentrations of the enzyme, in both hippocampus (Figure 14 A) and amygdala (Figure 14 B). These experiments indicate a high sensitivity of EphA4 to cleavage by plasmin. With regards to the native form of EphA4, the decrease is dose-dependent and it becomes statistically significant at higher concentrations of plasminogen (552.07 nM and 1104.14 nM) (Figure 14, n = 3-4.

ANOVA, $F(4,11) = 92.48$, $p<0.0001$. Undigested EphA4 (UD): 1.000±0.0359 a.u. vs. $tPA^{+}Plg^{++}$ treated: 0.496±0.044 a.u., $p<0.001$; vs. $tPA^{+}Plg^{+++}$ treated: 0.362±0.013 a.u., $p<0.001$). However, the cleavage of EphA4 is evidenced at even lower concentrations of plasminogen due to the appearance and disappearance of lower molecular weight EphA4 bands corresponding to EphA4's cleavage products (Figure 14, C2. ANOVA $F(4,11) = 208.1$, $p<0.001$. Undigested EphA4 (UD): 1.000±0.016 vs. $tPA^{+}Plg^{+}$ treated: 4.171±0.722 a.u., $p<0.002$; vs. $tPA^{+}Plg^{++}$ treated: 9.809±0.612 a.u., $p<0.001$; vs. $tPA^{+}Plg^{+++}$ 14.218±1.347 a.u., $p<0.001$).

These brain homogenates were probed with an antibody recognising a C-terminus epitope of the EphA4 receptor (intracellular domain) (Figure 14). The cleavage sites produced by plasmin generated a C-terminal fragment of about 40 kDa, which, compared to the molecular weight of the protein, would indicate the existence of a specific extracellular cleavage site close to the cell membrane. The blot was also probed with an antibody against plasminogen (data not shown), which indicated that there are small amounts of native plasminogen present in the homogenates. However, despite the presence of endogenous plasminogen in these homogenates, the incubation of the samples with exogenous tPA (in the absence of exogenous plasminogen) did not cause a significant cleavage of native EphA4 (Figure 14. N, C1 and C2) at 15 min of incubation and 40 nM of tPA.

Nevertheless, the incubation of amygdala homogenates with increased concentrations of exogenous tPA (>160 nM) and longer times of incubation (2 h) produced the increase of EphA4 lower molecular weight bands (Figure 15. C1, ANOVA, $F(3,9) = 0.356$, $p>0.05$. C2, ANOVA, $F(3,9) = 20.49$, $p<0.01$; Bonferroni: NT: 1.000±0.059 vs. tPA^{+}: 1.500±0.047, $p<0.05$; vs. tPA^{++}: 1.853±0.048, $p<0.01$; tPA^{+}: 1.908±0.024, $p<0.001$. C3, ANOVA, $F(3,9) = 27.07$, $p<0.01$; Bonferroni: NT: 1.000±0.0147 vs. tPA^{+}: 1.309±0.034, $p<0.05$; vs. tPA^{++}: 1.485±0.058 $p<0.01$. C4, ANOVA, $F(3,9) = 35.37$, $p<0.001$; Bonferroni: NT: 1±0.066 vs. tPA^{+}: 1.225±0.025 $p>0.05$; vs. tPA^{++}: 1.506±0.027, $p<0.001$; vs. tPA^{+++}: 1.629±0.056, $p<0.001$. C5, ANOVA, $F(3,9) = 9.28$, $p<0.05$; Bonferroni: NT: 1±0.052 vs. tPA^{+}: 1.332±0.154, $p>0.05$; vs. tPA^{++}: 1.646±0.092 $p<0.05$; vs. tPA^{+++}: 1.657±0.081, $p<0.05$. C6,

ANOVA, $F(3,9) = 17.57$, $p<0.05$; Bonferroni: NT: 1±0.047 vs. tPA^{+}: 0.903±0.024, $p>0.05$; vs. tPA^{++}: 1.030±0.016, $p>0.05$; vs. tPA^{+++}: 1.187±0.005 $p<0.05$, indicating that tPA, without the presence of exogenous plasminogen, is able to produce the cleavage of EphA4 in amygdala tissue homogenates.

Sufficiency of cleavage by tPA/plasmin and identification of the cleavage sites of EphA4 by tPA and plasmin

In order to assess the sufficiency of tPA/plasmin to cleave extracellular domains of EphA4 and to further characterise the plasmin cleavage sites, there was a need to use a purified *in vitro* system in which the essential components of the system would be isolated from other interacting proteins. This system features purified proteins as its components. Commercially-purified tPA and plasmin were incubated with a chimeric form of EphA4, namely EphA4-Fc. EphA4-Fc is a recombinant protein containing the extracellular domain of EphA4 fused to the Fc fragment of a human IgG.

The cleavage pattern after tPA/plasmin digestion (Figure 17 B) resembles the pattern observed in brain homogenates (Figure 14 B). However, cleavage products detected in homogenates and in the purified system do not present the same molecular weight because, in EphA4-Fc, the C-terminus domains are substituted for an IgG, which displays a different molecular weight than the native EphA4 receptor's intracellular domains. In a similar manner to that in brain homogenates, the EphA4-Fc cleavage bands increased with increasing plasminogen concentrations (Figure 17 A. N, ANOVA, $F(4,11) = 0.748$, $p>0.05$. C1, ANOVA $F(4,11) = 9.687$, $p<0.05$; Bonferroni: NT 1.000±0.125 vs. tPA 1.092±0.03475; vs. $tPA+Plg^{+}$ 1.006±0.03042; vs. $tPA+Plg^{++}$ 1.233±0.1951; vs. $tPA+Plg^{+++}$ 1.252±0.204. C2, ANOVA, $F(4,11) = 22.99$, $p<0.001$; Bonferroni: NT 1.000±0.110 vs. tPA 0.9411±0.1043; vs. $tPA+Plg^{+}$ 0.778±0.0811; vs. $tPA+Plg^{++}$ 1.952±0.1617; vs. $tPA+Plg^{+++}$ 2.255±0.419. C3, ANOVA, $F(4,11) = 3.752$, $p>0.05$; Bonferroni: NT 1.000±0.171 vs. tPA 0.7014±0.0381; vs. $tPA+Plg^{+}$ 0.6113±0.03937; vs. $tPA+Plg^{++}$ 1.074±0.1467; vs. $tPA+Plg^{+++}$ 1.236±0.1931. C4, ANOVA, $F(4,11) = 26.48$, $p<0.001$; Bonferroni: NT 1.000±0.107 vs. tPA

0.371±0.0276; vs. tPA+Plg^{+} 0.266±0.0245; vs. tPA+Plg^{++} 0.337±0.0334; vs. tPA+Plg^{+++} 0.311±0.0608. C5, ANOVA, $F(4,11) = 26.86$, $p<0.001$; Bonferroni: NT 1.000±0.164 vs. tPA 0.884±0.02693; vs. tPA+Plg^{+} 0.765±0.106; vs. tPA+Plg^{++} 1.201±0.242; vs. tPA+Plg^{+++} 2.702±0.142). Other novel lower molecular weight C-terminal EphA4 band was identified at approximately 33 kDa and other secondary bands were found at lower molecular weights (Figure 17 B).

In a parallel experiment, EphA4-Fc was incubated with tPA at increasing concentrations for 2 h. Western blot technique detecting the N-terminal part of EphA4 shows a non-native lower molecular weight band (Figure 16 A, “C” arrow) that becomes more intense with increasing concentrations of tPA (Figure 16 B; n = 3. ANOVA $F(4,10) = 112.402$; Bonferroni: NT:1.000±0.160 vs. tPA: 5.392±1.666, $p>0.05$; tPA^{+}: 14.283±2.728 $p<0.001$; vs. tPA^{++}: 22.262±2.493, $p<0.001$; vs. tPA^{+++}: 29.690±1.458, $p<0.001$). This demonstrates the cleavage *in vitro* of the extracellular part of EphA4 by tPA without the presence of plasmin. These two experiments also prove the sufficiency of either, tPA/plasmin or tPA alone, to cleave EphA4 without the need of further components in the proteolytic system.

Collaborative work of our lab performed by Dr Małgorzata Bajor at the Polish Academy of Sciences (Poland) shed some light regarding the precise cleavage sites produced by tPA and plasmin within the structure of EphA4. A comprehensive mapping of the cleavage sites produced by tPA/plasmin was performed using a technique called amino terminal oriented mass spectrometry of the substrates (ATOMS). Mass spectrometry detection and analysis of the fragments allowed the identification of five cleavage sites produced by plasmin in the extracellular domain of EphA4 (P1-P5) (Figure 18 A and B). Three of which were also produced by tPA (P1, P2 and P5) (Figure 18 A), showing an interesting overlap in the consensus sequences of both enzymes. In addition, this study provided evidence of the sensitivity of EphA4 towards tPA and plasmin cleavage. Among all the detected cleavage sites, the closest to the transmembrane domain is the most efficiently cleaved at low doses of the enzyme (P5). This cleavage site is located within the fibronectin type III domain and the rest of the cleavage sites are located within the ligand binding domain. This fact also sets out a

possible difference in these cleavage sites' functionality based on the structural domain affected by the shedding (Figure 18 B).

To test whether these consensus sites were unique to the cleavage by tPA and plasmin, I studied the cleavage pattern of another protease that belongs to the family of trypsin-like serine proteases but is not active in the brain, namely thrombin. Thrombin can cleave the EphA4 receptor in its extracellular part (Figure 19 A and B) but its cleavage fingerprint is clearly different from that produced by tPA and plasmin (Figure 19 C).

Cleavage of EphA4 in tPA- and plasminogen-deficient mice

To further investigate the possibility of a direct implication of tPA and plasmin in the cleavage of EphA4, amygdalae from unstressed wild-type, $tPA^{-/-}$ and $plasminogen^{-/-}$ C57BL/6J mice were homogenised and analysed by Western blot. This revealed a significant lower cleavage of EphA4 in the amygdala of animals lacking plasminogen in the brain but no substantial change in the cleavage of $tPA^{-/-}$ animals (Figure 20, Kruskall-Wallis test, $p = 0.018$. WT: 1.000±0.068 vs. $Plg^{-/-}$:0.547±0.031, Dunn's Multiple Comparison $p = 0.009$), suggesting that the cleavage of EphA4 in central amygdala could be produced, in a significant percentage, by plasmin and that plasminogen's absence would not be compensated by other mechanisms. Furthermore, the cleavage of EphA4 in the amygdala can be produced in a similar magnitude in spite of a lower/null presence of tPA, which suggests this enzyme is not crucial for the direct cleavage of EphA4 and that an alternative mechanism of plasminogen activation exists, perhaps through the urokinase-type of plasminogen activator.

FIGURES

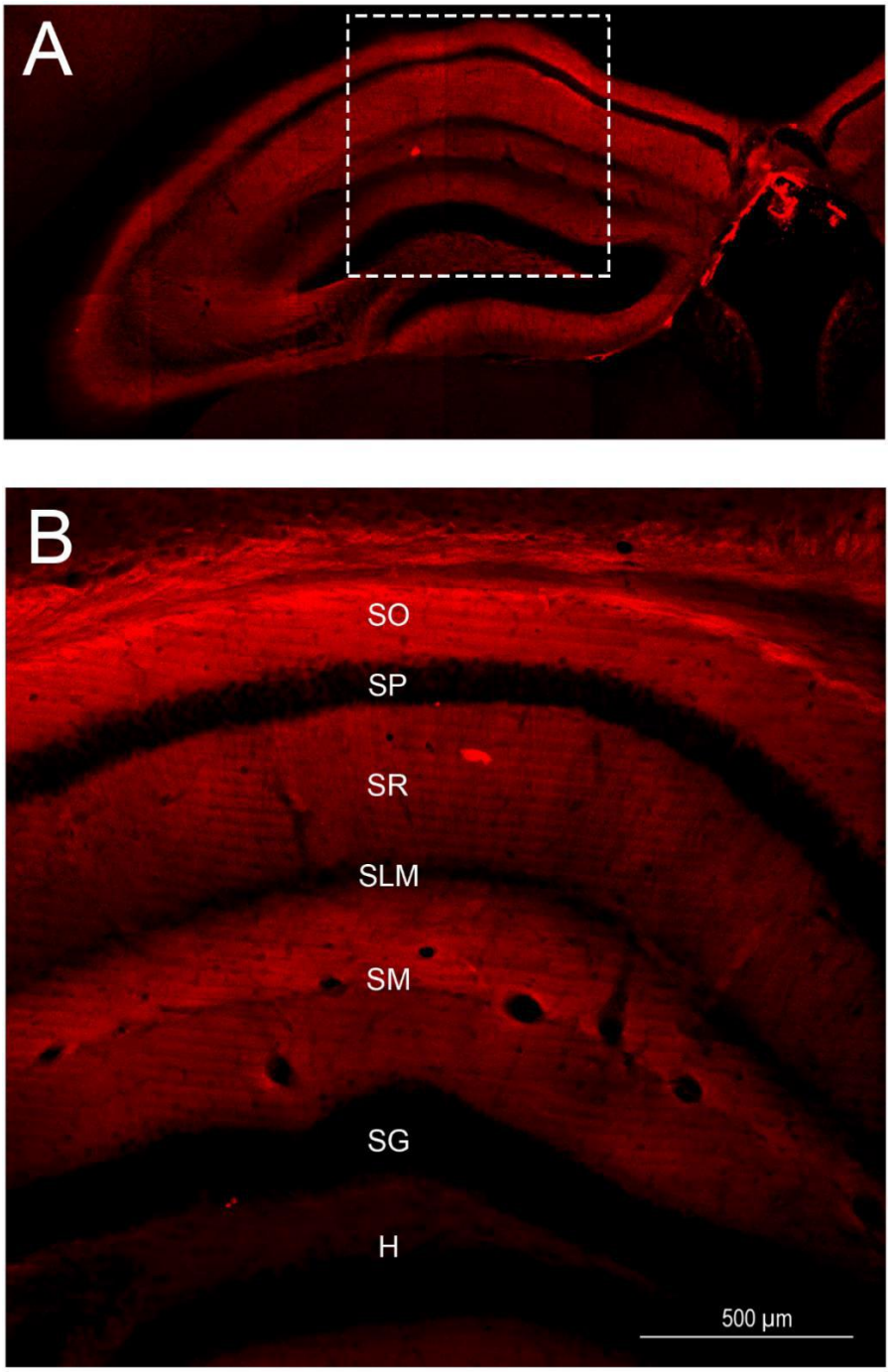


Figure 1. Fluorescence immunohistochemistry showing the expression of EphA4 protein in a cross-section of a hippocampal coronal slice of the mouse brain using an antibody raised against the C-term of EphA4 protein. SO = stratum oriens; SP = stratum pyramidale; SR = stratum radiatum; SLM = stratum lacunosum moleculare; SM = stratum moleculare; SG = stratum granulosum; H = hilus. Coordinates: approx. Bregma -1.82 mm (n = 3). A) Overview of the mouse hippocampus. B panel boundaries are marked with a dashed line. B) Representative detail of the hippocampal layers. The most intense signal is detected in layers mainly formed by processes from basal dendrites (SO, SM and SR).

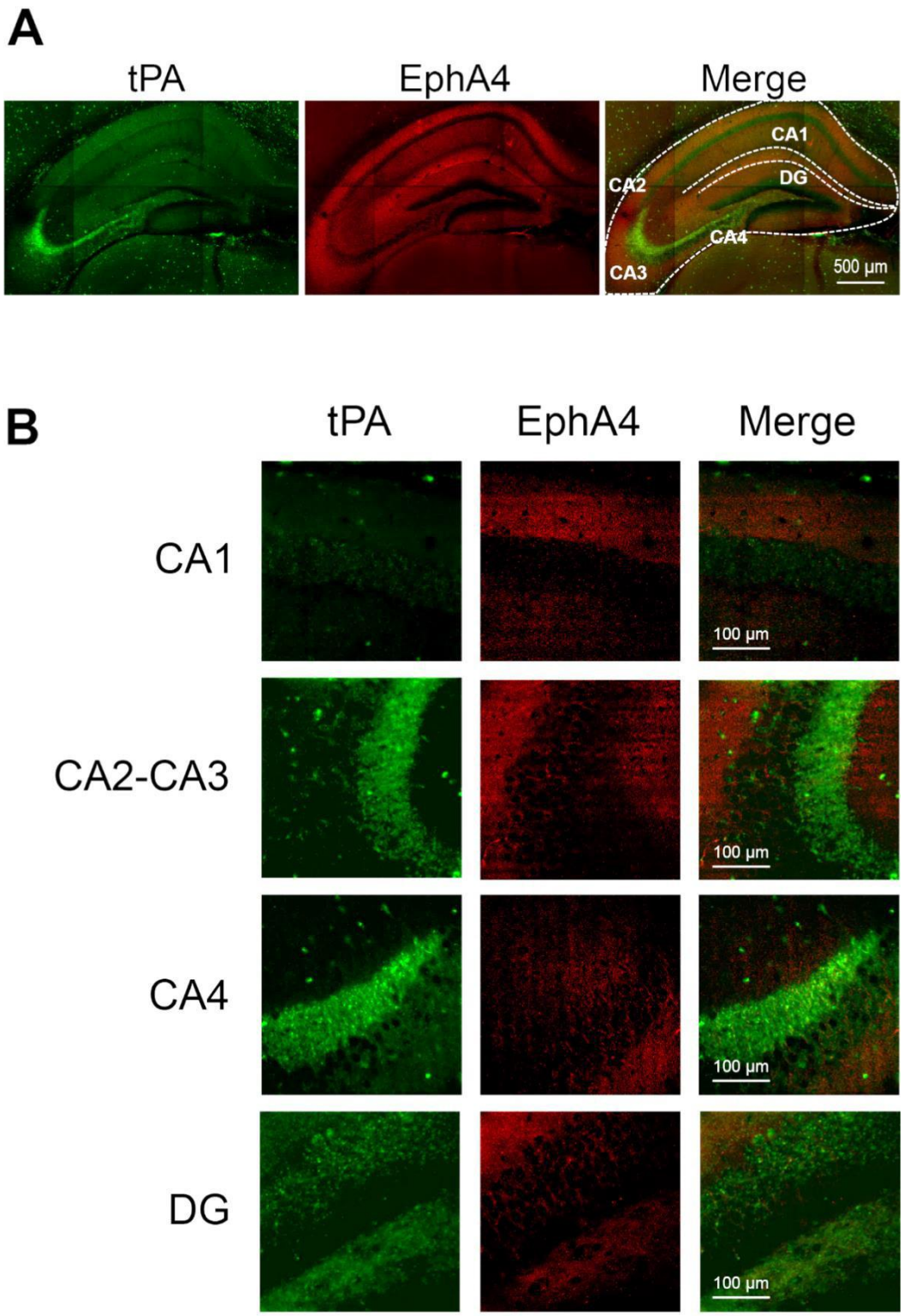


Figure 2. Fluorescence immunohistochemistry showing the co-localisation of tPA and EphA4 in the mouse hippocampus. The image shows a coronal section at approx. Bregma -1.82 mm (n = 3). Hippocampal areas (CA1-CA4, DG) are indicated in both panels. A) tPA and EphA4 co-localisation in the mouse hippocampus. Hippocampal areas are delineated with a dashed line. B) Detail of the co-localisation of tPA and EphA4 in the CA1, CA2-CA3, CA4 and DG areas of the hippocampus.

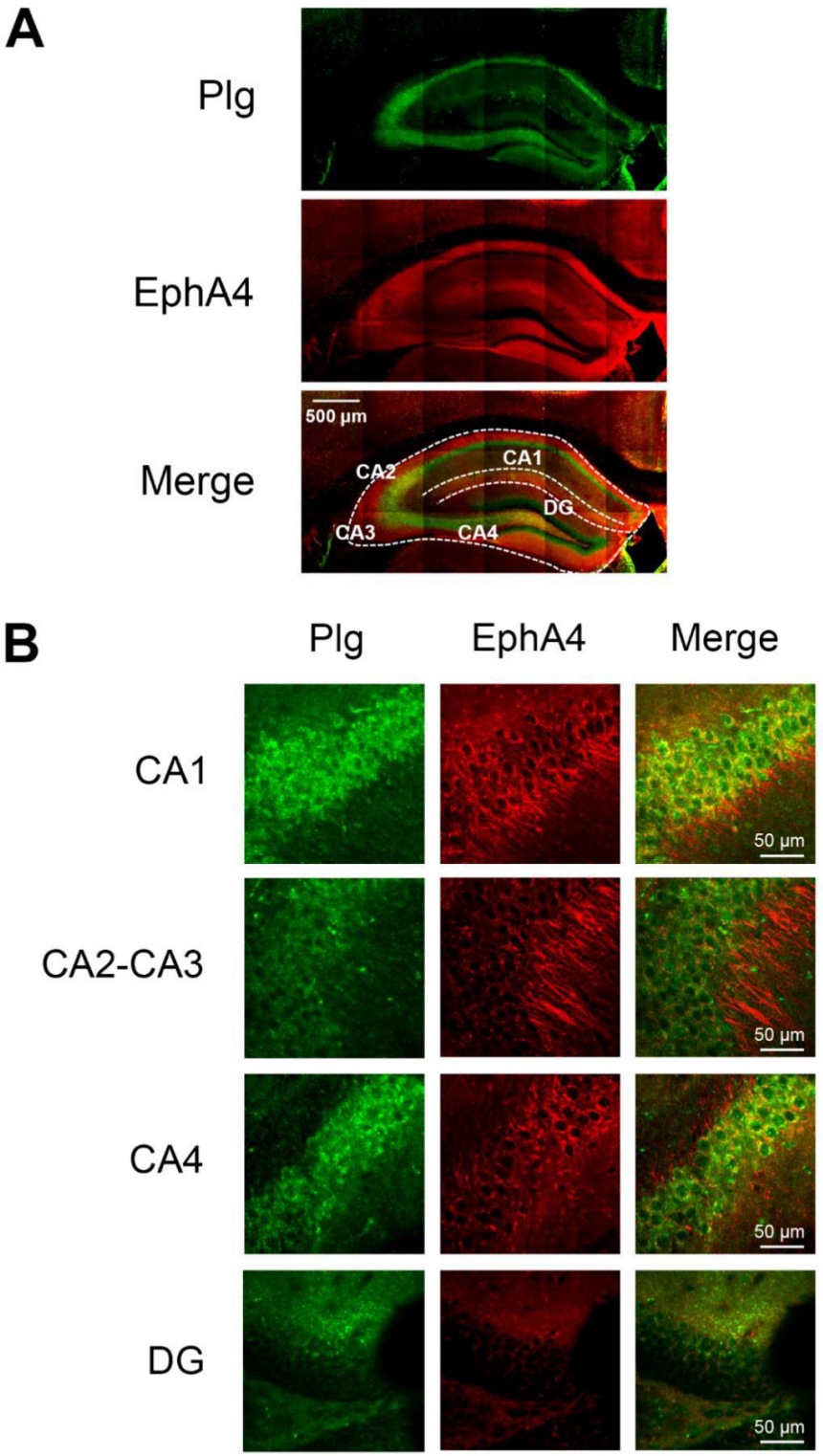


Figure 3. Fluorescence immunohistochemistry showing the co-localisation of plasmin(ogen) and EphA4 in the mouse hippocampus. The image shows a coronal section at approx. Bregma -1.82 mm (n = 3). Hippocampal areas (CA1-CA4, DG) are indicated in both panels. A) Plasmin(ogen) and EphA4 co-localisation in the mouse hippocampus. Hippocampal areas are delineated with a dashed line. B) Detail of the co-localisation of plasmin(ogen) and EphA4 in the CA1, CA2-CA3, CA4 and DG areas of the hippocampus.

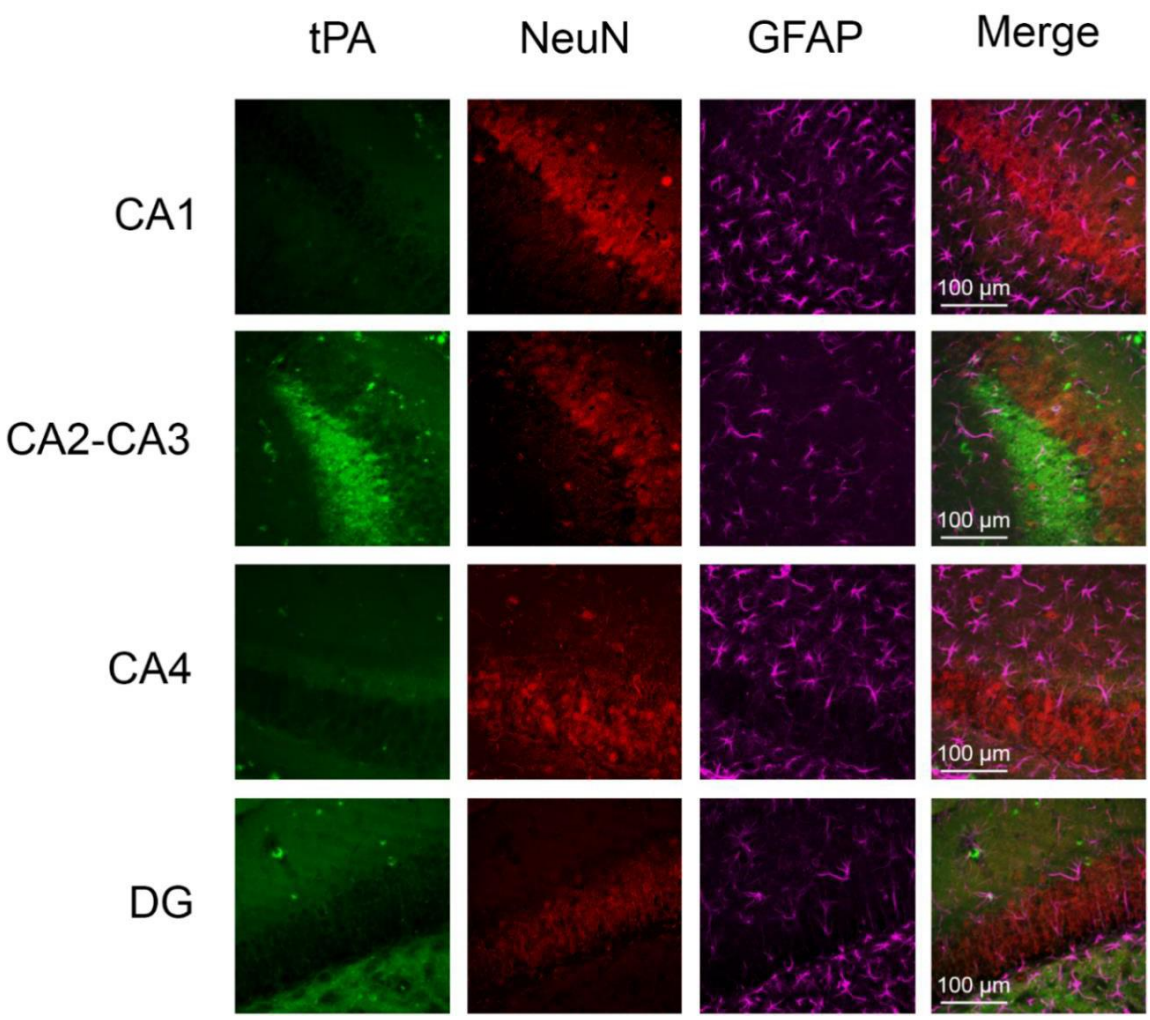


Figure 4. tPA co-localises with NeuN neuronal marker but not with GFAP glial marker in the hippocampus. The figure shows a high magnification of a fluorescence immunohistochemistry technique using antibodies for tPA detection plus the neuronal marker, NeuN and the glial marker, GFAP, in the wild type mouse hippocampus. The image shows a coronal section at approx. Bregma -1.82 mm (n = 3). Hippocampal areas (CA1-CA4, DG) are indicated accordingly.

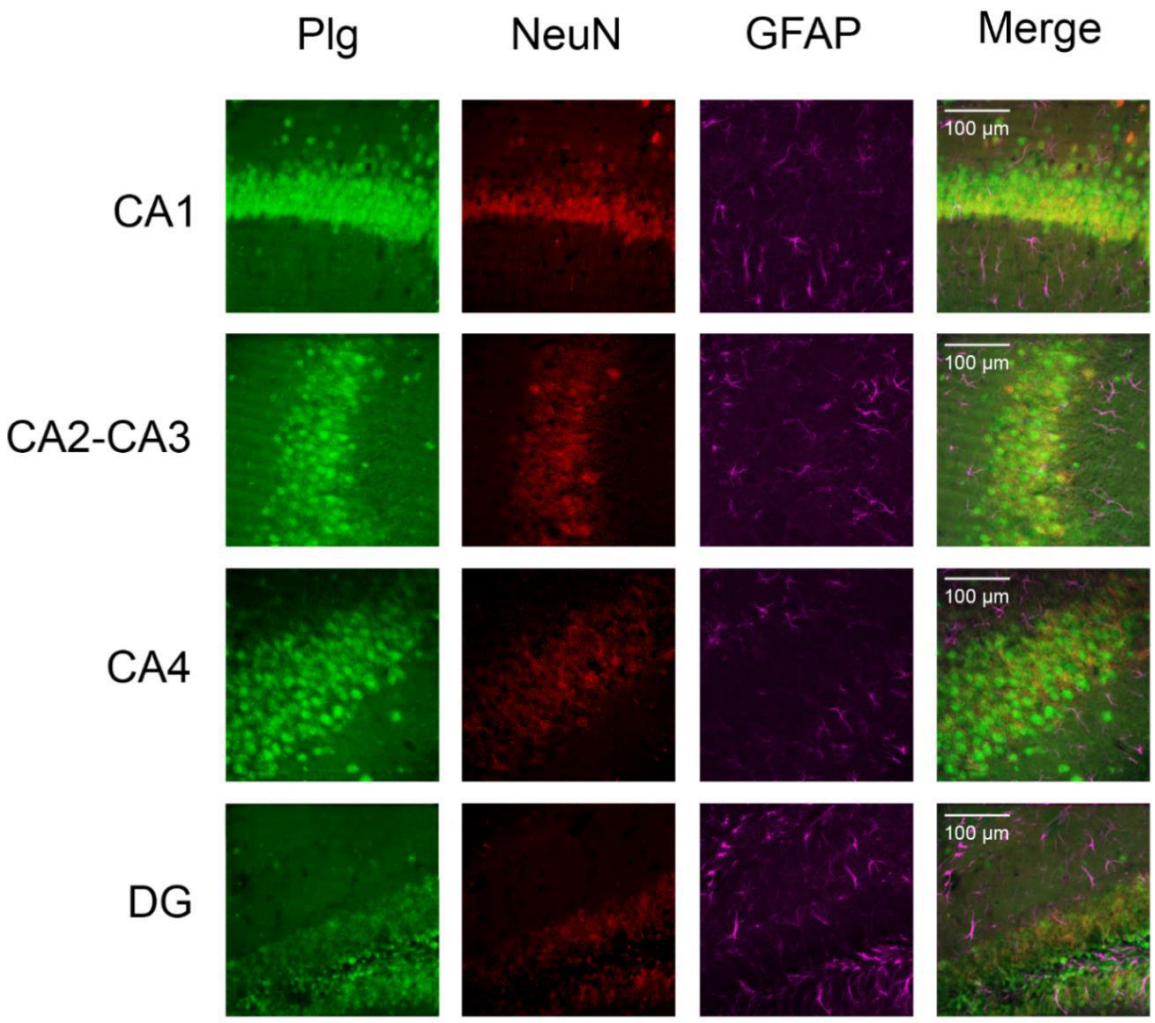


Figure *5*. Plasmin(ogen) co-localises with NeuN neuronal marker but not with GFAP glial marker in the hippocampus. The figure shows a high magnification of a fluorescence immunohistochemistry technique using antibodies for plasmin(ogen) detection plus the neuronal marker, NeuN and the glial marker, GFAP, in the wild type mouse hippocampus. The image shows a coronal section at approx. Bregma -1.82 mm (n = 3). Hippocampal areas (CA1-CA4, DG) are indicated accordingly.

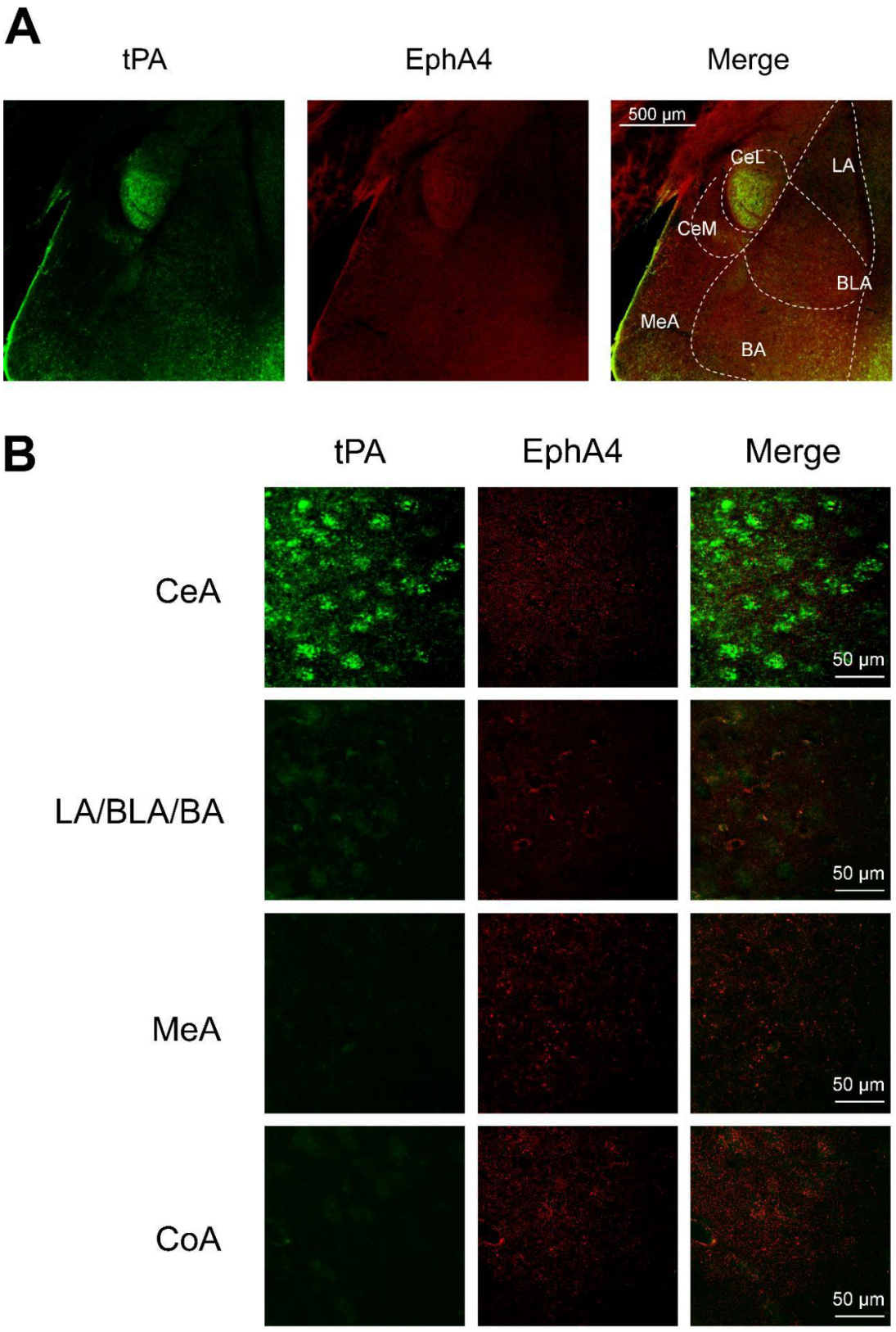


Figure 6. Fluorescence immunohistochemistry showing the co-localisation of tPA and EphA4 in the wild type mouse amygdala. CeA = central amygdala; LA = lateral amygdala; BLA = basolateral amygdala; BA = basal amygdala; MeA = medial amygdala; CoA = cortical amygdala. Both panels show a coronal section at approx. Bregma -1.82 mm (n = 3). Amygdalar nuclei are delineated with a dashed line. A) tPA and EphA4 co-localisation in the mouse amygdala. B) Detail of the co-localisation of tPA and EphA4 in the CeA, LA/BLA/BA, MeA and CoA.

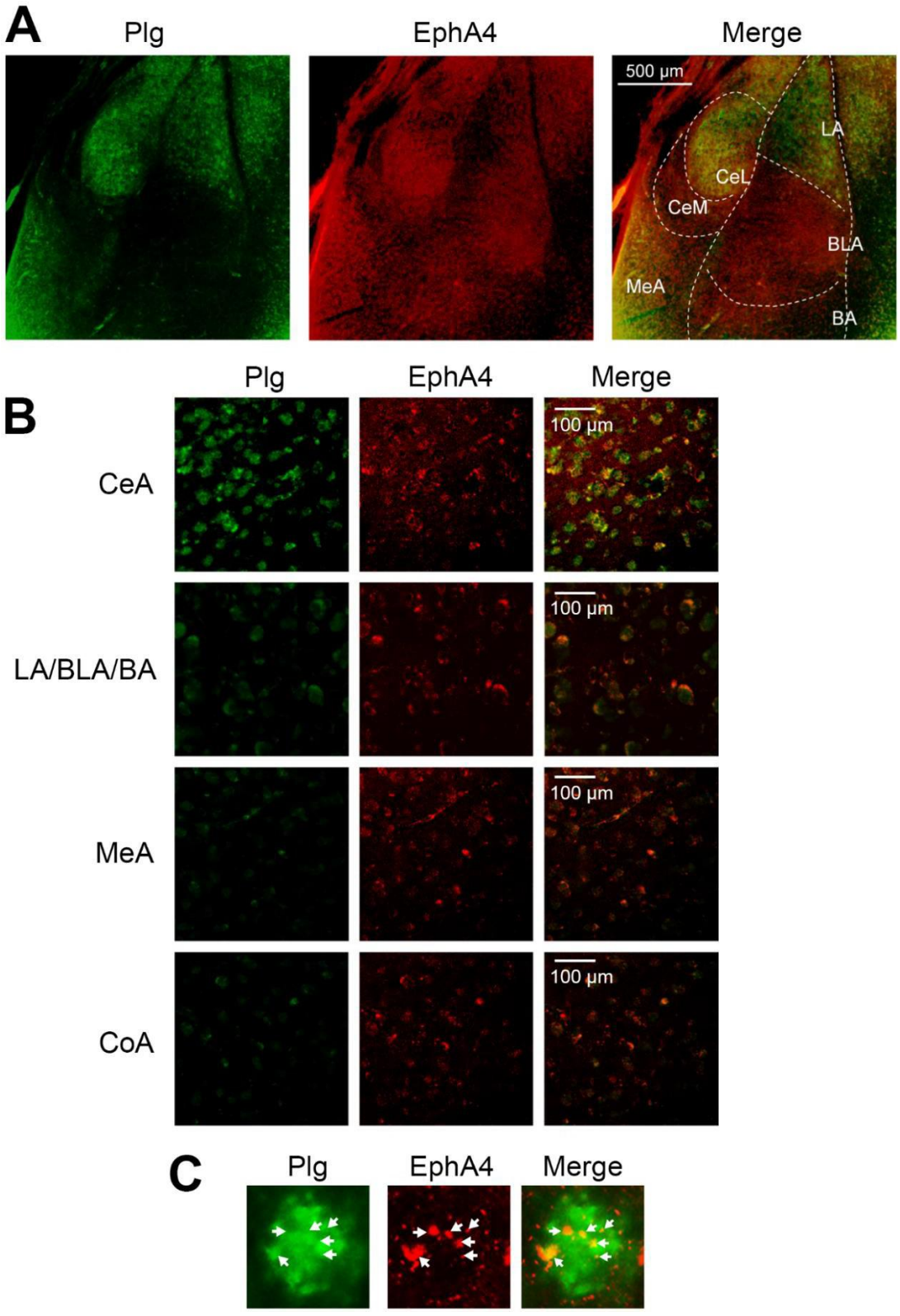


Figure 7. Fluorescence immunohistochemistry showing the co-localisation of plasmin(ogen) and EphA4 in the wild type mouse amygdala. CeA = central amygdala; LA = lateral amygdala; BLA = basolateral amygdala; BA = basal amygdala; MeA = medial amygdala; CoA = cortical amygdala. Both panels show a coronal section at approx. Bregma -1.82 mm (n = 3). A) Plasmin(ogen) and EphA4 co-localisation. Amygdalar nuclei are delineated with a dashed line. B) Detail of the co-localisation of tPA and EphA4 in the CeA, LA/ BLA/BA, MeA and CoA. C) Detail of co-localisation of plasmin(ogen) and EphA4 on a single neuron.

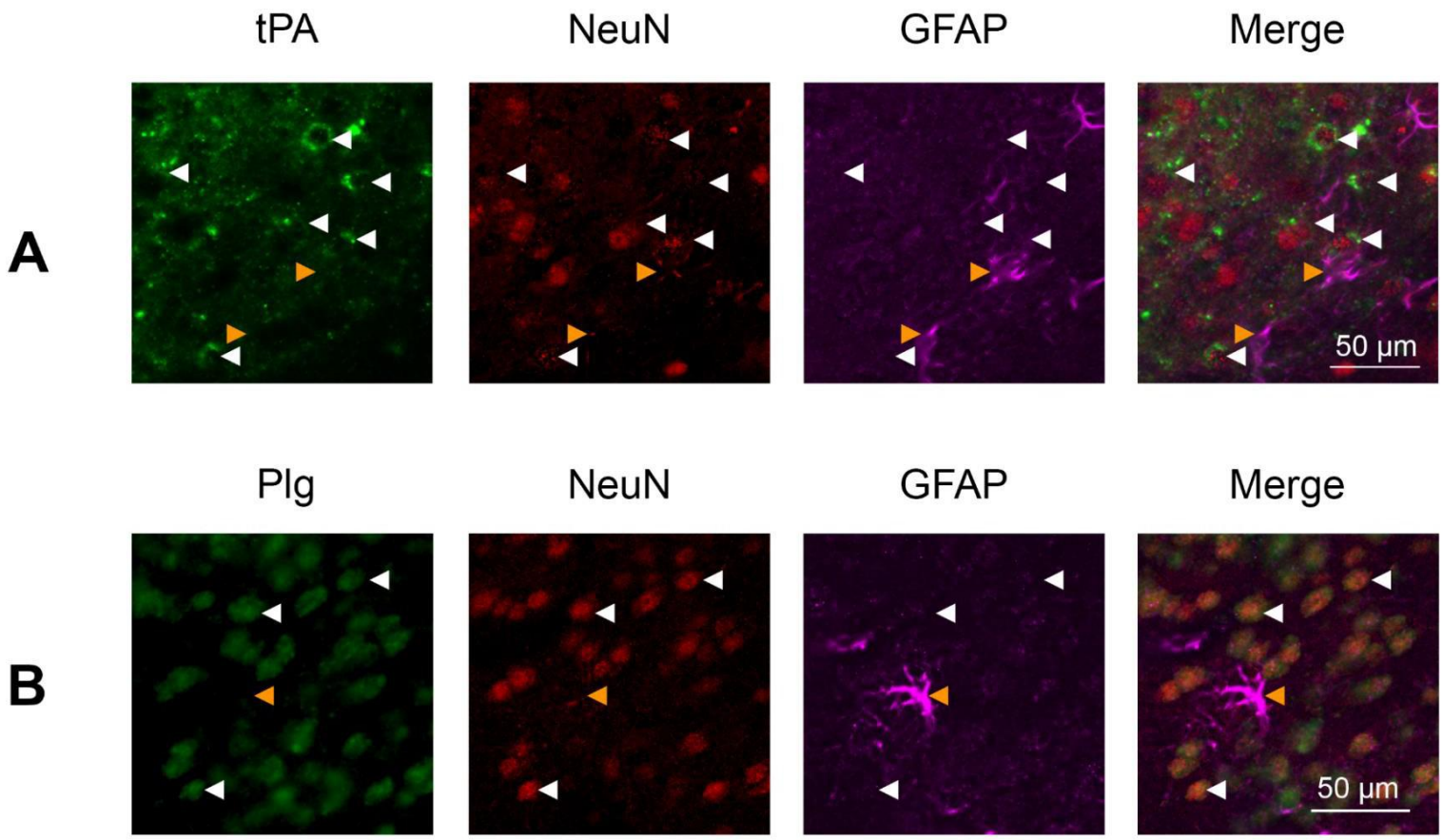


Figure 8. TPA and plasmin(ogen) co-localise with NeuN neuronal marker but not with GFAP glial marker in the central amygdala. tPA = tissue plasminogen activator; Plg = plasminogen; GFAP = glial fibrillary acidic protein. The image shows a coronal section at approx. Bregma -1.82 mm (n = 3). The figure shows a high magnification of a fluorescence immunohistochemistry technique using antibodies against tPA or plasmin(ogen) plus antibodies against the neuronal marker, NeuN and the glial marker, GFAP, in the wild type mouse central amygdala. White arrows point to NeuN+ cells (neurons), whereas orange arrows point GFAP+ cells.

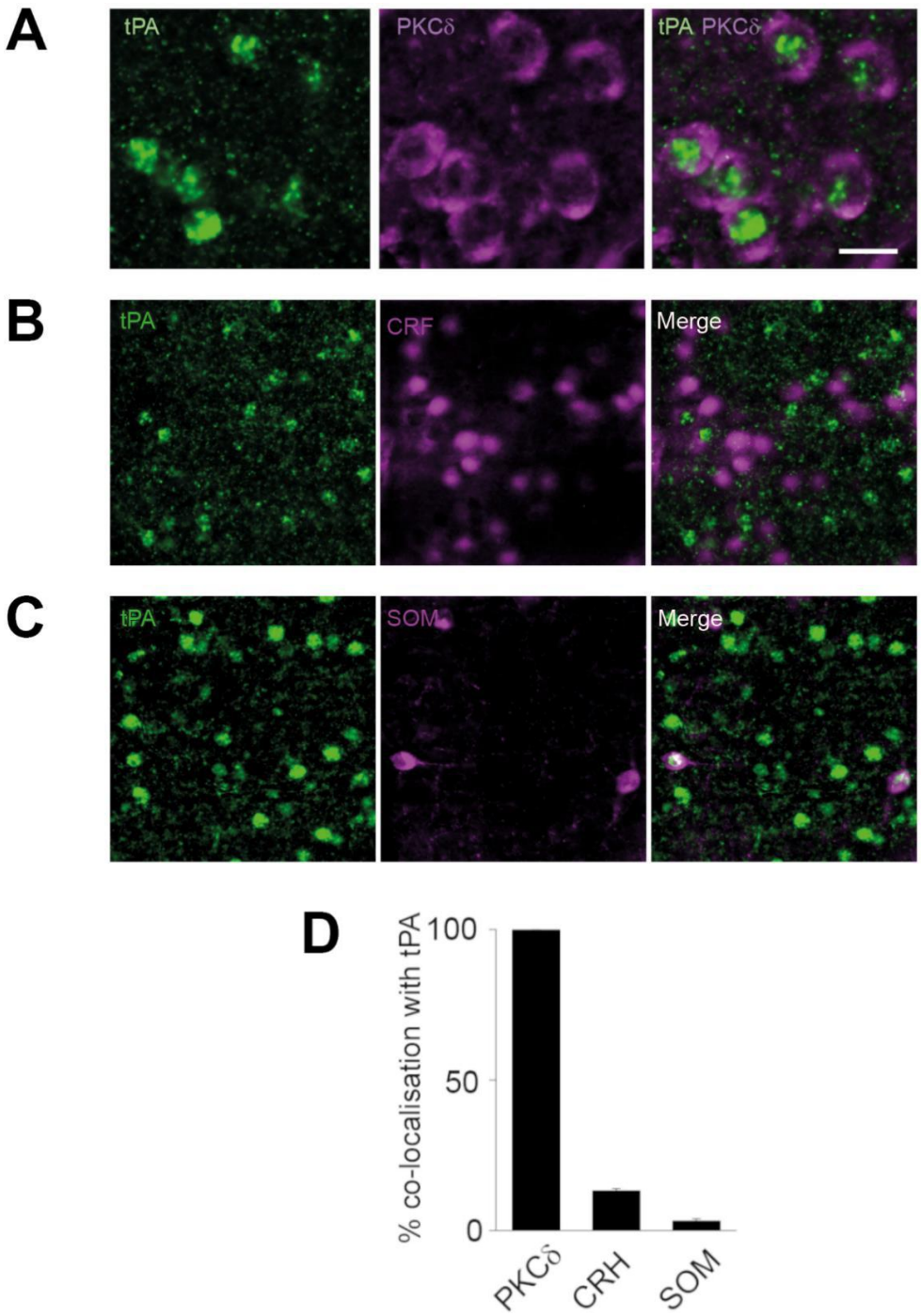


Figure 9. Different types of inhibitory neurons present tPA mRNA in the central amygdala, especially PKCδ+ inhibitory neurons. The figure shows the co-localisation tPA mRNA (green) in the CeA (detected by FISH technique) with different protein markers for interneurons (magenta) (detected with fluorescent immunostaining); i.e. A) Protein kinase C δ (PKCδ); B) corticotrophin-releasing factor (CRF) and C) somatostatin (SOM). D) Quantification shows that almost all PKCδ+ neurons were also tPA+ in the CeA, in contrast with CRF and SOM, that show a low number of cells expressing tPA mRNA. This manifest presence of tPA mRNA+ among PKCδ+ indicates that these latter type of cells could be regulating cell processes through tPA activity.

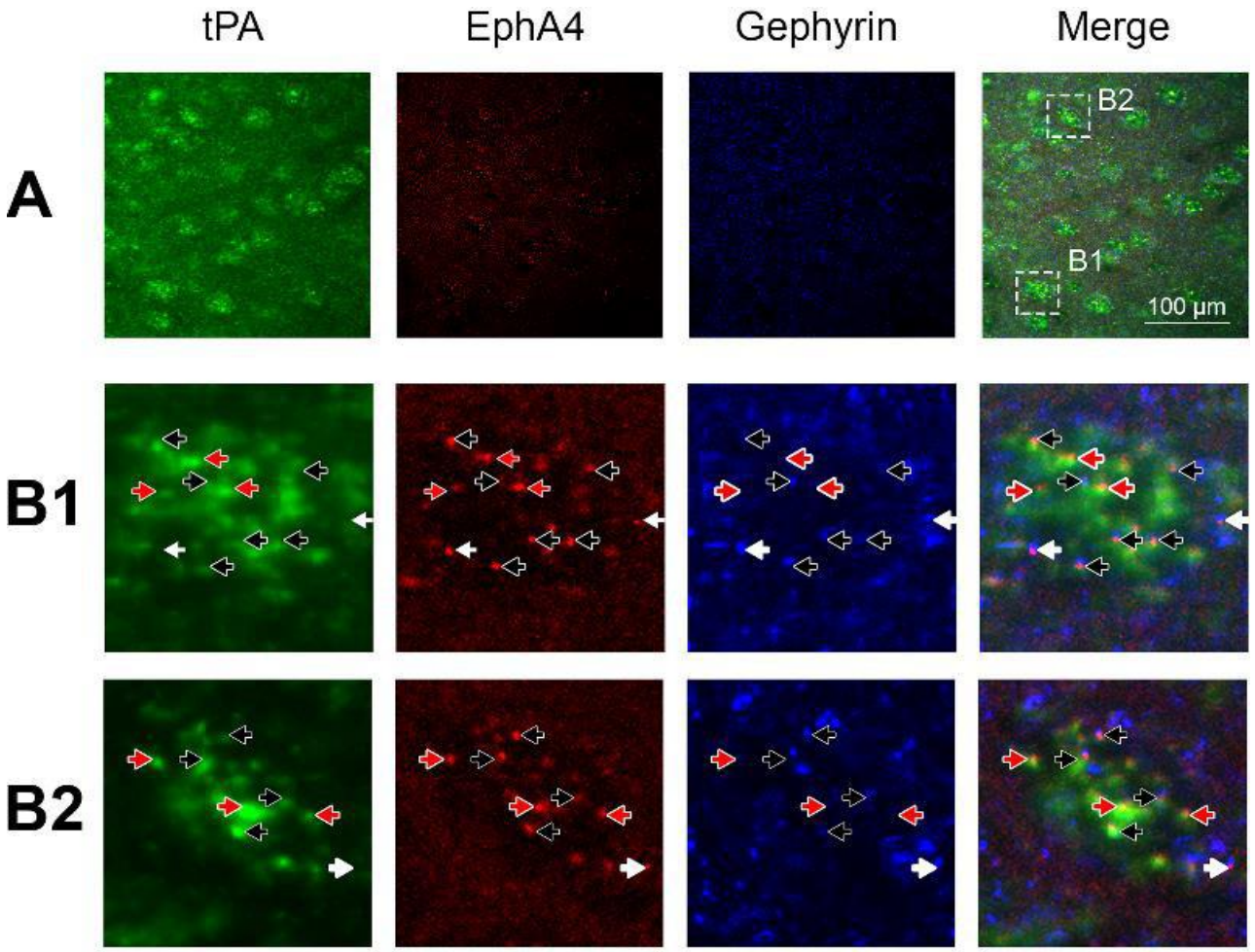


Figure 10. Fluorescence immunohistochemistry showing the co-localisation of tPA, EphA4 and the postsynaptic marker for GABAergic synapses, gephyrin, in the mouse CeA. Dashed-line squares in "merge" panel A are shown in more detail in panels B1 and B2 respectively. Black arrows point possible synapses in which tPA, EphA4 and gephyrin colocalise. Red arrows denote synapses in which tPA and EphA4 colocalise but not gephyrin. White arrows indicate synapses in which EphA4 and gephyrin colocalise but not tPA.

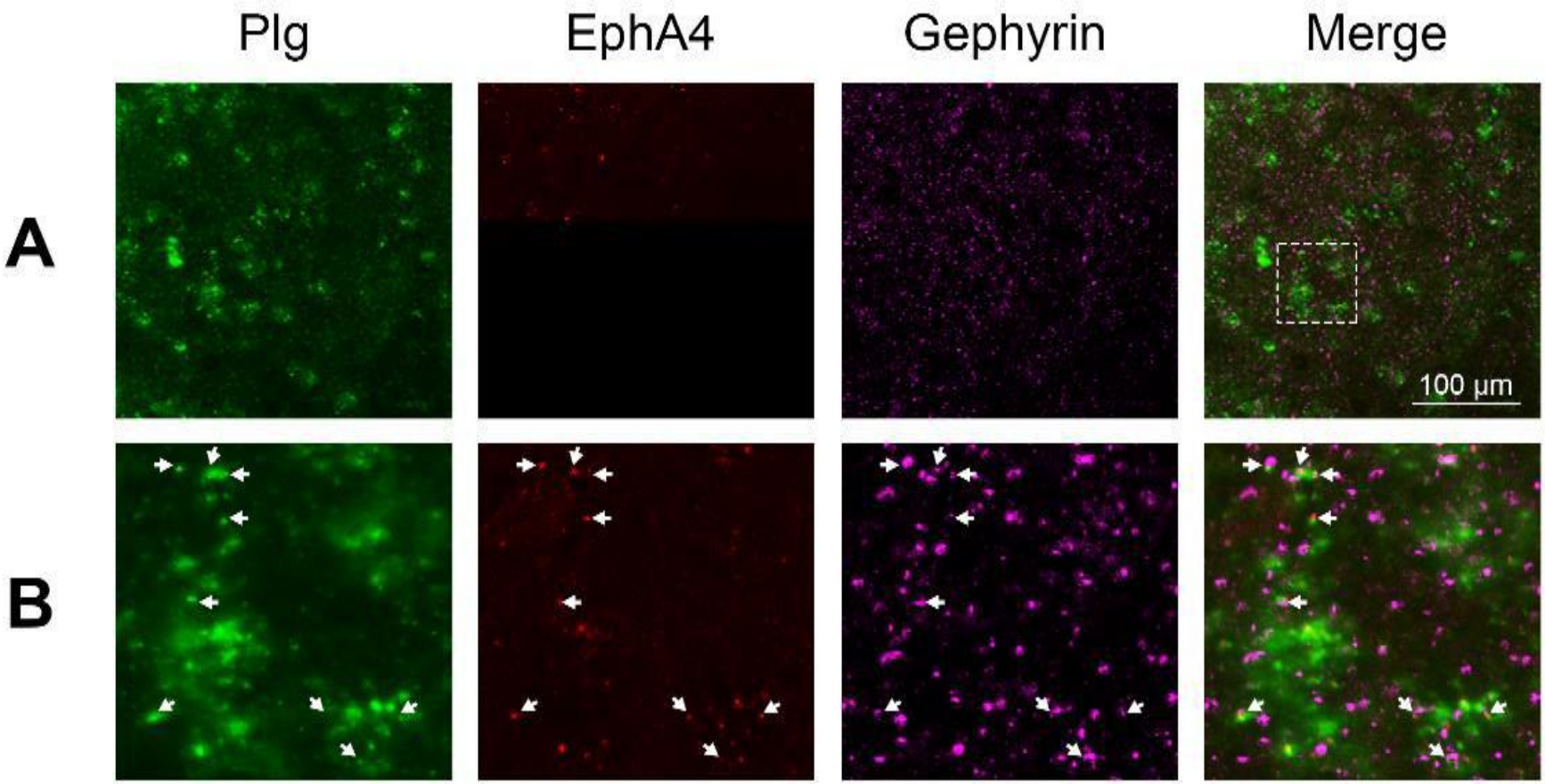


Figure 11. Fluorescence immunohistochemistry showing co-localisation of plasmin(ogen), EphA4 and gephyrin in the mouse central amygdala. A) Low magnification and B) at high magnification. Dashed-line square in A delimits the high magnification shown in panel B. White arrows point synapses in which EphA4 and gephyrin and plasmin(ogen) co-localisation.

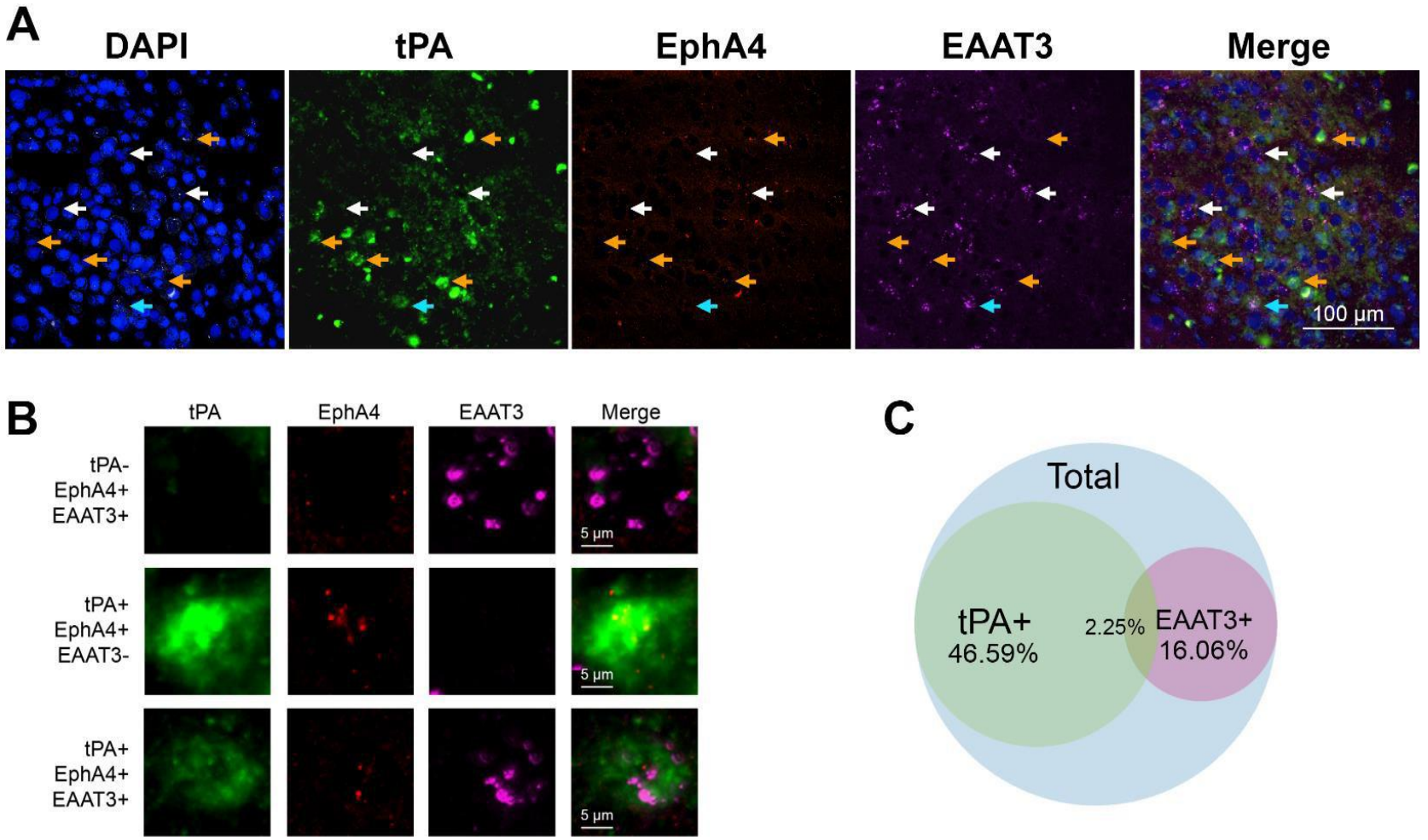


Figure 12. Cells targeted by tPA are mainly non-glutamatergic. tPA = tissue plasminogen activator; EAAT3 = excitatory amino acid transporter 3. A) Fluorescence immunohistochemistry showing the co-localisation of tPA, EphA4 and EAAT3 in the wild-type mouse central amygdala. The DNA staining, DAPI was included to observe cell nuclei. The vast majority of tPA^+ cells (orange arrows) lack EAAT3, although some examples of tPA^+ and $EAAT3^+$ cells can be found (cyan arrow). tPA+ and EAAT3+ populations are almost totally non-overlapping, meaning that the primary target of tPA activity is not the glutamatergic synapse. B) Detail of single cells examples of the cell types counted. C) V ann's d iagram o f tPA+ a nd EAAT3+ populations.

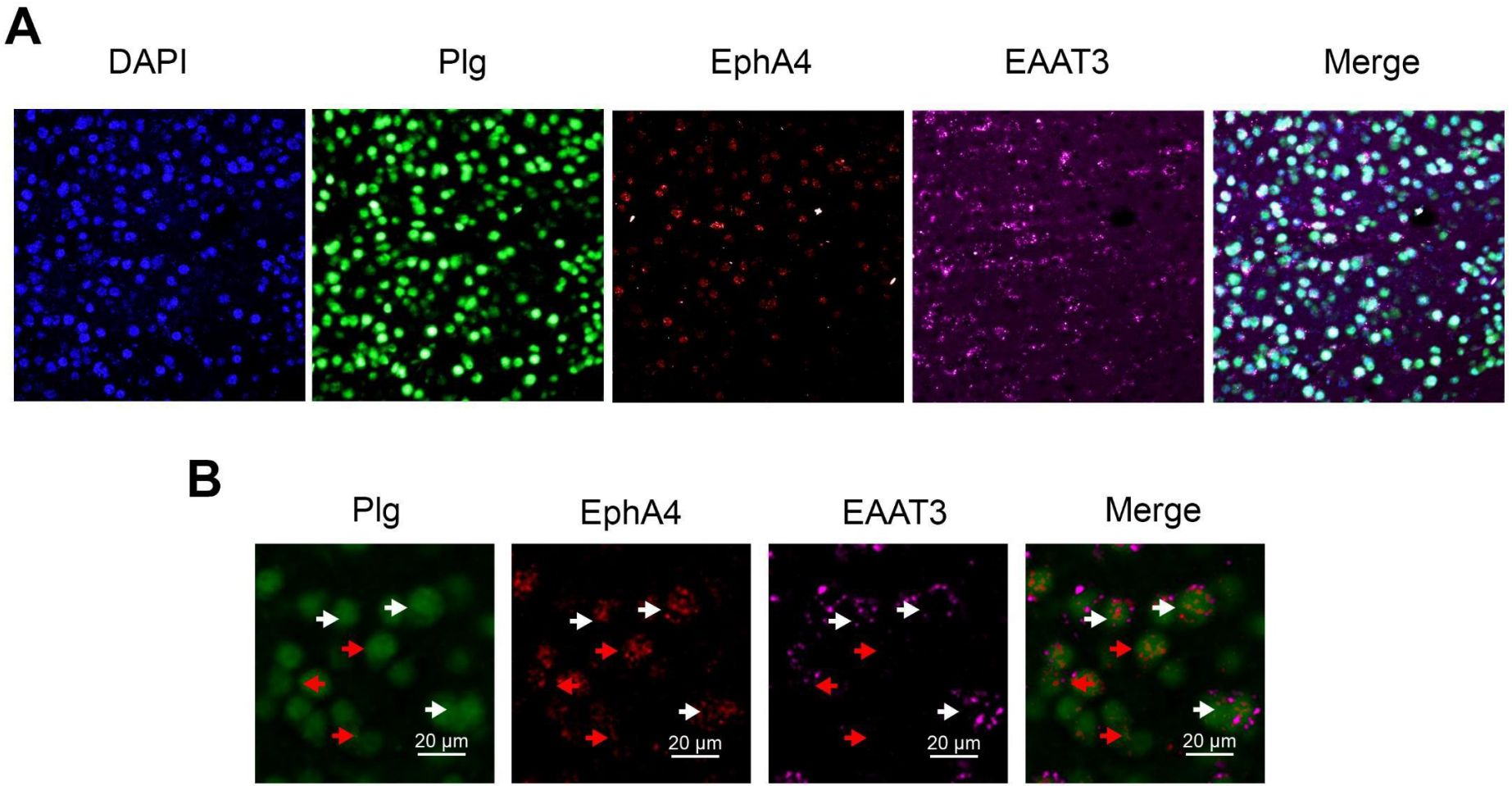


Figure 13. Fluorescence immunohistochemistry showing co-localisation of plasmin(ogen), EphA4 and EAAT3 in the mouse central amygdala. Plg = plasmin(ogen); EAAT3 = excitatory amino acid transporter 3. A) Low magnification and B) high magnification shows that all cells detected present plasminogen. However, opposite to tPA, all $EAAT3^{+}$ cells are also Plg^{+} (although not all Plg^{+} are $EAAT3^{+}$) indicating that tPA may be responsible for the specificity of the proteolytic system. White arrows show $EAAT3^{+}$ cells and red arrows show $EAAT3^{-}$ cells.

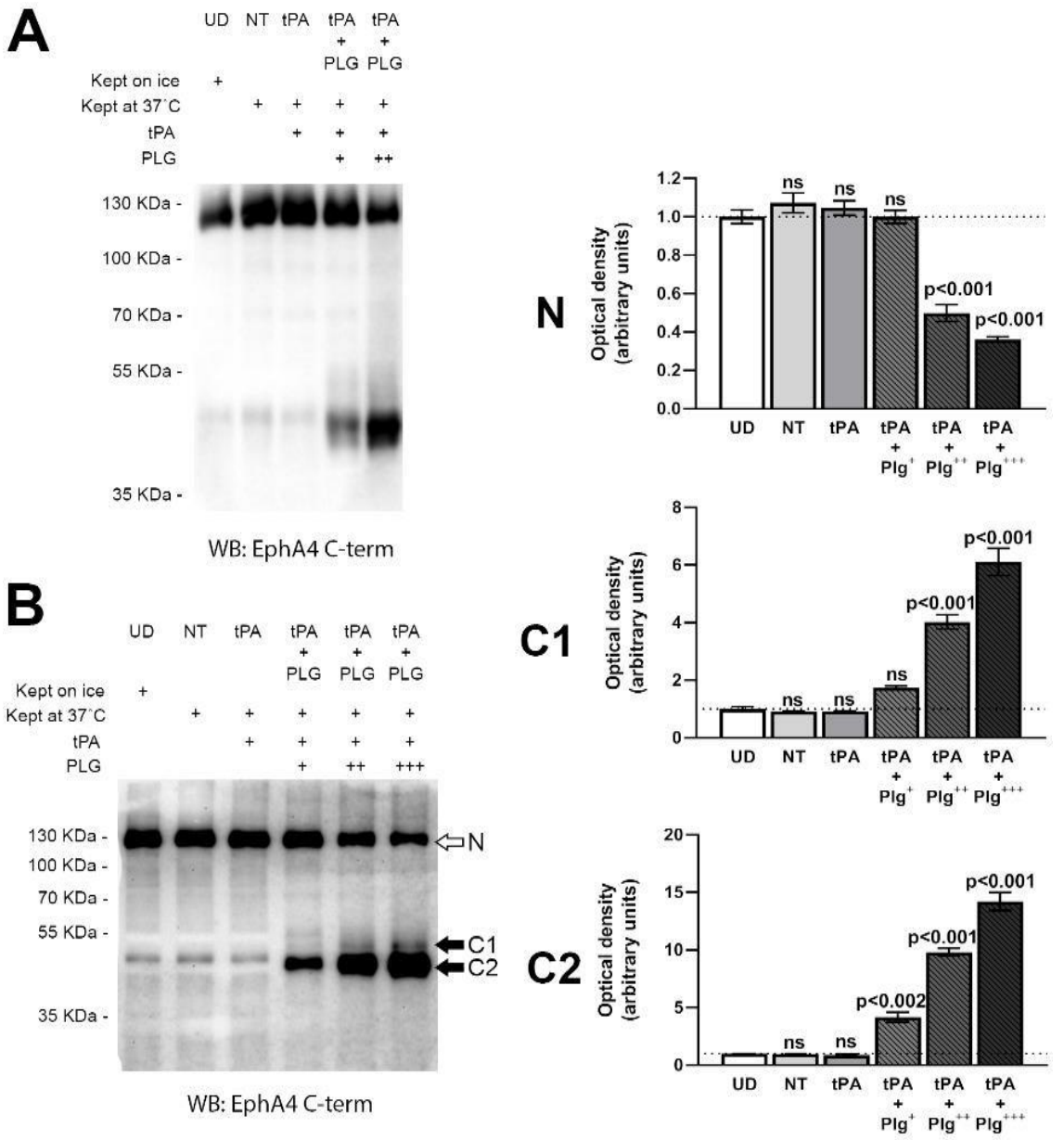


Figure 14. Plasmin and tPA cleave EphA4 receptor *ex vivo*. (UD = undigested; NT = non-treated; tPA = tissue plasminogen activator; PLG = plasminogen). The figure shows representative blots, generated by Western blot technique, of EphA4 protein and its C-terminal fragments as a result of tPA and plasmin treatment of mouse hippocampus homogenates (A) and mouse amygdala homogenates (B). Homogenates were treated for 15 min with tPA (+ = 2.5 µg/mL [39.73 nM]) and plasminogen (+ = 10 µg/mL [110.41 nM], ++ = 50 µg/mL [552.07 nM] or +++ = 100 µg/mL [1104.14 nM]). The treatment resulted in a decrease of native EphA4 (N arrow) along with an increase of lower molecular weight bands or appearance of new bands (C1 and C2 arrows), which demonstrates the cleavage of EphA4 by tPA and plasmin in the mouse amygdala. Quantifications for the native EphA4 band and its two main cleavage products in the amygdala (N, C1 and C2) are included. All data are presented as optical density (O.D.) mean ± SEM (n = 3-4). p values are included over the corresponding chart bars for Bonferroni's comparison with the contro l group.

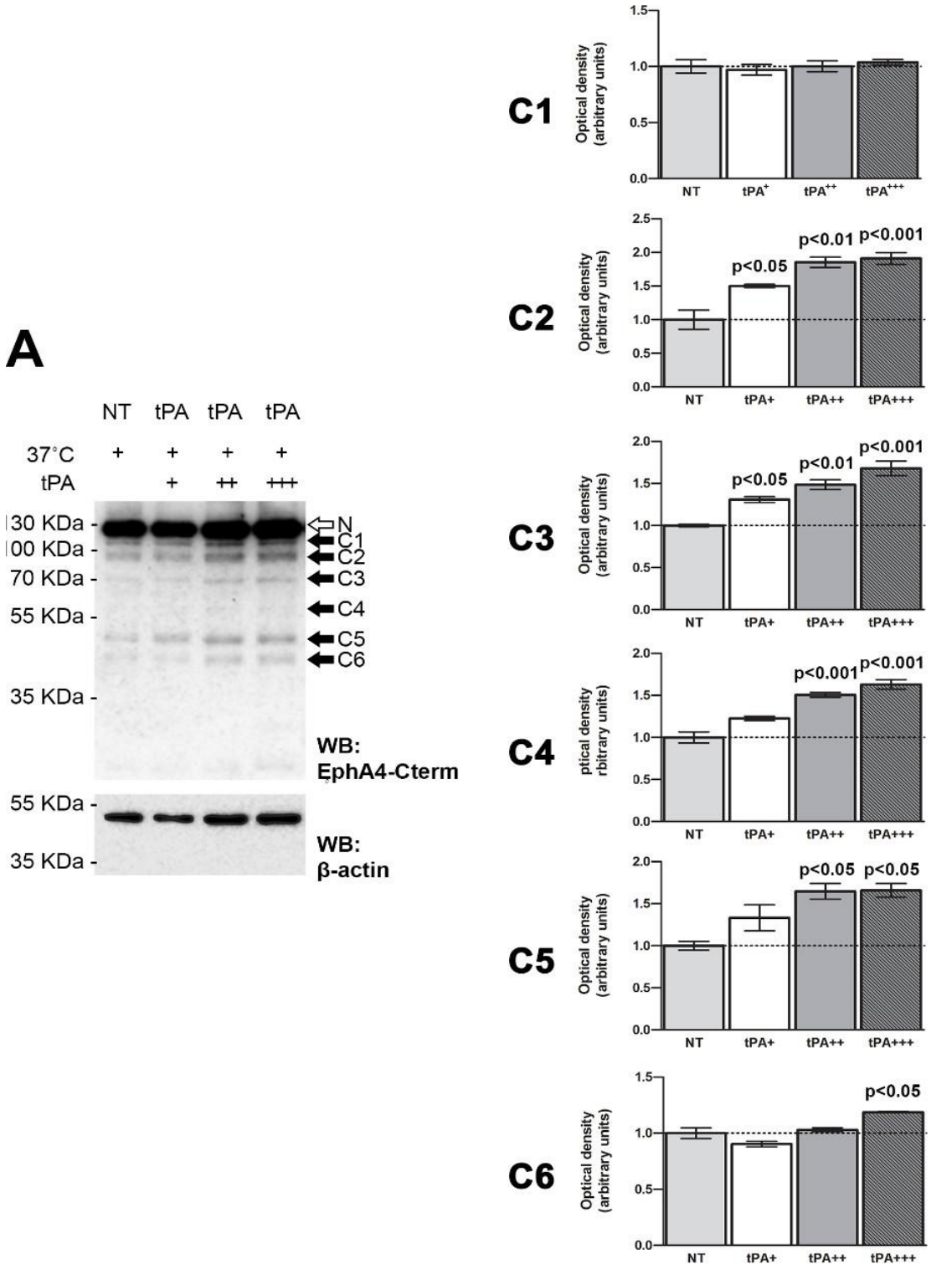


Figure 15. tPA cleaves EphA4 receptor *ex vivo*. (NT = non-treated; tPA = tissue plasminogen activator). The figure shows A) Western blot of the protein levels of EphA4 resulting from tPA treatment of mouse amygdala homogenates. Homogenates were treated for 2 hrs with tPA (+ = 5 µg/mL [79.47 nM], ++ = 25 µg/mL [397.35 nM], +++ = 50 µg/mL [794.7 nM], ++++ = 150 µg/mL [2.38 µM]). The treatment resulted in the increase of lower molecular weight bands or appearance of new bands (C1 to C6 arrows) without affecting the native band (N), which demonstrates the cleavage of EphA4 by tPA without the necessity of plasmin. C1 - C6) show the quantification of the optical density of the cleavage bands corresponding to C1-6 arrows. p values are included over the corresponding chart bars for Bonferroni's comparison with the NT control group.

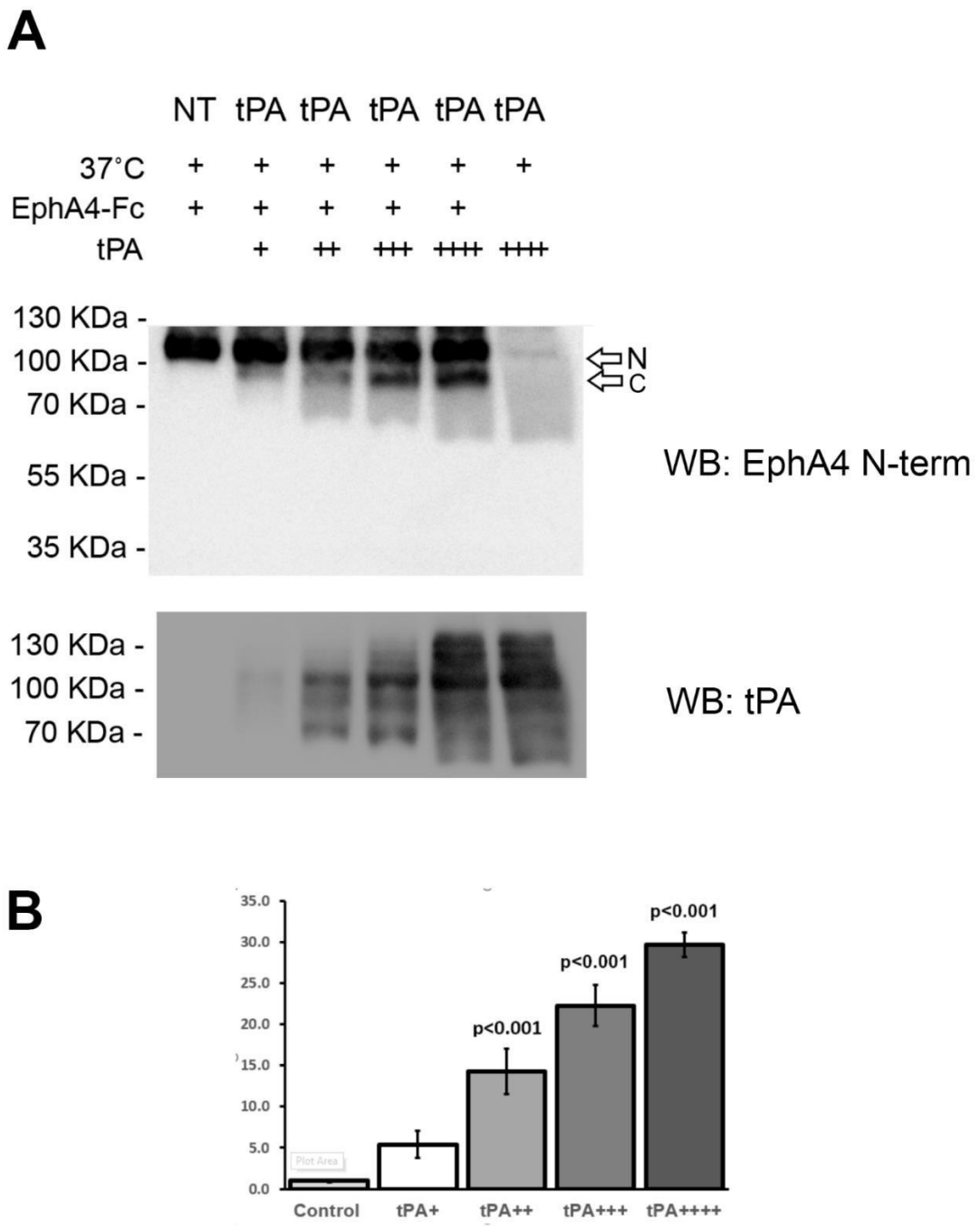


Figure 16. tPA cleaves EphA4 receptor *in vitro* in a dose-dependent manner. (NT = non-treated; tPA = tissue plasminogen activator; N = native EphA4; C = cleaved EphA4). The figure shows representative blots, generated by Western blot technique, of EphA4-Fc protein and its N-terminal fragments as a result of tPA treatment. EphA4-Fc was treated for 2 hrs with tPA (tPA+ = 5 µg/mL [79.47 nM] of tPA++ = 25 µg/mL [397.35 nM], tPA+++ = 50 µg/mL [794.7 nM], tPA++++ = 150 µg/mL [2.38 µM]). A) Representative blots of EphA4 protein (N) and its cleaved N-terminal fragments (C) as a result of tPA treatment. The treatment resulted in the appearance and increase of new of lower molecular weight bands (C), which demonstrates the cleavage of EphA4 by tPA without the necessity of plasmin. The lower blot shows tPA protein levels. B) Quantification of the N-terminal cleavage product (C) from panel A. p values are included over the corresponding chart bars for Bonferroni's comparison with the control group.

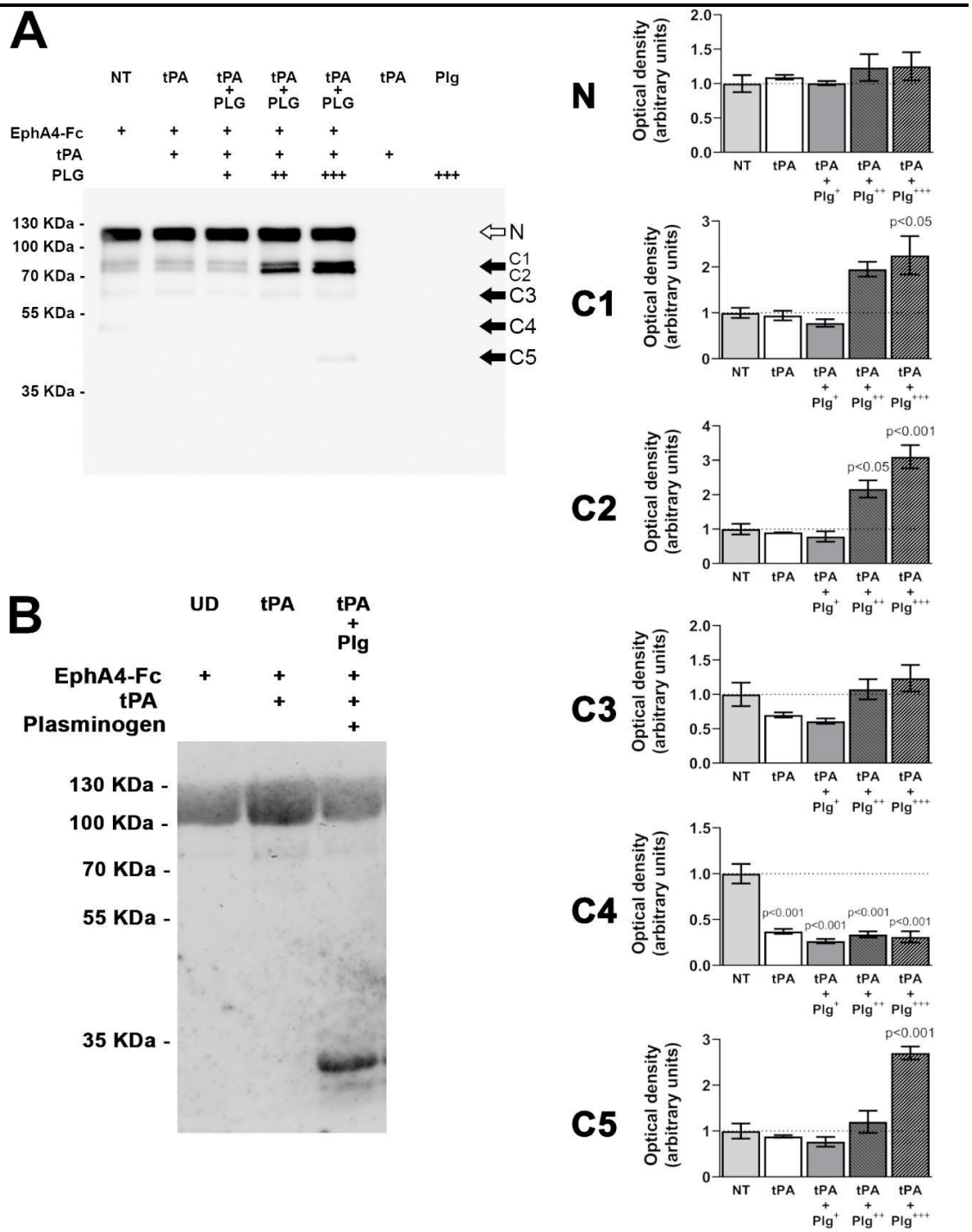


Figure 17. Plasmin and tPA cleave EphA4 receptor *in vitro* in a dose-dependent manner. (UD = undigested; tPA = tissue plasminogen activator; PLG = plasminogen; N = native EphA4; C = cleaved EphA4). The figure shows protein levels of EphA4 resulting from tPA and plasmin treatment of EphA4-Fc. EphA4-Fc was treated for 15 min with tPA (+ = 2.5 µg/mL [39.73 nmol]) and plasminogen (+ = 10 µg/mL [110.41 nM], ++ = 50 µg/mL [552.07 nM] or +++ = 100 µg/mL [1104.14 nM]). The resulting cleavage was analysed by Western blot technique. Representative blots are shown for A) EphA4 and its fragments using EphA4 antibody directed to its N-terminal segment and B) Fc antibody (C-terminal fragments). Histograms depicting the quantifications for A blot bands are included (N, C1-6). The treatment increased lower molecular weight bands or made new bands appear or disappear. This demonstrates the cleavage of EphA4 by tPA and plasmin. All data are presented as optical density (O.D.) mean ± SEM (n = 3-4). p values are included over the corresponding chart bars for Bonferroni's comparison with the NT control.

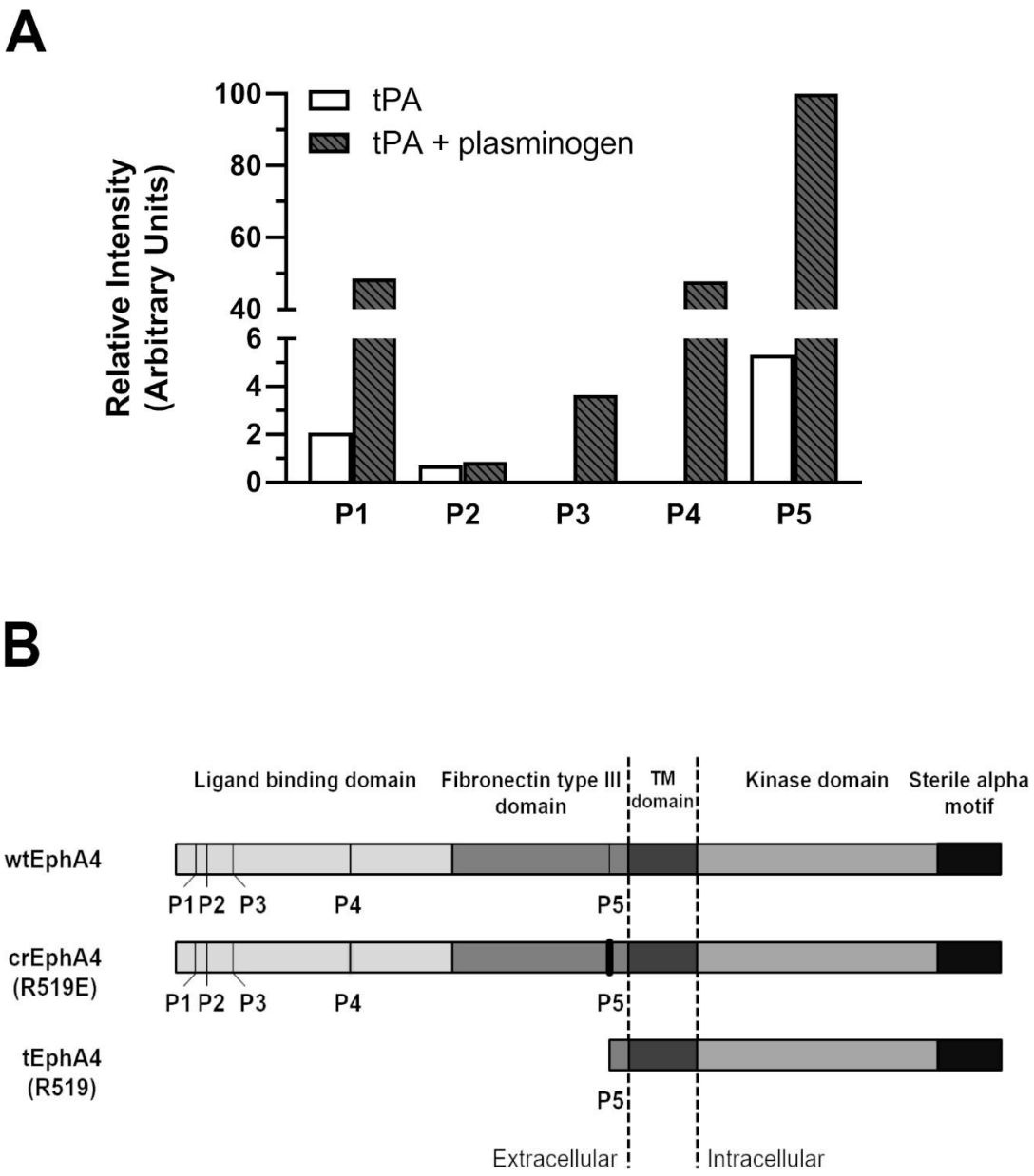


Figure 18. Cleavage products of the proteolysis of EphA4 by tPA and plasmin detected by the amino-terminal oriented mass spectrometry of the substrates (ATOMS) technique. (TM = transmembrane). This technique allowed to identify five tPA and plasmin cleavage sites (P1-P5), of which, three of them are also cleavage sites for tPA (P1, P2 and P5). A) Estimation of the sensitivity of the plasmin cleavage sites towards cleavage by plasmin and tPA. The study revealed that P5, located within the fibronectin type III domain, was most efficiently cleaved by low doses of this protease, which matches the cleavage sites observed on the Western blot results (Figure 14). B) Localisation of the cleavage sites relative to the structure of the mouse EphA4 (wtEphA4), EphA4 mutant resistant to cleavage by plasmin at P5 cleavage site (crEphA4) and a generated EphA4 variant truncated at P5 cleavage site (tEphA4).

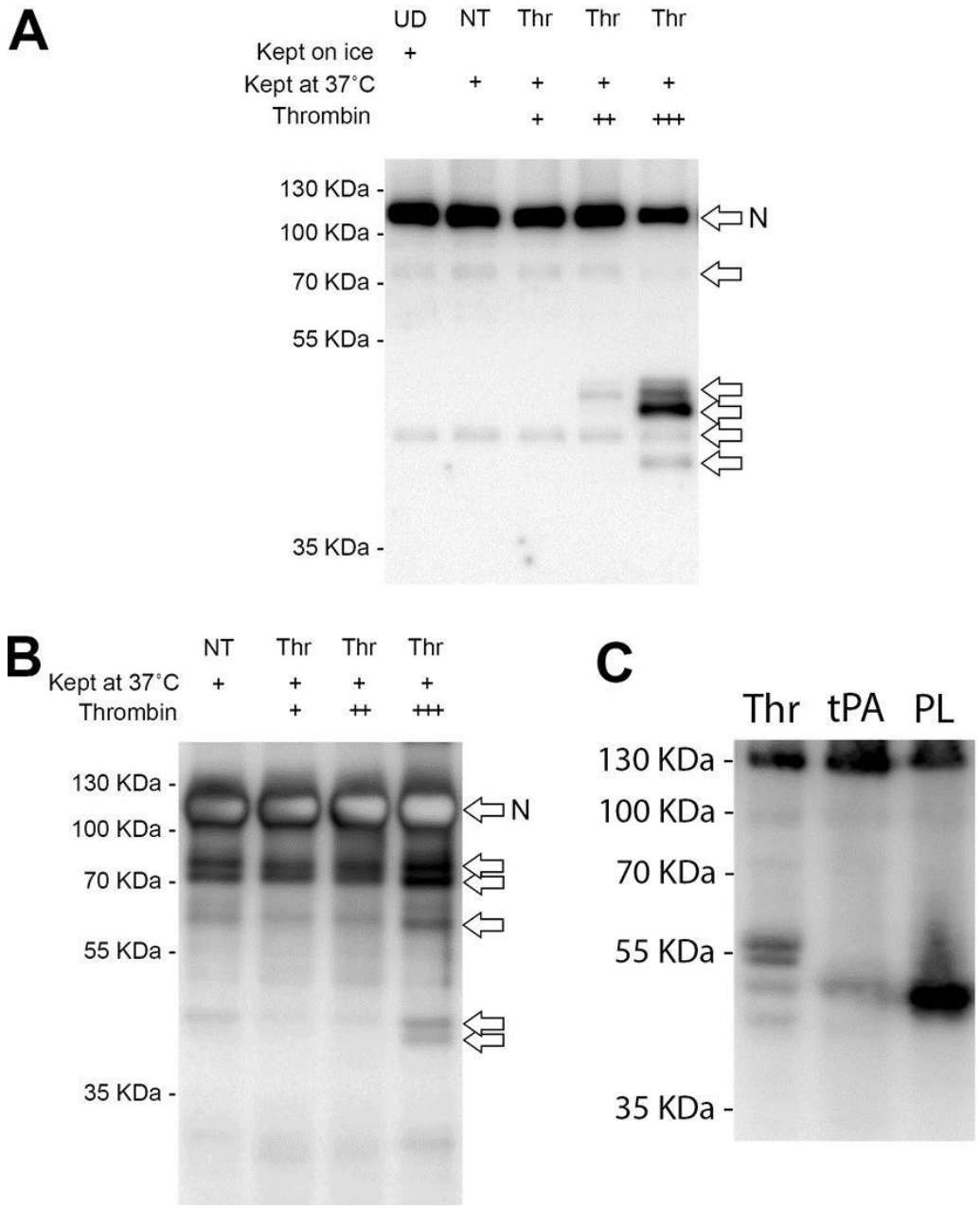


Figure 19. Thrombin cleaves EphA4 receptor *ex vivo* and *in vitro*. (UD = undigested; NT = non-treated; Thr = thrombin; tPA = tissue plasminogen activator; PL = plasmin; N = native). The figure shows representative blots, generated by Western blot technique, of EphA4 protein levels resulting from thrombin treatment of A) mouse amygdala homogenates (anti-EphA4 C-term antibody) and B) mouse EphA4-Fc (anti-EphA4 N-term antibody). Homogenates were treated for 15 min with thrombin (+ = 2 NIH units/mL, ++ = 20 NIH units/mL or +++ = 30 NIH units/mL). The treatment in the homogenates resulted in a decrease of the native EphA4 band (N) along with an increase of lower molecular weight bands or appearance of new bands (white arrows) (A), which demonstrates the cleavage of EphA4 by thrombin. C) Comparison of the bar codes for EphA4 cleavage mediated by thrombin, tissue plasminogen activator or plasmin indicates different processing by these proteases.

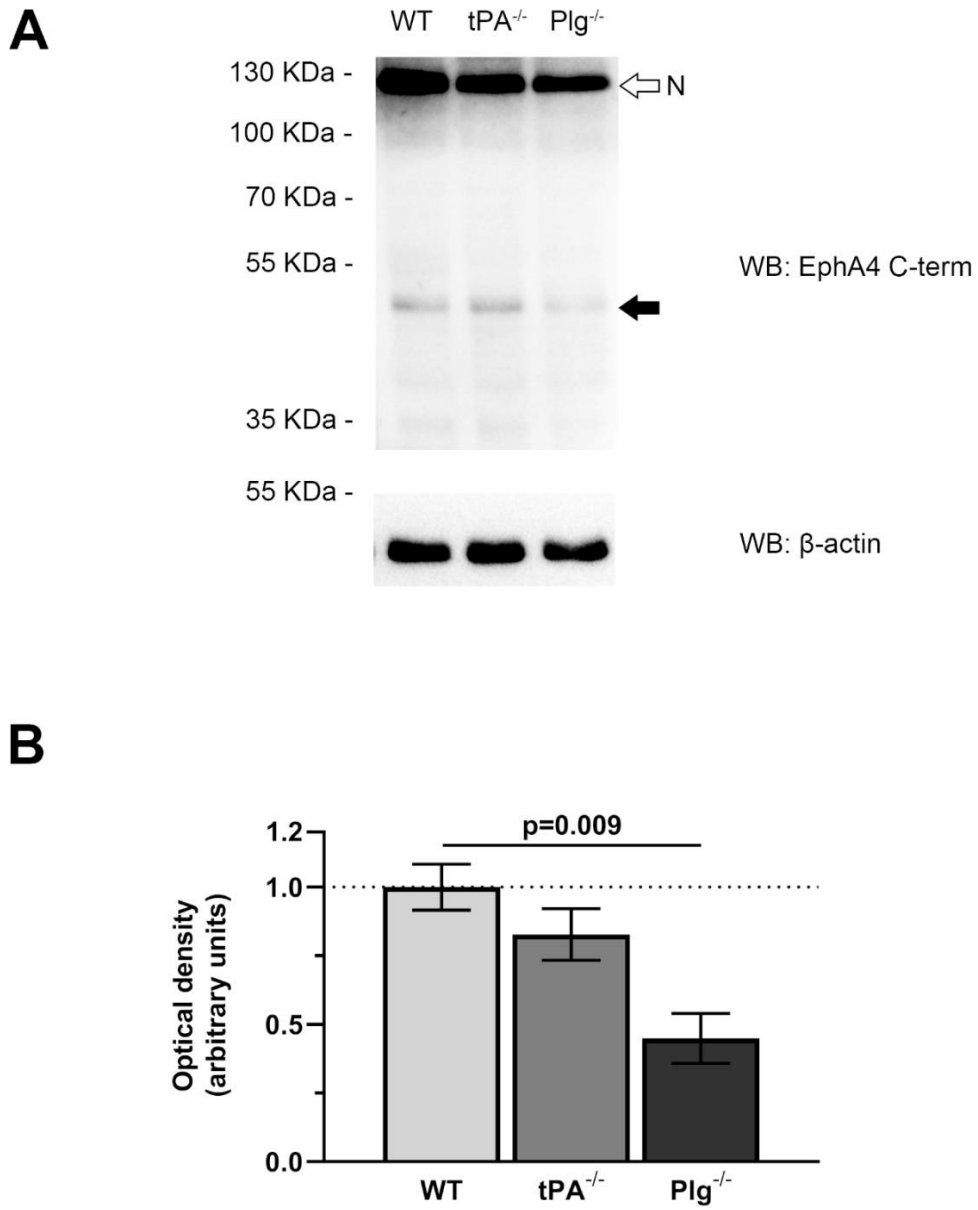


Figure 20. EphA4 cleavage is reduced in the amygdala of unstressed plasminogen knockout mice. (WT = wild type; $tPA^{-/-}$ = tPA knockout; $Plg^{-/-}$ = plasminogen knockout). The figure shows A) a representative blot, generated by Western blot technique, of EphA4 protein levels in the amygdala homogenates of different unstressed mouse strains and B) quantification of EphA4 main cleavage (black arrow on A panel). $Plg^{-/-}$ animals present lower levels of the main EphA4 cleavage product while the native EphA4 band (N) remains unchanged (data not shown), indicating that plasmin may play a role in EphA4 cleavage in the mouse amygdala. All data are displayed here as optical density (O.D.) mean ± SEM (n = 3-4). p values are included over the corresponding chart bars for Bonferroni's co mparison.

DISCUSSION

Introduction to previous works and feasibility of the research

Preliminary work from other members of our lab revealed that the gene and protein expression of Eph receptors are regulated by stress (Attwood, 2016). A piece of this previous work from our lab demonstrated that plasmin cleaves EphA4 in a specific, effective and dose-dependent manner in a human cell line and brain homogenates. Therefore, this seminal work was able to effectively show the cleavage of the human EphA4 receptor by tPA/plasmin. However, in order to test the plausibility of the system *in vivo*, there was a need to translate these observations into a model relevant for the human brain.

The previously-observed biochemical feasibility of the system in a human cell line (SHSY-5Y line) is indicative of a potential cleavage of EphA4 by tPA/plasmin in other phylogenetically closely related mammal species, which are suitable as a model in the studies of the biology of the CNS and behaviour. This is the case of the common house mouse (*Mus musculus*). Rodents are, by far, the most used models in CNS biology. When compared to the human genome, the mouse genome presents a large percentage (85%) of protein-coding regions that produce identical or orthologous proteins (Yue et al., 2014). Among them, all the components of the system examined in this study can be found with a high percentage of homology in the mouse brain: tPA (79.57%), plasminogen (79.06%) and EphA4 (98.58%). The catalytic sites of the two proteases are totally conserved in both species and their catalytic efficiencies are highly comparable when analysing mouse and human correlates (Matsuo et al., 2007). Also, EphA4 receptor presents minimal structural differences in the two species. The interspecies changes in EphA4 structure affect a total of less than 20 amino acids (out of 986) spread throughout the ligand binding domain, the fibronectin type III (FN3) domain furthest from the cell membrane and the SAM domain. Moreover, EphA4's kinase domain and the FN3 domain closest to the membrane remain equal in both species throughout evolution, which could be a sign of conservation of the function developed by those two domains. Of note, no changes in the cDNA

sequence can be detected in the amino acids corresponding to potential cleavage sites in EphA4 by tPA and plasmin, checked with the MEROPS database. Therefore, the close structural and activity similarities between these proteins in *Homo sapiens* and *Mus musculus* makes this latter species an, a priori, suitable animal model for the purpose of our study.

Apart from the biochemical point of view, our laboratory is particularly interested in the study of biological correlates of fear, anxiety and other forms of stress-related conditions. With regard to these brain states of the mind, mice have been proven to satisfy the requirements for face validity, construct validity and predictive validity when various aspects of these conditions were attempted to be reproduced (Hall, 1934; Rodgers et al., 1997; Cryan and Holmes, 2005; Ohl, 2005; Gerlai, Blanchard and Blanchard, 2006; Steimer, 2011; Campos et al., 2013; Andrews, Papakosta and Barnes, 2014). These characteristics (and the cost, accessibility and convenience) made *Mus musculus* an optimal species to study in relation to a potential tPA/plasmin/EphA4 system. With these premises in mind I set to examine this system in the mouse central nervous system.

Tissue plasminogen activator / plasmin cascade and EphA4 in the mouse brain: location and cleavage

The first necessary step of this research was to examine location of the components of the system in the mouse brain. I studied the two brain structures where the system had more potential to be present and physiologically active, i.e. the hippocampus and the amygdala. The rationale behind this choice is that enzymatic activity of tPA (the initiator of the cascade) is the highest in some regions of these structures upon different paradigms, including the restraint stress model of anxiety or kindling (Matys et al., 2004; Melchor and Strickland, 2005; Skrzypiec, Buczko and Pawlak, 2008). Therefore, these areas held a higher potential for a physiologically active cascade involving tPA. Additionally, EphA4 presents its highest protein levels in the hippocampus, cortex, cerebellum and amygdala, which potentially coincided with the location of the serine proteases studied here.

Of note, the theoretical lateral resolution of the used imaging setup has been calculated to be 0.08 μm per pixel for high magnification images and 0.63 μm per pixel for low magnification images. Therefore, this would be the limit co-localisation of proteins in our experiments and this distance should be taken into account when evaluating the results presented here.

Generally speaking, the localisation of the individual components of the cascade described in the “Results” section was in line with previous reports that analysed their activity/protein/mRNA levels (reviewed in the introduction of this chapter). Nevertheless, to my knowledge, this is the first time that immunofluorescence has been employed to localise and co-localise the low protein levels of tPA and plasmin(ogen) in the mouse brain without the expression of any chimeric protein and the use of commercial antibodies.

EphA4 showed high co-localisation with both proteolytic components of the cascade, tPA and plasmin(ogen), in various parts of the amygdala and hippocampus (Figures 2, 3, 6 and 7). Specifically, the higher levels of tPA and plasmin(ogen) in the central amygdala, dentate gyrus and CA2-CA4 areas of the hippocampus made these areas to be favoured in terms of co-localisation with EphA4, and thus in terms of likelihood of interaction (Figures 2 and 6).

In the CeA, tPA was remarkably located in the extrasomal space (Figure 10) which suggests a potential for higher interaction of the components of the tPA-plasmin-EphA4 proteolytic system in that location when compared to neuronal somata or distant extracellular spaces. Additionally, the higher activity of tPA in CeA after restraint stress (Matys et al., 2004; Skrzypiec, Buczko and Pawlak, 2008) may suggest a more influential role of this cascade in this area of the amygdala during stress. CeM is the main output nucleus of the amygdala and it participates in processing information and activating brain areas that control behavioural and physiological responses usually associated with fear and anxiety. Consequently, it is logical that biochemical and signalling changes in this area could affect these kinds of behaviours. The possible outcomes of the involvement of tPA activity in CeA will be further discussed in Chapter 6.

The co-localisation of the components of tPA/plasmin/EphA4 system was also

present in the mouse hippocampus (CA2-CA4 areas and dentate gyrus) (Figure 2), which indicates that the system has the potential to function in areas other than the amygdala. This topological information about the enzymes may entail some information about its function as well. The heterogeneous hippocampus structure along its longitudinal axis has been related to different functions. Previous lesion studies have provided evidence that the dorsal (septal) pole of the hippocampus is implicated in learning and spatial memory, whilst the ventral (temporal) pole regulates emotional and motivated behaviours (Bannerman et al., 2004; Fanselow and Dong, 2010). Ventral hippocampus has strong connections with the amygdala and its accessory nuclei, i.e. the nucleus accumbens, the bed nucleus of stria terminalis, and structures associated with the hypothalamic-pituitary-adrenal axis (Swanson and Cowan, 1977; Witter et al., 1989; Jay and Witter, 1991; Herman et al., 1995; Pitkänen et al., 2000; Butler et al., 2010) and these projections are generally reciprocal. For example, amygdalar afferents to hippocampus selectively target ventral portions of CA3, CA1 and the subiculum (Petrovich, Canteras and Swanson, 2001). Expectedly, altered hippocampal function has been demonstrated to have an impact in the activity of structures associated with emotions, such as the amygdala, the prefrontal cortex or the nucleus accumbens (O'Donnell and Grace, 1995; Moser and Moser, 1998; Seidenbecher et al., 2003; Lisman and Grace, 2005; Maren and Hobin, 2007; Jimenez et al., 2018). And last but not least, the hippocampus also influences the hypothalamic-pituitary-adrenal axis, since hippocampal lesions deregulate hormonal stress response (Jacobson and Sapolsky, 1991; Dedovic et al., 2009); and vice versa, increase in stress hormones can also produce hippocampal dysfunction in both humans and rodents (McEwen and Magarinos, 1997; Herman et al., 2005). Therefore, changes in the activity of the hippocampus by tPA/plasmin/EphA4 cascade could be likely modifying fear and anxiety-like behaviours and will be an interesting potential target for future experiments.

The primary presence of EphA4 in neuronal processes was confirmed by high magnification microscopy. At this level of magnification, EphA4 was observed, mainly, in puncta around cell bodies, which is in agreement with this transmembrane receptor's presence in the synapses belonging to the neuropil, a location that is likely shared with tPA and plasmin (Figure 2, 3, 6 and 7).

Accordingly, lower levels of magnification showed that EphA4 was more abundant in areas primarily occupied by cell processes, disregarding their origin (pyramidal or granule cell layers) (Figure 2 and 6). This is in agreement with EphA4's functions taking place in the cellular processes rather than in the cell body. In particular, changes in synaptic communication upon modifications EphA4 have been revealed and reviewed so far by various groups (Martone et al., 1997; Torres et al., 1998; Buchert et al., 1999; Grunwald et al., 2001; Murai et al., 2003).

In high-resolution images, puncta corresponding to EphA4 overlapped with tPA signal or were located in the close vicinities of the protease, which establishes the plausibility of physical interaction between both proteins (Figure 3, 7 and 10). In addition, numerous tPA and EphA4 puncta were adjacent to each other but not 100% co-localising (Figure 10 B1 and B2). This pattern is very characteristic of proteins belonging to the same synapse but located in the pre and postsynaptic compartments, respectively (Schneider Gasser et al., 2006). Thus, the synapse is a plausible subcellular space for tPA and EphA4 interaction. Indeed, the known location of these enzymes at the subcellular level would support this idea. tPA has been described to be secreted from neuronal growth cones (Krystosek and Seeds, 1981; Wu et al., 2012) and EphA4 has been described to be enriched in the active zone fraction and post-synaptic density fraction (Bouvier et al., 2008). Thus, this localisation makes synapses probable anatomo-functional structures to harbour the interaction between EphA4 and tPA/plasmin system (Krystosek and Seeds, 1981; Tremblay et al., 2007; Wu et al., 2012). Moreover, since areas with the strongest tPA fluorescence also had a strong EphA4 fluorescence, it is plausible that both proteins are expressed following a parallel (if not the same) mechanism of expression, which makes them good candidates to be associated genes in the mouse central amygdala. Further experiments will need to be performed to confirm this hypothesis.

Gephyrin (a postsynaptic marker of GABAergic synapses) was present in synapses of the CeA that exhibited EphA4-tPA or EphA4-plasmin co-localisation (Figures 10 and 11), suggesting that the interplay of these proteins is likely taking place at the GABAergic synapses of this area of the amygdala. This would be

consistent with a model in which plasminogen is located extracellularly at the GABAergic synapse (in the vicinities of EphA4 and tPA), in a way that, after the release of tPA upon neuronal activation, plasminogen is transformed into plasmin and thus this active enzyme can cleave extramembranous proteins to develop its activity. Alternatively, some researchers argue that, in neurons, tPA and plasminogen are incorporated in dense-core granules (DCGs) of dendritic spines and presynaptic boutons (Lochner et al., 2006, 2008; Scalettar et al., 2012). Some of these DCGs contain both tPA and plasminogen, which could suggest an intra-granular activation of plasminogen into plasmin is also possible (Lochner et al., 2008). The exocytosis of tPA from DCGs would be, then, induced by neuronal depolarisation (Gualandris et al., 1996; Lochner et al., 2006).

Adding up to the notion that tPA/plasmin/EphA4 cascade is likely to occur in the GABAergic synapse of the CeA, the EAAT3 glutamate transporter barely colocalises with the initiator of the cascade (tPA), meaning that glutamatergic synapses could be a secondary place for the cascade (Figure 12). Additionally, all EAAT3$^+$ cells seem to be plasmin(ogen)$^+$ (Figure 13); therefore, as the zymogen (plasminogen) can be found in all types of synapses, the specificity for the location of the proteolytic cascade would be limited by tPA.

With regard to the identity of tPA-expressing cells in the CeL, immunostaining experiments performed by Dr Mucha showed that all *tPA*-positive neurons co-expressed different interneuron (GABAergic) markers. PKCδ$^+$ neurons were 100±0% *tPA*+, while a significantly smaller proportion co-expressed corticotropin-releasing factor (13.4±0.5%) or somatostatin (3.3±0.6%). Furthermore, as mentioned in the introduction, the CeL hosts a neuronal population which produces modulation of anxiety-like behaviours upon activation and mainly expresses PKCδ$^+$. Optical stimulation of this population of PKCδ$^+$ neurons provoked increased anxiety-like behaviours accompanied by a decreased α_5-$GABA_AR$-mediated conductance (Tye et al., 2011; Cai et al., 2014; Botta et al., 2015; Wolff et al., 2014). Hence, PKCδ$^+$ (and thus *tPA*+) subpopulation projections present potential anxiogenic properties when they are targeting GABAergic synapses of the CeA (Botta et al., 2015; Wolff et al., 2014).

Eph receptors seem to be mainly associated with dendritic spines and

postsynaptic densities (PSDs) (Martone et al., 1997; Torres et al., 1998; Buchert et al., 1999; Murai et al., 2003; Grunwald et al., 2004). Various works have shown detailed information about the localisation of EphA4 in both pre and postsynaptic terminals. In a seminal work, Bouvier et al. (2008) showed that this receptor was expressed in axon terminals, dendritic shafts and synapses, both pre- and post-synaptically. However, these pieces of information do not show the localisation of EphA4 in amygdala tissue or PSDs of inhibitory GABAergic synapses. Our research provides the first evidence that EphA4 is present postsynaptically in GABAergic inhibitory synapses and that this presence can also be found in the CeL amygdala (Figures 10 and 11). After Bouvier et al., some works on hippocampal cultures have shown that EphA4 is associated with presynaptic vesicles in axon terminals co-localising with synaptophysin, vesicular glutamate transporter (VGLUT) and vesicular GABA transporter (VGAT) synaptic vesicles of cells immunopositive for clathrin-coated vesicles (CCVs). It is known, from previous reports, that membranous EphA4 expression is increased in less than 2 min following potassium chloride depolarisation, indicating an activity-dependent membrane expression (Murai et al., 2003; Tremblay et al., 2007), which is likely related to the described intracellular vesicles containing EphA4 (Bouvier et al., 2010). TPA expeditious release (Lochner et al., 2006) and membranous-EphA4 rapid increase upon neuronal depolarisation (Bouvier et al., 2010) indicate that indeed, tPA-EphA4 interaction could be immediate upon neuronal activity and have important functions driven by experience, which is especially relevant for this project since tPA is liberated after anxiety-related paradigms (Pawlak et al., 2003).

To sum up, our findings present a conspicuous spatial correlation between areas with high EphA4 expression and areas with an inducible tPA/plasmin(ogen) system. The central thesis of this discussion about the localisation of tPA, plasmin(ogen) and EphA4 shows that there is sufficient evidence about their location to support the existence of the tPA-plasmin-EphA4 cascade within the centrolateral amygdala and the CA2-CA4 areas and dentate gyrus in the hippocampus.

In conclusion, our findings suggest a model in which EphA4, plasminogen and

tPA are produced in neighbouring spaces of specific regions, so as, in the appropriate conditions, plasminogen could serve as a substrate for the activity-released tPA and hence develop its functions through EphA4 receptor.

Cleavage of EphA4 by plasmin and tPA

Seminal work from our laboratory provided in vitro biochemical evidence for the cleavage of EphA4 receptor in SHSY-5Y human neuroblastoma cell line using a mix of purified tPA and plasmin. The mix was generated by incubating tPA and plasminogen previous to the treatment of the cells, which allows tPA to cleave the precursor (plasminogen) and transform it into the active form, plasmin. This method was also used in the present work to induce the cleavage of EphA4. The cleavage of EphA4 in SHSY-5Y cells is produced at low concentrations of the enzymes and increases with rising concentrations of plasmin. Of note, other Eph receptors tested were not cleaved *in vitro* at low doses of the protease. Therefore, EphA4 cleavage by plasmin is seemingly specific, effective and dose-dependent in the human SHSY-5Y cell line. This is suggestive of limited extracellular proteolysis that is currently known as “ectodomain shedding”.

Ectodomain shedding affects a large number of transmembrane proteins and regulates a large number of the cellular functions mediated by them. This is especially interesting because previous works from our lab on the serine protease, neuropsin, have demonstrated that the shedding of an Eph receptor (i.e. EphB2) can affect stress and anxiety-like behaviours. Other processes, such as Eph-ephrin interaction role in axon guidance, are also modified by this cleavage. For example, mutations impeding the shedding of ephrin-A2, delay axon withdrawal during the axon guidance, indicating that the ectodomain shedding of ephrins could be a mechanism mediating axon detachment from the ephrin-expressing surfaces (Hattori, Osterfield and Flanagan, 2000). However, the functional significance of an ectodomain shedding is not unique. It depends on various factors that also vary with the sequence of the protein affected and its function. In particular, EphA4 shedding has been shown to affect spinal motor axon guidance (Gatto et al., 2014) and dendritic spine plasticity (Inoue et al.,

2009).

A putative plasmin cleavage site in EphA4

As Figures 14 and 17 illustrate, I was able to demonstrate the direct cleavage of extracellular EphA4 by tPA/plasmin system *ex vivo* (from hippocampus and amygdala homogenates) and *in vitro*. In the literature, the only information to date that shows direct proof of the cleavage of EphA4 by any protease is the initial work from our laboratory presenting tPA/plasmin cleavage (Attwood, 2016). Apart from this document, only two publications related to the cleavage of EphA4 exist. These papers show only indirect evidence of a possible cleavage of EphA4. The first one, by Inoue et al., uses a γ-secretase inhibitor (compound E) and an MMP inhibitor (GM6001) to show a decrease in the processing of an intermediate C-terminal fragment (CTF) of EphA4 (Inoue et al., 2009). The second study, by Gatto et al., shows the reduction of the processing of EphA4 by mutating a group of 15 amino acids in one of the extracellular FN3 domains of the receptor (Gatto et al., 2014). Interestingly, data extracted from the mass spectrometry analysis of EphA4 fragments (Figure 18) shows that the most efficiently processed cleavage site by plasmin and tPA belongs to this group of 15 amino acids which reinforces the likelihood of a *bona fide* cleavage site affected by these proteases at R516. This fact increases the evidence that this particular amino acid hub may be of special importance to the processing and function of EphA4.

Ex vivo experiments in the present thesis also reflect the feasibility of the cleavage in the brain milieu of the hippocampus and amygdala, but it did not exclude the possibility of more necessary actors in this system. To assess the sufficiency of the tPA/plasmin system to cleave EphA4, former members of the lab initiated a series of experiments *in vitro* in which the only actors present in a proteolytic reaction are both enzymes and the extracellular fragment of EphA4. This series of experiments and work from the current project confirmed that these three components (tPA, plasmin and EphA4) were sufficient to produce the extracellular cleavage of EphA4 (Figures 15 and 16). In addition, the present work also provides evidence of the sufficiency of tPA to cleave EphA4 without the

presence of plasmin *in vitro* and *ex vivo* in a cleavage site highly similar to plasmin (Figure 16). Then, EphA4 adds up to the list of the restricted number of proteins that tPA is able to cleave. Nevertheless, the amount of enzyme needed to achieve a cleavage comparable to plasmin was about five times more concentrated (~0.5 μM vs 2.5 μM) and the time of the reaction was eight times longer (2 h), which poses serious objections about the viability of a cleavage only led by tPA in a living system and indicates that the mechanism behind this cleavage *in vivo* would be mainly mediated by plasmin.

Further concurrent evidence supports the hypothesis of EphA4 being cleaved by plasmin in the living mouse brain. A notable piece of evidence from the present work is that the protein levels of the main cleavage product of EphA4 found in the amygdala (which is also the main one produced by tPA/plasmin cleavage) is significantly decreased in unstressed plasminogen-deficient C57BL/6J mice when compared with tPA-deficient or wild type controls (Figure 20). This indicates that plasmin, but not tPA, plays a significant role in the cleavage of EphA4 *in vivo* in the amygdala. It is noteworthy that the cleavage of EphA4 is not entirely absent in the plasminogen knockout animals (Figure 20 A), suggesting that plasmin is not the only enzyme processing EphA4 in the mouse amygdala, which would be in line with previous works in which other classes of proteases are suggested to regulate the cleavage of EphA4 (Inoue et al., 2009). Furthermore, the feasibility of the cleavage in the absence of tPA gene in our experiments (Figure 20) indicates that there must be a different system that allows the activation of plasmin or other compensatory mechanisms to produce the cleavage of EphA4. A possible explanation is that uPA is involved in the process but no cases have been described to date in which uPA is active in the brain parenchyma.

Taken together, the pieces of evidence described so far demonstrate the cleavage of EphA4 by tPA/plasmin cascade in the living mouse brain and suggest an essential role of plasmin in cleaving EphA4 *in vitro, ex vivo* and *in vivo*.

The different cleavage sites produced by tPA and plasmin were identified and localised within the EphA4 ectodomain by mass spectrometry (Figure 18). As expected from the *ex vivo* and *in vitro* data, the most efficiently processed cleavage site (by both tPA and plasmin) is located close to the cell membrane,

indicating a preference of these enzymes for this cleavage point. This tendency to cleave at specific distinct sites close to transmembrane regions is a common feature shared with many other proteolytic systems in which transmembrane proteins are involved. These events are often related to proteases processing the ECM; however, they are also an important source of signalling towards the intracellular space (Werb, 1997; Nava, Kamekura and Nusrat, 2013). Another interesting fact extracted from mass spectrometry data is that the most effectively produced fragments are the same for tPA and plasmin, which implies some biological redundancy with different efficacies. This redundant cleavage with different shedding magnitude could be explained by different requirements on cleavage intensity for alternative mechanisms that might be differently controlled (Delattre and Félix, 2009).

The detected cleavage sites of both plasmin and tPA are in agreement with the cleavage pattern described as their consensus in previous reports compiled in MEROPS and Cutdb databases. To be more specific, the cleavage site is produced N-terminal to arginine residues in all of the cases described in this thesis. Additionally, the most efficiently cleaved site is followed by isoleucine a common amino acid found in this position. Arginine residues in tPA/plasmin´s cleavage sites are also preceded by serine, one of the most common amino acids present in this position (Rawlings, Barrett and Finn, 2016). Therefore, the common pattern on the sequence of amino acids detected in our experiments increases the plausibility of the detected cleavage sites to be *bona fide* excision points.

The postulated main cleavage site in EphA4 structure by tPA/plasmin is located within a FN3 very close to the transmembrane domain. Fibronectin (FN) is a large modular domain composed of homologous repeats that make it a very versatile domain. It is known to be involved in the cell adhesion, migration, differentiation and proliferation. FN3 is the most common type of FN domain and it interacts with different proteins in the extracellular space, such as collagen, heparin, fibrin and cell membrane receptors (Bencharit et al., 2007). FN3 domains tolerate considerable sequence variation without affecting their overall structure or properties. That characteristic makes this type of domain a suitable target for

experimental modifications without affecting its structural properties and interactions. As an example of this robustness, extensive changes in loop lengths up to the insertion of eight amino acids do not change overall stability or entropic properties; with the exception of for proline residues, which result in substantial changes for nucleating and folding characteristics (Steward, Adhya and Clarke, 2002). Moreover, the combination of different loops results in only minor destabilisation (Bloom and Calabro, 2009).

The use of mass spectrometry technique in the present work implies some confounding factors, such as basal proteolysis, which concerns any proteolytic event occurring before the ATOMS experiments. In this case, the use of commercial highly purified target proteins and enzymes ruled out this possibility. Moreover, in the ATOMS process, some repeated small peptides can be generated and these multiplets can be misidentified as one single cleavage site and be erroneously dismissed. This possibility in the current case is very unlikely since the fragments that have been generated with the ATOMS technique would be long enough to not be misidentified.

A further proof that the analysed structures are putative cleavage sites produced by plasmin is that the processing by a different serine protease, namely thrombin, yielded a completely different EphA4 cleavage barcode (i.e. the cleavage pattern of bands created by the fragments produced and normally measured by Western blot) (Figure 19). Importantly, thrombin is generated by cleavage of prothrombin circulating in plasma (Crawley et al., 2007; Licari and Kovacic, 2009); and although prothrombin protein is expressed in neurons, the presence of thrombin or a relevant activation of prothrombin in non-pathological brain has never been proved, even in transgenic mice overexpressing the prothrombin gene (Xi, Reiser and Keep, 2002; Shiosaka, 2004; Arai et al., 2006). Thus, this expression pattern would suggest that the cleavage of EphA4 led by thrombin is highly unlikely in this environment under physiological conditions in the adult mouse brain. Nevertheless, it could become possible in pathologic conditions in which thrombin can be extravasated from the bloodstream (e.g. seizures, stroke, traumatic injuries) (Gingrich and Traynelis, 2000; Gingrich et al., 2000; Sinnreich et al., 2004). Of note, the thrombin-mediated cleavage of ligand-binding domain of

murine EphA4 and the intracellular domain of human EphA4 has been demonstrated in the past (Binns et al., 2000; Qin et al., 2008; Singla et al., 2010; Lamberto et al., 2012); however, the molecular weight of the fragments produced with the murine EphA4-Fc in the present work suggests that thrombin-produced cleavage sites can be closer to the cellular membrane than the sites revealed in previous works (Figure 19). This could have implications for works studying extravasation of blood into brain tissue and its contact with the brain parenchyma.

CONCLUSIONS

EphA4, in the hippocampus, is mainly present in structures formed by basal dendrites rather than axons, although a lesser presence is also detected in areas predominant in cell bodies. EphA4 and tPA macroscopically co-localise to a high degree in the areas in which tPA is more abundant, i.e. projections making input and output connections in CA2-CA4 and dentate gyrus areas. Moreover. subcellular puncta corresponding to EphA4 overlap with tPA signal or are located in the close vicinities of the enzyme, which indicates that these two molecules are likely to be notably interacting in the mouse hippocampus. Immunostainings also indicate that plasmin(ogen) is localised around the cell perimeter in all these areas and co-localises with EphA4, which enables the proteolytic cascade.

Although EphA4 levels in the amygdala are not comparable with the higher levels in the hippocampus, this receptor is present in all sub-nuclei of the amygdala. A marginally more intense signal is observed in the central amygdala. The subcellular location coincides in hippocampus and amygdala, i.e. in scattered puncta or groups of puncta surrounding cell bodies, which suggests its presence in the neuropil.

Co-localisation of EphA4 and tPA is macroscopically evident in the area in which both proteins are most expressed, i.e. the centrolateral amygdala, but minimal or null in other amygdalar areas. The presence of EphA4 and tPA in each other's vicinities indicates that they likely accumulate in spatially related structures, such

as the pre and postsynaptic components of the synapse. Plasmin(ogen) and tPA show a similar pattern of expression in spines of the central amygdala, which indicates that the proteolytic cascade can take place in this structure. Biochemical characterisation of tPA-targeted cells suggests that a portion of this proteolytic process can take place in inhibitory synapses of GABAergic neurons of the centrolateral amygdala, due to the co-localisation of the components of the cascade and the GABAergic postsynaptic biomarker, gephyrin.

The allocation of the components of the cascade would be consistent with a model in which plasminogen is located extracellularly at the GABAergic synapse (in the vicinities of EphA4 and tPA) in a way that, after tPA is released to activate its connate proenzyme, the active enzyme (plasmin) can cleave extramembranous proteins, such as EphA4, to regulate their activities.

Additionally, *in situ* hybridisation experiments show that tPA-mRNA-expressing (*tPA*+) cells in the centrolateral amygdala co-express PKCδ (an interneuron class marker) and that a significantly smaller proportion co-express other interneuron classes, namely corticotrophin-releasing factor or somatostatin. This finding is in connection with previous works where neurons linking the basolateral with central amygdala were demonstrated to be mainly $PKC\delta^{+}$. Data from the present work suggest a model in which tPA and plasmin from $PKC\delta^{+}$ cells have the capacity to cleave EphA4 in GABAergic synapses of centrolateral amygdala, which are, according to the literature, potentially anxiogenic.

It is known from previous works that tPA and plasminogen are upregulated in the hippocampus and amygdala following chronic stress; and that plasminogen can be converted into plasmin in the hippocampus, the amygdala and other areas of the mouse brain. Here, I demonstrated that a distinct cleavage of EphA4 by tPA and by plasmin is possible in amygdalar tissue *ex vivo* (in mouse amygdala homogenised tissue) and *in vitro*. Additionally, *in vivo* analysis of EphA4 cleavage in the amygdala of plasminogen$^{-/-}$ and tPA$^{-/-}$ mice indicate that a fraction of EphA4 endogenous cleavage in the amygdala is due to plasmin but not to tPA presence.

The data presented so far in this work indicates the existence of a tPA-plasmin-

EphA4 system in the brain, and it demonstrates that fibronectin type-III domain of EphA4 would be the preferred topological substrate of a putative endogenous extracellular cleavage by plasmin. The tPA/plasmin cascade's unique cleavage sites on the EphA4 structure are different from the cleavage produced by other thrombin-like proteases, such as thrombin, which argues in favour of the singularity of tPA/plasmin cleavage of this Eph receptor. Further chapters will also describe the effect of this cleavage *in vivo*, especially in stress-induced events.

FUTURE WORKS

Although the present work revealed the existence of the tPA/plasmin/EphA4 cascade at the molecular level, a relevant subsequent question is what are the underpinnings at the circuit level that underlie brain functions (e.g. anxiety-like behaviours) related to the cascade. Knowing which neuronal populations are presynaptically and postsynaptically connected to *tPA*+ neurons will allow to precisely define and modify such circuits and decipher their functionality. These goals can be achieved by using viral tracers. Viral tracers have been extensively used to map input and output connections of defined neuronal populations. They include genetically engineered strains of rabies virus (RABV), which are used to interrogate retrogradely connected networks. When a neuron is infected with RABV, the virus is transferred transneuronally and presynaptically in one direction only from the infected neuron. Then, adequate expression of fluorescent markers (using the virus as a vector) can reveal presynaptic connections with a particular neuron (i.e. *tPA*+ neurons in our case). Analogous transsynaptic transmission of fluorescent markers can be achieved in an anterograde manner in order to tag postsynaptically tPA-targeted neurons by using, lipophilic dyes, radioactively-labelled amino acids or different strains of anterogradely-infecting viruses (e.g. AAV). The main focus of this future work would be those areas in connection with neurons of CeA/hippocampus, expressing tPA and related to stress and anxiety. These would include areas of the amygdala and hippocampus themselves, but also BNST or the hypothalamus.

Amygdala and hippocampus are not the only regions of the brain in which tPA is highly expressed and active. Data not shown in this thesis indicates that the tPA/plasmin system could be present and active in other regions related to stress and anxiety, such as localised neuronal populations of the BNST and hypothalamus. There is evidence that the BNST may be involved in unconditioned fear responses; particularly those responses where there is a less explicit threat stimulus, such as exposure to the elevated plus maze, which would have interesting implications for anxiety behaviours. Consequently, exploration of the tPA/plasmin/EphA4 cascade in these areas would be of interest for further research. These location studies can be combined with functional ones by using optogenetic and pharmacogenetic tools to identify and target individual cell types based on their molecular profile or connectivity (such as GABAergic neurons in CeA).

All classic neuroscientific techniques used to interfere with circuit activity (lesions, electrical stimulation and micro-injections) miss the temporal and spatial resolution to observe how particular changes in one area of the brain tend to affect activity in different systems, circuits or even the brain as a whole. In this sense, overgeneralisation of conditioned fear and deficits in the extinction of conditioned fear are hypothesised to contribute to the development of anxiety disorders. Therefore, brain imaging studies would be useful to study the overactivation of fear/anxiety areas and other patterns of activity affected by tPA/plasmin/EphA4 cascade. Studies on the pathophysiology of anxiety using brain imaging studies (such as functional MRI), tend to suggest overactivity in limbic regions (including the amygdala) during the processing of emotional stimuli, and aberrant functional connectivity between these regions. Therefore, a closer look at the activation of the amygdala and other related regions (hippocampus, mPFC) is a necessary step to understand the influence of EphA4 in stress and anxiety.

As mentioned, areas with the strongest tPA fluorescence also have a strong EphA4 fluorescence and it would be plausible that both proteins are expressed following interconnected mechanisms of expression. It would be of interest to know how tPA and plasmin genes and expression are regulated upon cleavage

of EphA4. Additionally, genetic screenings for up or downregulation of other related genes would be of interest for our research.

Cleavage of EphA4 is not completely missing in the plasminogen knockout animals, which suggests that plasmin could be only one of the various enzymes processing EphA4 in the mouse amygdala. Therefore, identifying other enzymes able to cleave EphA4 would be paramount to understand the regulation of its functions. The use of protease inhibitors or genetic manipulations directed to knock-down specific protease genes will help to address this question.

CHAPTER 4: SYNAPTIC PLASTICITY

INTRODUCTION

The term “plasticity” in relation to the brain was first used in 1890 by the American psychologist William James in *Principles of Psychology*, although others, like Dumont in 1876, and Carpenter in 1874, spoke in terms of “l'Habitude” (the habit) and “adaptive unconscious” respectively before that (Blanco, 2014). Even Freud can be associated with the term in reference to the nervous system and learning (Centonze et al., 2004). In any case, these authors described an idea of inherent modifiability of human behaviour and they linked it to the brain’s physical structure and its nervous paths, pointing to the reinforcing connections of that system as the cause of adopting habits and, hence, as a source of plasticity (Berlucchi, 2002).

Soon after, some prominent researchers endeavoured to find the mechanism that underlies brain plasticity. They all shared the view of the prominent neuroanatomist and Nobel laureate, Santiago Ramón y Cajal, about his neuron theory, which describes the nervous system as an aggregate of neurons separated by very small distances (Blanco, 2014). In particular, Tanzi (in 1893, following an earlier suggestion from Spencer in 1862), proposed that the functional modifiability of interneuronal distances would be the mechanism behind brain’s plasticity, which implies that changes in existing neuronal connections may underlie the mechanism of information storage in the brain. For him, the repetitious activity of a neuronal path (produced as a consequence of changes in the environment) could cause hypertrophy of the neurons involved in that path, which would reduce the interneuronal distance; thus making it less difficult for nervous excitation to cross that distance. Sherrington, in 1897, named that interneuronal space “synapse” and described the unidirectionality of transmission through the neural pathway. After that, Tanzi’s disciple, Lugaro, added to his mentor’s hypothesis proposing the chemical nature of the synaptic transmission and finally connecting it to the concept of plasticity (Berlucchi, 2002; Berlucchi and Buchtel, 2009).

The early ideas of James, Cajal, Tanzi and Lugaro were temporarily neglected until Konorski and Hebb, in a paradigmatic change of ideas, incorporated these old concepts into postulates suggesting that long-lasting alterations in cellular

processes (such as processes' growth or metabolic changes), as well as formation of novel synapses, may change as a result of neuronal excitation (Berlucchi, 2002; Berlucchi and Buchtel, 2009). Long-lasting changes in neurons captivated great attention after the work carried out by Bliss and Lømo (Bliss and Lømo, 1973). They discovered that a brief tetanic stimulation (high-frequency electrical stimulation) of neurons produces a long-lasting form of electrophysiological potentiation of postsynaptic neurons. This phenomenon was termed long-term potentiation or LTP and can last for hours or days in the mammalian hippocampus. Since then, many laboratories have been studying LTP as a cellular model for information storage in the brain. For example, similar changes have been described after fear conditioning within the amygdala (McKernan and Shinnick-Gallagher, 1997). In addition to LTP, Lynch et al., in 1977, first described a long-lasting decrease in the electrophysiological response upon neuronal stimulation, which was termed long-term depression (LTD). These findings and other supporting evidence precipitated the idea of LTP (or LTD) being a process that produces structural changes that give rise to some aspects of information processing and therefore, plasticity. Thus, LTP and LTD can be considered as plastic changes since these lasting functional changes are accompanied by lasting structural changes such as the integration of new AMPA receptors on the cell membrane or the growth of new synaptic contacts (see Yuste and Bonhoeffer, 2001).

Furthermore, dendritic spines are thorn-like structures that receive input from a single axon at the synapse. Before the 1980's it was commonly assumed that spines were relatively stable structures. However, in 1982, Crick proposed that spines can move ("twitch") in response to synaptic stimulation (Crick, 1982). This has been later confirmed with novel techniques and it opened the question of the functionality of this motility element (Chen and Sabatini, 2012). Moreover, there is increasing evidence that the morphology, number and density of spines directly reflect functional characteristics of neurons and synapses (Yuste and Bonhoeffer, 2001 for review). Extensive literature has been produced about this topic and it would be difficult to cover it here in detail; however, I will mention some notable works that will provide the reader with examples to approach this large body of research in the next sections. In particular, regarding the work performed in this

thesis about the spine morphology modification upon EphA4 cleavage.

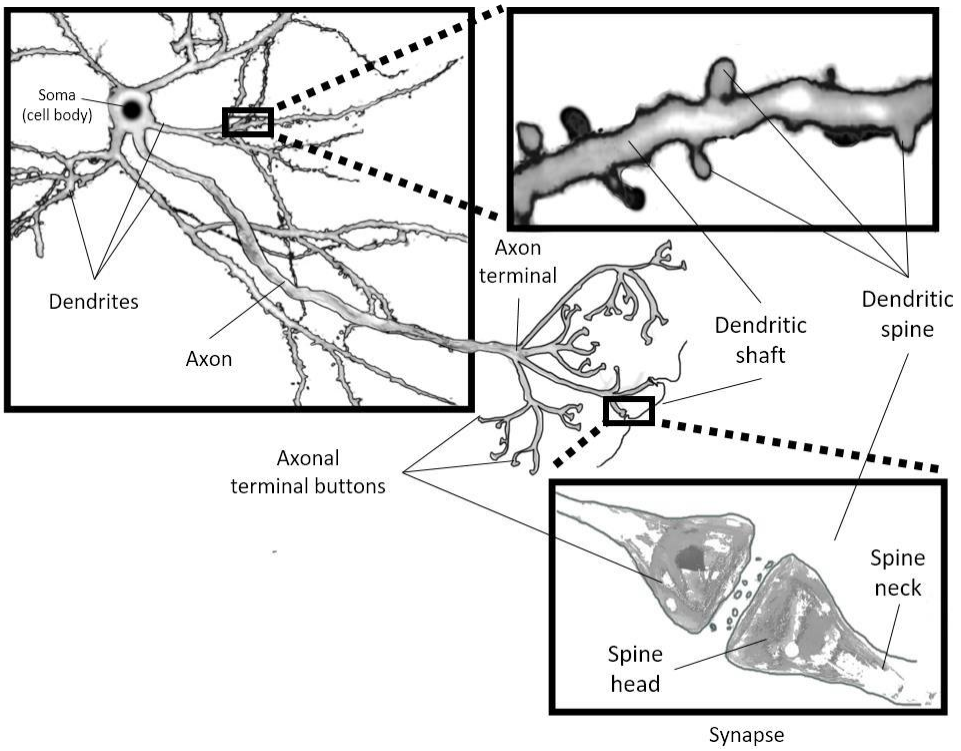


Image 8. Anatomy of the neuron.

Dendritic spine morphology

Dendritic spines were first acknowledged as a natural neuronal structure (rather than a Golgi's technique artefact) by Cajal. He described dendritic spines as "thorns or short spines" emanating from dendrites in his work about the avian (i.e. chicken and duck) cerebellar cortex. Cajal also observed that dendritic spines were present in various neuronal subtypes and brain regions in a similar way across several animal species, including the human being (Yuste, 2015; Gipson and Olive, 2017). Nowadays it is well known that spine-containing ("spiny") neurons can be found in numerous human brain regions. The most intensively studied are the pyramidal cells of the cerebral cortex, the medium spiny neurons of the dorsal and ventral striatum and the Purkinje cells of the cerebellum (which were initially studied by Cajal). However, some amygdalar cell types have also been a target for these studies. It is also known that the vast majority of excitatory

inputs that neurons receive are located at the dendritic spines (more than 100,000 dendritic spines can be found in a single neuron) whereas dendritic shafts are practically missing these connections (Yuste, 2010).

The posterior development of high-resolution microscopy techniques (e.g. confocal microscopy or two-dimensional transmission electron microscopy) and three-dimensional reconstructions enabled the observation of the high variability in dendritic spine morphology in space and time. Typically, spines consist of a rounded “head” or “head apparatus” (which is an enlarged tip of the spine that receives primarily excitatory synaptic inputs) atop a thinner “neck” or “neck apparatus” emanating from the dendritic shaft. Most common dendritic spines are about 1–3 μm in length, from which ~1 μm corresponds to the head diameter and ~1 μm to the spine neck, which presents a width of about 100 nm (Yuste, 2010; Harris and Spacek in Stuart, Spruston and Häusser, 2016).

During the development of the nervous system, there is an abundance of immature long “filopodia-like” spines (between 2 and 10 μm long). They are considered “immature” or a “transient” phenotype because they contain fewer organelles, no differentiable head apparatus, they present fewer membrane receptors and lack synaptic inputs, which makes them dysfunctional (or “inhibited”) in terms of synaptic transmission. However, this type of spines is highly dynamic, since it is able to extend or retract within minutes, or even seconds, of chemical stimulation (Fischer et al., 1998; Harris, 1999). Additionally, upon increased synaptic input (either chemical or electric), this type of dendritic spines is able to develop more “mature” and “stable” phenotypes (Bourne and Harris, 2007).

Dendritic spines in adult neurons can be classified into subtypes according to their morphological and physiological properties. The most used criteria used to classify spines use categories based on their overall morphology. For example, simple dendritic spines often include “stubby” or “sessile” spines, which are only different in length. They are relatively short (<0.5 μm), miss neck apparatus and are characterised as being less variable in time. This categorisation also includes “pedunculated” spines, which means that their head apparatus is wider in diameter than the neck. They are often classified as “mushroom” spines;

however, the same pedunculated spines with narrower diameters are often called “thin” spines. Additional less-frequent types can be found in specific neurons (e.g. crook thorns in cerebellum or gemmules in the olfactory bulb). Sometimes, mature spines present two (bifurcated) or more (multi-branched) processes with fully functional heads, thus allowing for a less classical classification related to complex morphologies. These include synaptic crests, claw-like glomerular endings, brush endings and thorny or coralline excrescences. Of note, the frequency of these structures is less than simple dendritic spines (Harris and Spacek in Stuart, Spruston and Häusser, 2016).

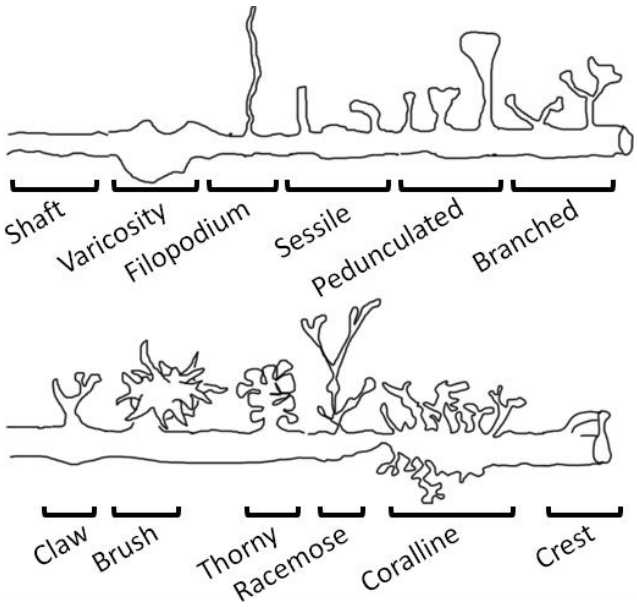


Image 9. Types of spines. Adapted from Stuart, Spruston and Häusser, 2016.

Nevertheless, many authors argue that this way of classification may not be adequate to describe the constant and subtle changes in the dynamics of spines (Gipson and Olive, 2017). Categorical approaches to quantifying spine plasticity (for instance, number of “mushroom-shaped” versus “filopodia-like” spines, which is a parameter often used to interpret spine maturation) miss the perception of the many measurements of spine morphology (e.g. head and neck diameter, volume, length, ratio of head to neck diameter) that follow a continuum; and thus,

they are not suitable to measure subtle differences in spine morphology (Arellano et al., 2007). The “thin” and “filopodia-like” spines are a good example of this. They are similar in shape and may have similar diameters, but the length of the spine that makes them fall into one or another category is not totally agreed upon. Therefore, it is considered that measurements based on distributions of spine features are a more reliable method of classification when analysing spine morphology.

Hypothesised functions of dendritic spines

As described in the previous section, dendritic spines morphologies are diverse and dynamic; and since their discovery, their functional significance or the rationale behind their existence have been a source of speculation (Crick, 1982; Yuste, 2015). Cajal hypothesised spines were used to increase the surface area of dendrites and hence increase their capacity of receiving synaptic inputs (Yuste, 2015). This is an idea that is still present and defended. After that, Rall et al. ventured a quantitative prediction, wherein the morphology of the spine could influence synaptic function; specifically, whether shortening of the spine neck could lead to an increase in synaptic strength (Chang, 1952; Segev, Rinzel and Shepherd, 1994). Strength is defined as the amount of current or voltage produced in the postsynaptic terminal by an action potential in the presynaptic terminal. These initial works led to further work on activity-dependent changes in spines’ morphological structure (Fifková and Van Harreveld, 1977), and to more recent research that has described changes in spines after functional manipulations (reviewed in Bonhoeffer and Yuste, 2002 and Pickel and Segal, 2015).

For instance, it is known that the volume of the spine-head is directly proportional to the number of docked vesicles in the presynaptic terminal (Schikorski and Stevens, 1999) and to the number of receptors (Nusser et al., 1998) and PSD area (Harris and Stevens, 1989; Schikorski and Stevens, 1999) in the postsynaptic glutamatergic terminal. This data poses the spine head volume as a parameter that is highly likely to be directly proportional to the strength and

genuineness of a synapse. Postsynaptic enlargement can also be produced rapidly and selectively in stimulated spines in response to glutamate release and this is associated with an increase in the amplitude of AMPA receptor-mediated currents at the stimulated synapse but not at the neighbouring ones (Matsuzaki et al., 2004; Okamoto et al., 2004). Therefore, observations show that, in general, increased synaptic strength correlates with higher dendritic spine head diameter and volume (Sala and Segal, 2014; Ryan et al., 2015). On the contrary, depotentiating stimuli (either electrically or chemically induced) leads to spine retraction and shrinkage or even loss of the spines (Nägerl et al., 2004; Zhou, Homma and Poo, 2004; Wang, Yang and Zhou, 2007; Oh, Hill and Zito, 2013). Spine shrinkage is persistent but reversible, as it can be reverted by a potentiation stimulus (Bosch and Hayashi, 2012). Additionally, LTP- or LTD-induced changes seem to vary between brain regions, neuron subpopulations or even in the same dendritic shaft; however, these changes are more consistent within specific dendritic segments determined by the distance from the soma (Bosch and Hayashi, 2012; De Roo et al., 2008; Fortin, Srivastava and Soderling, 2012; Yuste and Bonhoeffer, 2001). In connection with these results, spine morphology has been reported to respond to synaptic activity through AMPA and NMDA receptors in dissociated culture (Fischer et al., 2000; Korkotian and Segal, 2001). Nonetheless, the same effects were not observed in organotypic slices (Dunaevsky et al., 1999), which poses doubts about this thesis.

Moreover, part of the literature on spine morphology has emphasised the relation between the size of the spine head and the length of the spine with the calcium dynamics in the spines (Majewska, Tashiro and Yuste, 2000; Yuste, Majewska and Holthoff, 2000; Hering and Sheng, 2001; Bonhoeffer and Yuste, 2002). Specifically, Majewska et al. related the spine neck length to the time constant of calcium compartmentalisation (Majewska, Tashiro and Yuste, 2000; Yuste, Majewska and Holthoff, 2000; Sabatini, Oertner and Svoboda, 2002) and to the filtering of electrical potentials (Svoboda, Tank and Denk, 1996; Araya et al., 2006). Therefore, the spine neck morphological parameters could also tell us about the strength of a synapse and about its calcium-dependent dynamics. Finally, more recent super-resolution microscopy and theoretical approaches argue about the effects of dendritic morphology on cytoplasmic and membrane

diffusion of proteins and whether that responds to a structural compartmentalisation (Adrian et al., 2014).

With respect to GABAergic synapses, there is a common conception that they are all symmetrical synapses (or type II) and locate on the surface of either the dendritic shafts or the soma of neuronal cells. However, there are reports of experiments *in vivo* in which inhibitory synapses residing on dendritic spines of pyramidal neurons of the mouse cortex can reach approximately 30% of the total of inhibitory synapses (Chen et al., 2012; van Versendaal et al., 2012). These dynamic synapses are always found next to an excitatory synapse on the same spine, and can directly inhibit excitation onto that synapse (Villa et al., 2016). Nonetheless, data from other brain regions and neuron types are still elusive for this type of spines. In any case, GABAergic synapses can also experience structural remodelling that results in the appearance or disappearance of inhibitory contacts and changes of synaptic size and morphology. These rearrangements have been recognised as an important mechanism of activity-dependent regulation of the GABAergic function. In GABAergic connexions, more stable and functionally stronger synapses are marked by increased density of $GABA_AR$ accompanied by enlargement of the synaptic area and the presynaptic bouton (Flores et al., 2014). Electron microscopy studies have shown that increased synaptic strength in inhibitory synapses produces a coordinated insertion of $GABA_ARs$ and enlargement of the PSD that precedes gephyrin insertion (Flores et al., 2014). However, specific morphological changes in GABAergic spines have not been described. Moreover, functional consequences of synaptic morphological plasticity depend on the depolarising effects of the presence of GABA receptors and these will be only understood once upstream and downstream signalling targets are understood.

Summarising, changes in size and shape of spines have been documented *in vitro* (Fischer et al., 1998, 2000; Dunaevsky et al., 1999; Korkotian and Segal, 2001) *and in vivo* (Lendvai et al., 2000; Grutzendler, Kasthuri and Gan, 2002; Trachtenberg et al., 2002; Majewska and Sur, 2003). However, the connection between morphological modifications of synaptic spines and specific functions remains correlational since systematic *in vivo* manipulation of specific

characteristics of spines has not been achieved so far. Notably, a recent pioneering study tackled this idea by using light-induced shrinkage of those spines that were potentiated during motor learning to disrupt newly acquired motor skills (Hayashi-Takagi et al., 2015).

All these findings are a substantial base of data indicating that the spine structure is tightly related to synaptic function.

Stress' influence on spine morphology of amygdalar structures

As reviewed in Chapter 1, it has been demonstrated that repeated aversive experiences can be a modifier of many processes in the brain, and dendritic spine morphology is not an exception. Preclinical models of stress (such as restraint stress, social stress or unpredictable stress in rodents) have helped to elucidate some of the influences of stress in dendritic spine morphology. In particular, acute restraint stress (ARS) and chronic restraint stress (CRS [repeated stress for 21 days]) have been suggested to produce functional and structural changes in the hippocampus, the PFC and the amygdala (McEwen, 2010).

The reason to study these areas is that PFC, amygdala and hippocampus are interconnected and influence each other via direct and indirect neural activity. In general, observations provide evidence that stress increases spine density and the excitability of amygdala and NAc neurons, whereas it reduces spine density and impairs LTP in the PFC and hippocampus neurons (Rosenkranz et al., 2011).

In the amygdala and the NAc of rodents (subcortical limbic structures involved in anxiety regulation), stress generally results in an increase in spine density, complexity and synaptic strength. Acute restraint stress for 2 h on male Wistar rats did not produce an increase of spine density or arborisation after 1 day of exposure but it did after 10 days. Simultaneously, ARS causes an increase in spine density without any effects on dendritic arbours in BLA spiny neurons (Mitra et al., 2005), which shows that these neurons could be sensitive to stress. The only work providing evidence of the effects of a single prolonged stress session in rat's CeA (2 h of restraint stress plus 20 min of forced swimming) reported no

change in this area, but it shows an increase of the dendritic arborisation in the BLA (Cui et al., 2008).

In turn, exposure to chronic stress has been profusely studied and literature shows that it can also modify spine dynamics. The most commonly used paradigms in experimental animals are either chronic unpredictable mild stress (CUMS) (14 days to 8 weeks) or restraint stress (2 h daily for 10-21 days) (Magariños and McEwen, 1995; Magariños et al., 1996; Lambert et al., 1998; Conrad et al., 1999; McKittrick et al., 2000; Sousa et al., 2000; Brunson et al., 2005; Pawlak et al., 2005; Donohue et al., 2006; Qiao et al., 2016). In the case of CRS in the rat brain, stress is related with an increase of dendritic length, hypertrophy of dendritic arborisation, increased spine density, enlarged synaptic connectivity and an increase in LTP in basolateral amygdalar spiny neurons receiving thalamo-amygdalar connections. Interestingly, recovery period after stress is not enough to reverse these changes (Vyas et al., 2002; Vyas, Bernal and Chattarji, 2003; Vyas, Pillai and Chattarji, 2004; Mitra et al., 2005; Liston et al., 2006; Vyas, Jadhav and Chattarji, 2006; Padival, Blume and Rosenkranz, 2013; Padival, Quinette and Rosenkranz, 2013; Suvrathan et al., 2013). CUMS in rats is associated with an increase of the synaptic area and increase of PSD thickness of the active zone in BLA (Li et al., 2015).

Male mice were also shown to undergo increase of dendritic length and density of dendritic spines in the BLA upon chronic stress (Bennur et al., 2007; Qin et al., 2011; Pillai, Anilkumar and Chattarji, 2012; Hill et al., 2013). These neurons are thought to be glutamatergic. In contrast, CRS causes a decrease in the spine density of medium spiny neurons (MSNs) in the medial amygdala, which is mainly composed of GABAergic neurons (Bennur et al., 2007). This latter compelling work also links anxiety-like behaviour to tPA in the mouse brain. Interestingly, $tPA^{-/-}$ mice showed attenuation of stress-induced reduction in spine density in the MeA, but not in the BLA, providing evidence that tPA could play a role in stress-induced amygdalar spine plasticity in GABAergic neurons. In mice, just as in rats' brains, CUMS is associated with increased spine density and length in the BLA (Sharma and Thakur, 2015). Chronic social defeat stress (CSDS) in mice also increases spine density, especially on stubby spines of BLA and the MSNs

of the NAc (Zahm, 2000; Sah et al., 2003).

Repeated exogenous administration of the stress-related glucocorticoid, corticosterone (CORT), has also been proposed as an animal model of stress-induced depression-like behaviours (Gregus et al., 2005). CORT is the main stress-related steroid released in rodents after HPA axis activation in the stress response. Long-term administration (21 days) of increased levels of this hormone induce anxiety and depression-like behaviours in male rats. Moreover, this chronic treatment in the drinking water produces an anxiogenic effect in mice (Gourley et al., 2008). A key advantage of CORT administration when compared to stress models is the reduced bias caused individual differences in the regulation of HPA axis (Gregus et al., 2005) and in the CUMS test (Vyas et al., 2002). However, despite CORT's relation with stress, in the literature, spine dynamics have been studied independently of actual stress exposure in animals. Along with many other brain regions, the amygdala and prefrontal cortex also contain adrenal steroid receptors (Ahima and Harlan, 1990; Ahima, Krozowski and Harlan, 1991) and excitatory amino acids that appear to play a role in stress-induced dendritic retraction (Martin and Wellman, 2011).

Short-term injection of CORT has not been profusely investigated. As mentioned, a single acute stress exposure produces increased spine density but not increased dendritic branching or length at 10 days after treatment (Mitra et al., 2005). This result could be reproduced by a single, acute injection of glucocorticoids (Mitra and Sapolsky, 2008). However, administration of glucocorticoids before a traumatic-stress event prevents the delayed increase (of 10 days) in the density dendritic spines (McEwen et al., 2012); and, at least one report, shows no difference in spine dynamics when comparing to sham controls (Mitra and Sapolsky, 2008; Zohar et al., 2011).

Stress-related hormones, even if they are not exogenously administered, have various effects on chronic stress. In rats, prolonged exposure to CORT (daily injections for 3 weeks) shows a limited effect on the neuronal plasticity of the BLA's pyramidal neurons (Morales-Medina et al., 2009; Monsey et al., 2014). However, in mice, CORT for 20 days mimics chronic stress, meaning that it produces increases in dendritic length and spine density in the BLA (Gourley,

Swanson and Koleske, 2013).

These different results pose the area of the brain, the timing and the magnitude of the stress response as important factors for the effects produced by stress and corticoids exposure in the amygdala.

The role of tPA in structural and functional synaptic plasticity

TPA has the potential to be a modulator of neurotransmission and synaptic plasticity as its spatial and temporal localisation demonstrate. Seminal experiments on LTP electrophysiology of hippocampal organotypic slices set the foundations for this hypothesis. As mentioned in this work, tPA's mRNA and its protein expression are upregulated after LTP paradigms. In a like manner, inhibition of tPA activity or absence of the tPA gene impaired the late-phase LTP (Frey, Müller and Kuhl, 1996; Huang et al., 1996; Baranes et al., 1998; Zhuo et al., 2000; Pawlak et al., 2002) and, in the case of $tPA^{-/-}$, also long-term depression (LTD) in striatal slices (Calabresi et al., 2000). On the opposite side of the spectrum, overexpressing tPA in neurons of hippocampal slices from $tPA^{-/-}$ mice, produces increases in both LTP and paired-pulse facilitation (increased postsynaptic potential evoked when that impulse closely follows a prior impulse) magnitudes compared to wild-type mice, which supports the idea that tPA activity can modify synaptic function (Madani et al., 1999; Pawlak et al., 2002).

Evidence from behavioural experiments also adds to the thesis that tPA modifies synaptic plasticity. Although $tPA^{-/-}$ mice do not exhibit obvious behavioural deficiencies, they display impairments in various learning and memory tasks. These include impaired escape latencies in active and step-down avoidance tests, modified reactivity to spatial and object novelty, lower freezing levels in contextual fear conditioning and altered acquisition of a cerebellum-dependent motor learning task (Huang et al., 1996; Madani et al., 1999; Calabresi et al., 2000; Pawlak et al., 2002; Seeds, Basham and Ferguson, 2003; Benchenane et al., 2007). In line with the *in vitro* experiments, mice overexpressing tPA in the CNS exhibit better scores in hippocampal-dependent spatial memory measured

by both Morris water maze and homing hole board tests (Madani et al., 1999; Pawlak et al., 2002); but the lack of tPA also seem to produce impairments in hippocampal-dependent memory tasks in $tPA^{-/-}$ mice (Huang et al., 1996; Calabresi et al., 2000).

Importantly, as mentioned in Chapter 1, absence of tPA can affect chronic stress paradigms in mice. $tPA^{-/-}$ animals show lower levels of anxiety In EPM and startle reflex) when compared with wild type animals. Moreover, although wild type mice present a stress-induced decrease in the spine density of CA1 pyramidal cells, this effect is not present in $tPA^{-/-}$. Both results correlate with impaired learning in the Morris water maze paradigm (Pawlak et al., 2005).

Other studies also indicate that tPA/plasmin activity allows for the reorganisation of connections in the visual cortex after monocular deprivation (a different type of plasticity-related paradigm), potentially by shortening dendritic spines and reorganising the ECM, which reinforces the idea that this proteolytic system contributes to synaptic plasticity (Melchor and Strickland, 2005).

Expanding the view of this system's synaptic plasticity functions, morphine and ethanol addictions are also considered a form of adaptive synaptic plasticity. Models for these disorders showed elevated tPA expression in the NAc (morphine) and limbic system (ethanol). Effects (seizures) produced by morphine addiction are markedly reduced in tPA-deficient and plasminogen-deficient mice. In line with these results, processes of ethanol consumption and withdrawal also increase tPA activity. Additionally, tPA-deficient mice also present reduced ethanol withdrawal seizures, which suggests that tPA may play a key role in these conditions (Melchor and Strickland, 2005).

As summarised here, a number of studies have implicated tPA as a modulator of synaptic activity. However, there is some debate surrounding the underlying mechanisms (see Samson and Medcalf, 2006), which are not entirely understood. A proposed mechanism involves the cleavage of the GluN1 subunit of NMDA receptors (Matys and Strickland, 2003; Samson and Medcalf, 2006; Nicole et al. 2001). In the proposed models, tPA treatment produces enhancement of NMDA signalling, which in turn generates an increase of calcium

permeability and intracellular calcium levels. In an *in vitro* model of excitotoxicity, these high levels of calcium eventually produce neuronal damage. It has been demonstrated that this tPA-dependent excitotoxicity is plasmin-dependent (e.g. Tsirka et al., 1997. Alternatively, tPA interacts with NMDA receptors in a non-proteolytic way through its affinity with the GluN2B subunit, which leads to an increased phosphorylation of GluN2B and the activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway (Pawlak et al., 2005; Norris and Strickland, 2007). In addition, tPA could also indirectly influence NMDAR function through the mediation of the low-density lipoprotein receptor (LDLR) (Samson and Medcalf, 2006; Samson et al., 2008). A recent study also reported that the proteolytic activity of tPA and the synaptic NMDARs can modulate changes in intracellular calcium levels (Robinson et al., 2015).

Other mechanisms studied to date imply the participation of tPA in dopaminergic neurotransmission, which has been evidenced from experiments on LTP and drug addiction paradigms. The striatum is a structure highly innervated by dopaminergic neurons. Striatal interneurons of $tPA^{-/-}$ mice show low sensitivity to dopamine-mediated depolarisation via D1 receptors. This deficiency can be reversed by increasing cAMP levels, meaning that tPA may facilitate D1-mediated signalling. In this line, D1 agonist-induced LTP in CA1 area is abolished in $tPA^{-/-}$ mice and, in a similar way, L-LTP maintenance induced in CA3 by forskolin (a dopaminergic activator) is weakened in $tPA^{-/-}$ mice; but this effect can be prevented with exogenous PAI-1 (Samson and Medcalf, 2006). Therefore, hippocampal spatial learning and memory are not visibly affected by the lack of tPA (Huang et al., 1996); instead, these defects may affect other plasticity-related behavioural processes like anxiety or addiction.

Interestingly, other substrates for tPA/plasmin system can modify LTP in a marked way. For example, the application of mature brain-derived neurotrophic factor (mBDNF) (which is produced by tPA/plasmin cleavage) to hippocampal slices can rescue impaired L-LTP in both, tPA-deficient and plasminogen-deficient mice (Pang et al., 2004).

Another example of a tPA-interacting receptor that affects LTP is LRP. An

antagonist of LRP, called receptor-associated protein (RAP), reduces tPA-induced L-LTP and prevents the rescue of synaptic potentiation via addition of tPA in tPA-deficient mice (Zhuo et al., 2000; Melchor and Strickland, 2005). Intriguingly, the LRP receptor can also affect tPA-dependent blood brain barrier (BBB) permeability (Yepes, 2003).

Summarising, while it is clear that tPA plays a vital role in normal brain function, its effects are likely not direct. tPA is a narrow spectrum protease and its primary substrate, plasminogen, is also found in the brain. It is, therefore, possible and likely that tPA's effects, or at least some of them, arise as a result of plasminogen activation.

The role of plasmin in structural and functional synaptic plasticity

tPA, which is secreted by neurons (Gualandris et al., 1996; Baranes et al., 1998; Fernández-Monreal et al., 2004), is potentially able to modify plasticity by converting extracellular plasminogen into the active protease, plasmin. Plasmin, then, can affect synaptic plasticity by degrading various components of the synapse (Hoffman, Martinez and Lynch, 1998; Endo et al., 1999; Nakagami et al., 2000; Wu et al., 2000; Hampel et al., 2011). Compared to tPA, plasmin presents a broader spectrum proteolytic activity but, like tPA, is able to produce significant changes in the CNS.

Plasmin is able to modulate some forms of LTP. For instance, the presence of either plasminogen or plasmin and a sub-threshold tetanus electric excitation protocol facilitated the induction of LTP (Mizutani, Saito and Matsuki, 1996; Melchor and Strickland, 2005). In this line, plasminogen, in a similar way to tPA, can produce NMDAR-mediated increase in intracellular calcium concentrations in neurons (Inoue et al., 1994). Plasmin also interacts with NMDA receptors in a proteolytic manner. It is able to cleave the amino-terminal domain of the GluN2A subunit (Melchor and Strickland, 2005; Yuan et al., 2009). That cleavage removes a high-affinity zinc binding site within the ATD, which impairs the zinc inhibition of NMDAR affecting LTP (Melchor and Strickland, 2005; Yuan et al., 2009).

Opposite to these studies, a different group suggests that plasmin could be negatively influencing LTP. These experiments show impaired LTP maintenance in organotypic hippocampal slices incubated with plasmin. This effect has been thought to be produced by plasmin-mediated degradation of laminin (an extracellular matrix component [ECM]) and subsequent destabilisation of ECM (Nakagami et al., 2000). Furthermore, plasmin-cleaved laminin fragments or anti-laminin antibodies injected into the hippocampus of either wild-type or plasminogen-deficient mice disrupt the ECM and make these animals more sensitive to kainate toxicity (Nakagami et al., 2000).

Behavioural studies have shown that microinjection of plasmin in the NAc (associated with substances-abuse) can potentiate morphine-induced dopamine release and hyperlocomotion in mice, suggesting a role for plasmin in addiction, a type of event shown to be profoundly affected by plasticity (Nagai et al., 2005; Nagai, Nabeshima and Yamada, 2008).

Other proteolytic substrates of plasmin are also involved in plasticity, but their mechanisms are not well understood. For instance, as mentioned before, plasmin cleavage of the pro-brain-derived neurotrophic factor (proBDNF) to generate mature BDNF is important for the expression of late-phase LTP (Pang et al., 2004). Interestingly, plasmin activation of BDNF in the suprachiasmatic nucleus can modulate circadian rhythms by modulating glutamate-induced phase shifts (Mou, Peterson and Prosser, 2009). In addition, some tPA/plasmin inhibitors, such as the plasmin inhibitor, α2-antiplasmin, can reduce LTP (Mizutani, Saito and Matsuki, 1996).

Eph receptors and ephrins in structural plasticity

Ephs and ephrins are known to play an important role in controlling spine morphology (Irie and Yamaguchi, 2004; Klein, 2009; Lai and Ip, 2009; Hruska and Dalva, 2012). For instance, EphB receptors are required for dendritic spines formation but also the control of spine maturation. Mice lacking some of these receptors fail to form dendritic spines *in vitro* and the few spines formed in the

hippocampus are shown to be headless or to present a small-head. Similarly, in the same study, it is shown that neurons presenting EphB2 receptors lacking the kinase domain also show an impaired formation of mature mushroom-like spines in hippocampal neurons *in vitro* and *in vivo*. Similarly, EphB1, EphB2 and EphB3 double and triple mutants lacking these receptors exhibit abnormal spine formation and a marked decrease in excitatory glutamatergic synapses and the clustering of NMDA and AMPA receptors (Henkemeyer et al., 2003).

Structurally speaking, the actin cytoskeleton dynamics are essential for memory formation (Dines and Lamprecht, 2014). The continuous reorganisation of the actin cytoskeleton leads morphological changes in spine formation and maturation (Matus, 2000; Luo, 2002; Ethell and Pasquale, 2005; Tada and Sheng, 2006; Schubert and Dotti, 2007; Honkura et al., 2008; Hotulainen and Hoogenraad, 2010). There are several members of the Eph/ephrin family that control downstream signalling molecules important for the dynamics of the actin cytoskeleton. Specifically, there are several members of the Rho family of small GTPases (RhoA, Rac and Cdc-42) related to the maintenance of spine morphology in the hippocampus (Nakayama, Harms and Luo, 2000). In this line, some laboratories reported that ephrin-B–mediated activation of EphB receptors triggers Rac1 and Cdc-42 pathways through different Rho-GEF factors namely Tiam1 (T-lymphoma invasion and metastasis-inducing protein 1), Kalirin-7 and intersectin (Irie and Yamaguchi, 2002; Penzes et al., 2003; Tolias et al., 2007). EphB receptors also activated serine/threonine kinases, like PAK (p21 activated kinases), and they regulate actin dynamics in filopodial motility and synapse formation (Kayser, Nolt and Dalva, 2008).

EphA receptors are also able to regulate spine morphology in the absence of EphBs (Henkemeyer et al., 2003). For example, dendritic spine morphology of neocortical neurons is markedly altered in mice lacking EphA5 or EphA6. These animals present irregular spine morphology of basal dendrites on layer 5, which present complex spine structures like bifurcated or multi-branched processes similar to thorny or coralline excrescences (Das et al., 2016).

Despite all the works mentioned about Eph receptors reviewed so far, most of the works related to the influence of EphA receptors in spine morphology refer to

EphA4. EphA4 is present and enriched in dendritic spines of pyramidal neurons in the adult mouse hippocampus, and its absence markedly changes spine morphology. Treatment with ephrin-A3-Fc (a chimaeric cognate EphA4 ligand fused with an immunoglobulin's heavy chain) decreases spine length and density in hippocampal slices, and EphA4-deficient mice or EphA4 inactivation results in longer, disorganised dendritic spines with spine shape abnormalities (Murai et al., 2003; Bourgin et al., 2007; Inoue et al., 2009). Also, *in vitro* overexpression of the EphA4 kinase domain augments dendritic spine density, while EphA4 knock-down reduces spine maturity (Inoue et al., 2009; Murai et al., 2003).

EphA downstream signalling affecting spine morphology is also related to actin cytoskeleton rearrangements. The main body of research is related to the Rho family GTPases, which are tightly linked to cytoskeleton changes. EphA4 interaction with its ligands leads to inactivation of Rap1 GAP, with the consequent inhibition of integrin signalling and reduced adhesion to the extracellular matrix (Bourgin et al., 2007; Richter et al., 2007) or activation of the Cdk5 serine/threonine kinase, the RhoA-specific GEF ephexin1 and RhoA (Fu et al., 2007). EphA4 activation can also modulate spine morphology through inhibiting β1-integrin activity (Bourgin et al., 2007). In mature neurons of the amygdala, EphA4 forward signalling activated by ephrin-B3 is necessary for the regulation of Rab5-GEF Rin1, which is involved in EphA4 internalisation and restricts LTP (Deininger et al., 2008). The regulation of the spine length and the retraction of spines may also involve other types of enzymes, like the ephrin-A3-mediated activation of PLCγ1, which reduces membrane association of the actin depolymerisation factor, cofilin (Zhou et al., 2007).

Eph receptors and ephrins in functional plasticity

Several works relate Ephs and ephrins with fine regulation of synaptic transmission and thereby regulation of synaptic efficacy. Both long-term potentiation (LTP) and long-term depression (LTD) are affected by these proteins (e.g. Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Martin, Barad and Kandel, 2000; Gerlai, 2001).

For instance, EphB2 deletion affects long-term potentiation (in late, rather than early phases of LTP) and long-term depression (LTD) (Henderson et al., 2001; Grunwald et al., 2001). Since this is a kinase-independent mechanism, EphB2 must modulate LTP and LTD through other interactions, such as the one that modulates NMDA receptor (Dalva et al., 2000; Murai and Pasquale, 2002; Takasu et al., 2002). Furthermore, Dalva and colleagues demonstrated that the binding of B-class ligands to their receptor induces a direct interaction between EphB and the extracellular regions of NMDA receptors and this produces reduced frequency but not amplitude of spontaneous miniature excitatory synaptic currents (Kayser et al., 2006).

Interference with the binding of EphB receptors to GRIP (a protein interacting with the PDZ domain of this receptor) also impairs the LTP produced at the synapses of mossy fibres with the hippocampal synapse in CA3, and the application of ephrin-B increases their basal excitatory transmission and hampers both tetanus-induced and forskolin-induced synaptic potentiation (Contractor et al., 2002). Liu et al. have also shown that some EphB receptor activators, such as EphB1-Fc and EphB2-Fc lower the threshold for LTP (Liu et al., 2009).

Previous reports indicate that ephrin-B2 naturally accumulates on the postsynaptic terminals of the hippocampus (Grunwald et al., 2004) and that ephrin-B2 and ephrin-B3 are required for LTP and LTD (Grunwald et al., 2004; Rodenas-Ruano et al., 2006). However, this latter statement is controversial since no impairment in LTP was found in ephrin-B3$^{-/-}$ mice, but was so when the cytoplasmic C-terminal is replaced with β-galactosidase (Armstrong et al., 2006). Additionally, when ephrin-B2 is in contact with its putative Ephs receptors, it is phosphorylated by Src family kinases (Palmer et al., 2002). It has been demonstrated that tyrosine phosphorylation sites in ephrin-B2 are required for hippocampal LTP, but not LTD, and that ephrin-B2 missing the C-terminal PDZ interaction site cannot produce such plasticity phenomena (Bouzioukh et al., 2007). In a different model (*Xenopus retinotectal* system), EphB2-Fc application produces ephrin-B1-mediated enhanced transmitter release and facilitates theta burst stimulation-induced LTP (Lim, Matsuda and Poo, 2008).

Knowledge about EphA receptors (other than EphA4) is based on research using

purified forms of ephrin-A5 or EphA5 extracellular domains (EphA5-Fc and ephrin-A5 Fc). As these ligands and receptors are very promiscuous, A5 receptor and ligand likely affect the function of all endogenous EphA receptors. In this context, rat hippocampal slices treated with EphA5-Fc (theoretically producing EphA inhibition) impairs LTP. In contrast, ephrin-A5-Fc (theoretically producing EphA activation) produces an LTP-like potentiation. Indeed, these effects have a correlation with behavioural performance in hippocampal-dependent learning tasks (Gerlai et al., 1999).

Specifically focusing on the functional aspects of EphA4 plasticity, the absence of EphA4 seems to impair hippocampal synaptic plasticity (Grunwald et al., 2004; Filosa et al., 2009). EphA4 is important for early stages of LTP at the CA3 to CA1 synapse, and this effect is produced in a kinase-independent manner (Grunwald et al., 2004), a quality that seems to be shared with EphB2 in the hippocampus (Grunwald et al., 2001). Dendritic EphA4 and ephrin-A3 in astrocytes of hippocampal CA1 region regulate glutamate concentrations in the vicinity of the synapses and promote LTP by inhibiting the expression of glutamate transporters (Filosa et al., 2009). In contrast, the interactions with ephrin-Bs at CA1 might reduce LTP (Grunwald et al., 2004). Moreover, organotypic slices lacking either ephrin-A3 or EphA4 present deficient depolarisation and impaired LTP in the same area, but the authors argue that this could be altered by ephrin expression in astrocytes (Carmona et al., 2009; Filosa et al., 2009). EphA4 receptor is also required for functional synaptic plasticity in the amygdala (Deininger et al., 2008). Importantly, the role of EphA4 in LTP is independent of forward signalling, but there are mechanisms, such as EphA4 phosphorylation that can affect this process (Grunwald et al., 2004; Filosa et al., 2009).

In conclusion, tPA, plasmin and EphA4 signalling control diverse forms of synaptic plasticity. Their coordinated interaction of action could underlie a mechanism of action that allows various forms of plasticity and therefore, spine morphology and density could reflect some of these changes.

RESULTS

In order to assess the resistance to cleavage of the crEphA4 variant, N2A cells overexpressing wtEphA4 or crEphA4 were treated with a tPA/plasmin mix as described in *Materials and Methods*. Western blot analysis of the homogenates produced an increase in the main cleavage product band intensity of EphA4 upon tPA/plasmin treatment when compared to untreated controls, whereas no significant cleavage was induced in the crEphA4 variant. This experiment confirms the cleavage resistance of crEphA4 variant at low concentrations of these proteases (Figure 21 C and D, n = 3. Figure 21 D, ANOVA, $F(3,8) = 16.549$, $p<0.001$. Bonferroni: wtEphA4: 1.000±0.058 vs. wtEphA4+tPA/Plg: 1.357±0.024, $p = 0.003$. wtEphA4+tPA/Plg: 1.357±0.024 vs. crEphA4 0.793±0.085, $p<0.002$. wtEphA4+tPA/Plg 1.357±0.024 vs. crEphA4+tPA/Plg 0.936±0.053, $p<0.002$).

To investigate the changes that the cleavage of EphA4 by tPA/plasmin may produce in morphological synaptic plasticity, EphA4 and its variants were overexpressed in neuronal primary cultures as described in *Materials and Methods.* This analysis revealed differences in the morphology of spines. Cleavable forms of EphA4 at P5 (i.e. wt EphA4 and tEphA4) develop longer spines when compared with vector-transfected controls (Figure 22 A. Kruskall-Wallis (3,4583). $H = 19.427$, $p<0.001$. Dunn's test: Vector-transfected control: 1.392±0.027 µm vs. wild-type EphA4: 1.473±0.028 µm, $p = 0.017$; vs. tEphA4: 1.545±0.030 µm, $p<0.001$). In addition, wild-type and crEphA4 mutant (full-length variants) have wider spine heads when they are compared with vector-transfected control, whereas truncated EphA4 (tEphA4) exhibit narrower spine heads (Figure 22 B. Kruskall-Wallis (3,4560), $H = 124.233$, $p<0.001$. Dunn's test: Vector control: 0.666±0.008 µm vs. wild type: 0.737±0.010 µm, $p<0.001$; vs. crEphA4: 0.772±0.011 µm, $p<0.001$; vs. tEphA4: 0.643±0.010 µm, $p = 0.022$). This change is more evident when a length/width ratio is used, which reveals opposite effects in crEphA4 mutant and truncated tEphA4 compared to vector-transfected control neurons and wild-type EphA4-transfected neurons (Figure 22 C. Kruskall-Wallis (3,4581), $H = 99.831$, $p<0.001$. Dunn's test: Vector control: 2.331±0.046 µm vs. crEphA4: 2.159±0.053 µm, $p = 0.002$; vs. tEphA4: 2.800±0.060 µm, $p<0.001$; WT: 2.241±0.046 µm vs. tEphA4: 2.800±0.060 µm,

p<0.001). Measurements of the area of the spines yield similar results to those observed in the spine width results; they show an increase of the spine area in wtEphA4 and crEphA4 comparing the vector and tEphA4 (Figure 22 D. Kruskall-Wallis (3,4585), H = 31.680, p<0.001. Dunn's test: Vector: 0.685±0.015 vs. WT: 0.769±.0.017 μm^2, p<0.001; vs. crEphA4: 0.779±0.017 μm^2, p<0.001. tEphA4: 0.695±0.016 μm^2 vs. WT: 0.769±.0.017 μm^2, p = 0.003; vs. crEphA4: 0.779±0.017 μm^2, p<0.001). Opposite to spine morphologies, the overexpression of these EphA4 constructs does not affect the spine density or the dendrite length (Figure 23 A AND B).

FIGURES

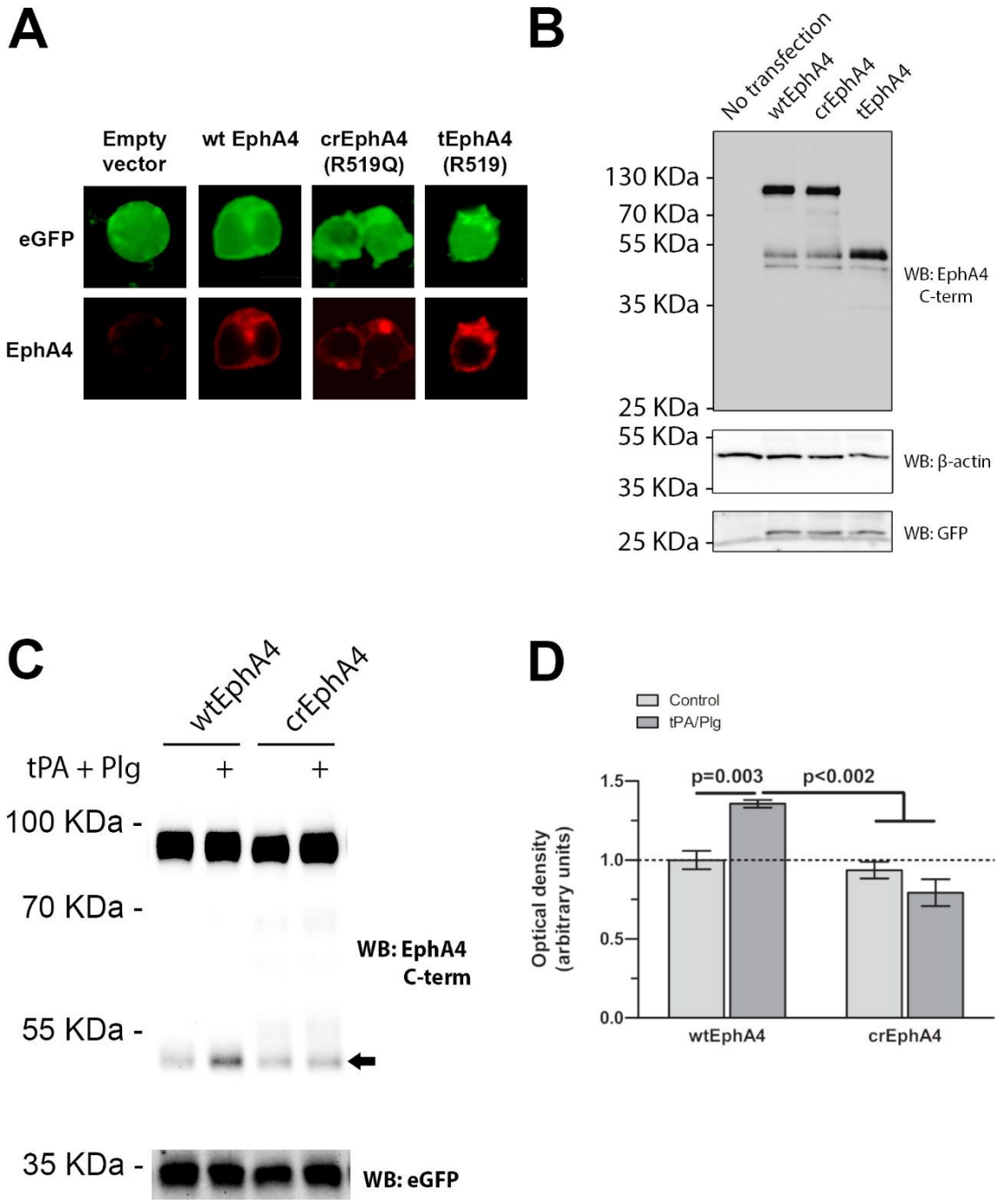


Figure 21. Cleavage-resistant EphA4 mutant (R516Q) is resistant to tPA and plasmin cleavage. wtEphA4 = wild type EphA4; crEphA4 = cleavage-resistant EphA4; tEphA4 = truncated EphA4 at P5 cleavage site; tPA = tissue plasminogen activator; Plg = plasminogen. A) Immunocytochemistry of the overexpression of the different EphA4 protein variants in Neuro-2A cell line. As expected, EphA4 receptor variants are mainly located on the boundaries of the cell body indicating a prominent cellular membrane presence. B) Representative blot of transfected N2A cells with the different constructs optimised for lentiviral expression. C) Representative blot of products resulting from the treatment of N2A cell lines for 20 min with + = 2.5 µg/mL (39.73 nmol) of tPA and + = 10 µg/mL (110.41 nM) of plasminogen. The black arrow points the main cleavage product. D) Quantification of the main EphA4 cleavage form (black arrow in C). Proteolytic treatment crEphA4 mutant does not produce a significant increment in the main EphA4 cleaved form, meaning that it is resistant to cleavage by tPA and plasmin. All data are presented as optical density (O.D.) mean ± SEM (n = 3-5). p values for Bonferroni's comparison test are included over the corresponding chart bars.

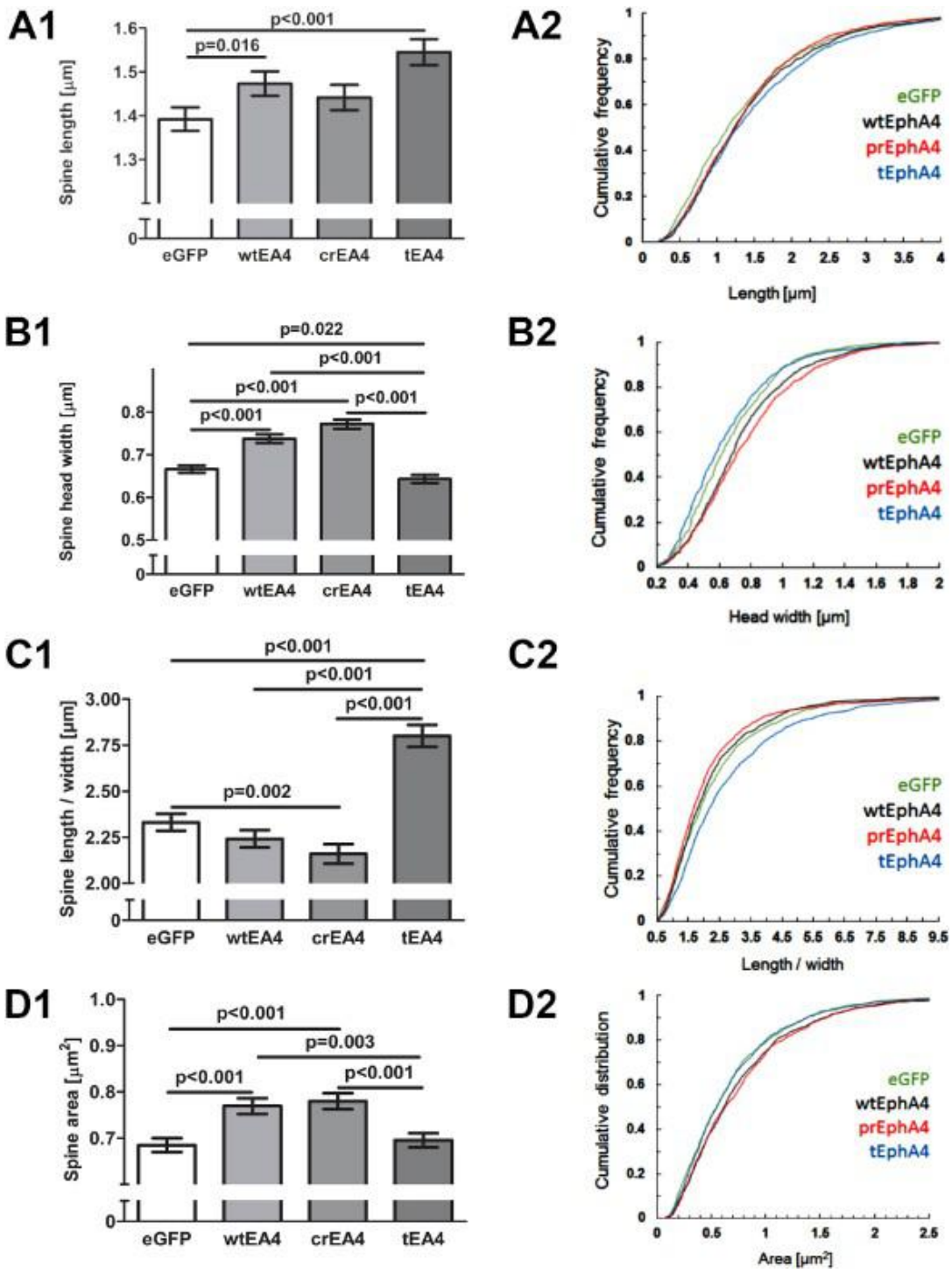


Figure 22. Truncated EphA4 variant at R516 induces longer and thinner-head spines. wtEphA4/wtEA4 = wild-type EphA4, crEphA4/crEA4 = cleavage-resistant EphA4 mutant, tEphA4/tEA4 = truncated variant at P5. This figure includes a quantitative morphological analysis of spines from P0 mice primary neurons transfected with the different EphA4 variants. Histograms of the average values (1) and cumulative frequencies (2) are included for each of them: A) The overexpression of wtEphA4 and tEphA4 produce longer spines when comparing to no-EphA4-transfected controls. B) wtEphA4 and crEphA4 present wider spine heads. C) Length/width ratio accentuates the effects produced by tEphA4. This parameter suggests that the cleavage of EphA4 at P5 induces longer and thinner spines when comparing to no-EphA4-transfected control, wtEphA4 and crEphA4 mutant, which may indicate a more immature and versatile kind of spines in the tEphA4 group. D) Spine area is more prominent in wtEphA4 and crEphA4, probably due to the wider spine heads of these groups. All data are presented as mean ± SEM (N = 9-15 cells; n = 1088-1241 spines). p values are included over the corresponding chart bars.

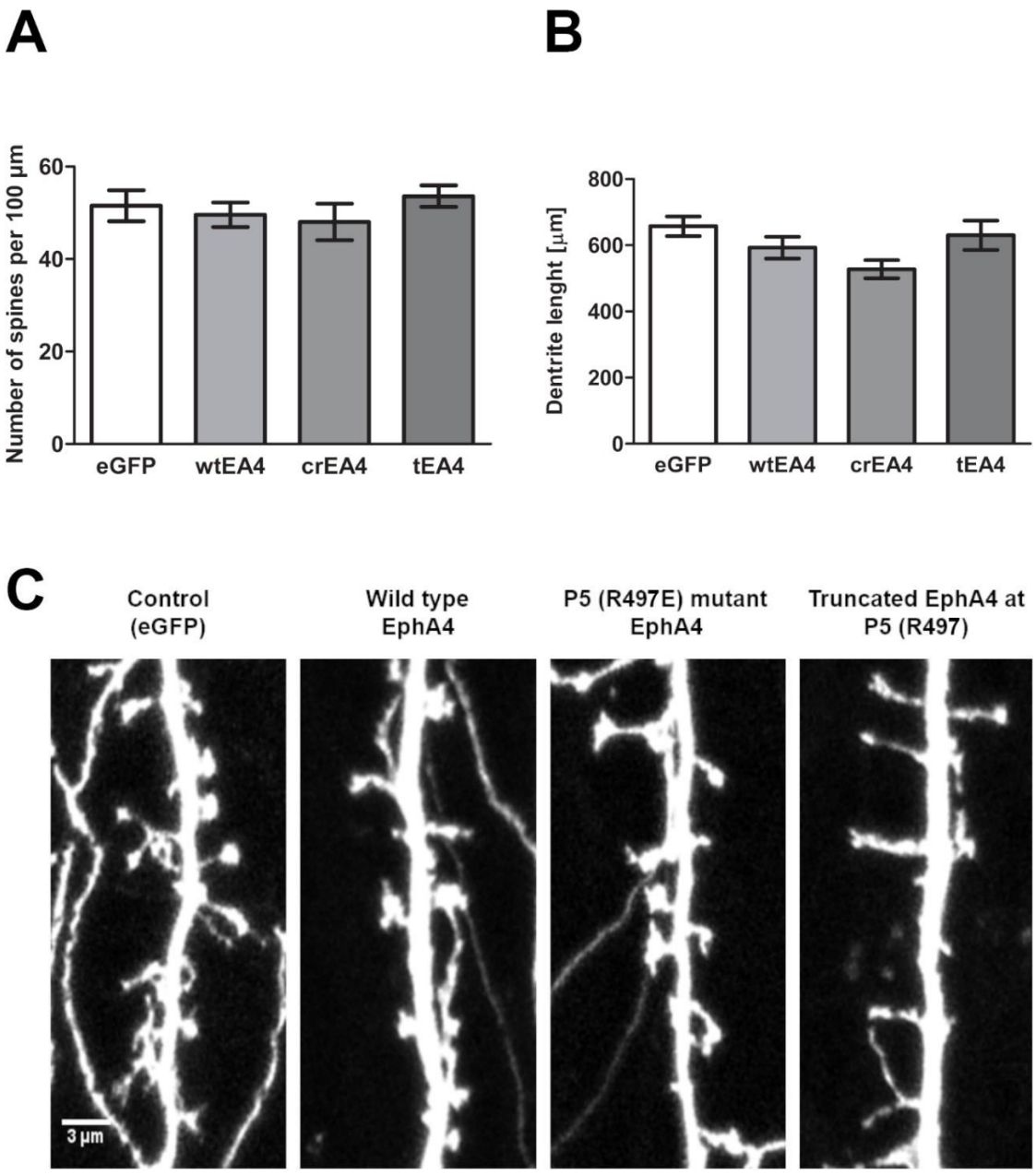


Figure 23. EphA4 variants do not affect spine density or dendritic length in primary neuronal cultures. Upper panels show the quantification for the spine density (A) and dendrite length (B), respectively. There is not an observable change in these parameters. Panel C shows representative images of dendrite sections of P0 mice primary neurons transfected with the different EphA4 variants. As confirmed by quantitative morphological analysis, the overexpression of EphA4 receptor truncated at P5 presents longer spines with a narrower head. All data are presented as mean ± SEM (N = 9-15 cells; n = 1088-1241 spines). p values are included over the corresponding chart bars.

DISCUSSION

Dendritic spines morphology is affected by EphA4 truncation or cleavage resistant forms

As reviewed in this chapter, tPA, plasmin, EphA4 and its downstream effectors can produce neuroplastic changes in the context of synaptic or dendritic morphology and their electrophysiological correlates. This fact raises the question of whether the plasmin/EphA4/effectors signalling unit can regulate the morphological correlates of plasticity produced in postsynaptic terminals downstream of *tPA*+ (PKCδ^{+}) interneurons from CeL. To address this hypothesis, disaggregated neuronal cultures from the amygdalae of P0 murine tissue were produced and transfected with the synthesised plasmids containing the code for two EphA4 variants related to plasmin cleavage. The first variant consisted of a mutant form of EphA4 receptor which is resistant to plasmin proteolysis at the P5 cleavage site. Of note, P5 is the most efficiently cleaved site at low doses of plasmin (Figure 18 A and B). The second variant was a truncated form of EphA4 at P5 cleavage site, which mimics a persistent cleavage of the receptor at P5 by tPA/plasmin (Figure 18 B). To assess the specific dendritic and spine morphologies, the transfected P0 primary cultures were blindly and semiautomatically analysed by our collaborators in Prof Jakub Wlodarczyk's laboratory at the Nencki Institute as described in *Materials and Methods.* Importantly, the theoretical lateral resolution of the used imaging setup has been calculated to be 0.07 µm per pixel. Therefore, this would be the limit for accurate measurements of dendritic length and spine shape in our experiments and this distance should be taken into account when evaluating the results presented here.

As previously reviewed in the introduction of this chapter, there are various features of spines and dendrites known to change neuronal plasticity. In this discussion, I focused on one particular feature, which is the high variability of their morphological organisation. This morphological variability includes changes in the shape of the dendritic arborisation of a neuron and changes in the morphology

of dendritic spines.

EphA4 variants do not change dendritic arborisation in amygdalar neuronal primary cultures

In our experiments, the dendritic arborisation (length [Figure 23] and complexity, measured by Sholl analysis [data not shown]) remains unaffected throughout the overexpression of the different variants. These were expected results since none of the Eph/ephrins studied in the literature has been shown to produce any changes in dendritic arborisation in the adult brain when they are expressed in the absence of ligand interaction (Whitford et al., 2002; Jan and Jan, 2003; Miller and Kaplan, 2003; Kim and Chiba, 2004). Nonetheless, there are various reports in which treatment of EphA4-expressing neurons with some of its cognate ligands (e.g. ephrin-A1 or A3) reduces their spine density and dendritic length (Fu et al., 2006; Bourgin et al., 2007; Richter et al., 2007; Carmona et al., 2009). These results contrast with experiments in which EphA4 is not interacting with ephrins. In these latter cases, no changes in spine density or length are observed, but the absence of EphA4 in mice produces spine irregularities in pyramidal neurons and in slices transfected with kinase-inactive EphA4 (Murai et al., 2002). This is especially interesting because, to date, EphA4 is one of the few EphA receptors shown to modulate spine morphology (Murai et al., 2003). These results add evidence in favour of the idea that the presence EphA4 modifies spine morphology without affecting the spine density or the dendritic morphology in the adult mouse brain, unless its known ligands activate the receptor. This idea would imply that EphA4 produces different effects (possibly through diverse pathways) depending on whether it is bound to its ligands.

Dendritic length and branching have been observed to be modified by anxiogenic paradigms in hippocampus and amygdala in the past (Faherty, Kerley and Smeyne, 2003; Pittenger and Duman, 2008). An EphA4-dependent mechanism could be producing these effects; however, as argued in the previous paragraph, such changes in dendritic morphology would likely be a ligand-activated mechanism and, therefore, further experiments would be needed to unveil the

real mechanism of these changes in anxiety-like behaviours.

As mentioned, in the present work, EphA4 did not produce any effect on arborisation. Generally speaking, arborisation regulates the function of a neuronal cell by determining how synaptic information is received and integrated. A paradigmatic example of this feature is the somatosensory system in which the shape of the dendritic arbour is determinant of the shape and extension of the receptive field and hence the region of the extracellular compartment from which a neuron receives an input (Peichl and Wässle, 1983). Also, the arbour's branching pattern determines the density with which a neuron examines a field; in other words, the more complex arbours (more branches), the more inputs a neuron can receive from presynaptic partners. Additionally, parameters such as dendrite diameter, distance from the soma, and the number of branch points that must be crossed to reach the soma also determine functionality by controlling the probability of a given excitatory postsynaptic potential (EPSP) to occur (London and Häusser, 2005; Spruston, 2008; Lefebvre, Sanes and Kay, 2015).

EphA4 variants influence dendritic spines morphology in amygdalar neuronal primary cultures

There is broadly accepted evidence that the CeA is mainly formed of GABAergic medium spiny neurons (McDonald 1982; Sah et al., 2003). Moreover, there is also compelling evidence that (GABAergic) interneurons from CeL target local circuits within the CeL and that two virtually not-overlapping interneuron populations (SOM^+ and $PKC\delta^+$) cover the majority of the neuronal population of this area (Ciocchi et al., 2010; Haubensak et al., 2010; Hunt et al., 2017; McCullough et al., 2018). Therefore, it is realistic to assume that $PKC\delta^+$ type of interneurons expressing tPA at the CeL target this area as well and that the targeted cells are likely medium spiny neurons.

However, there is a spread conception that dendritic spines are exclusively formed in the postsynaptic terminal of glutamatergic synapses and empirical data seems to confirm this in general terms. Hence, the spine population presenting

spines targetable by tPA would be restricted to the glutamatergic fraction of spines within the CeA. Taking these facts into account, the effects of EphA4 cleavage by tPA on spines would be relevant for synapses with a presynaptic GABAergic terminal and a postsynaptic glutamatergic one; in other words, heterosynaptic synapses. These connections are formed between two neurons that are regulated by a third neuron extrinsic to the putative synapse, which can be similar or different than the central synapse. Some authors argue that this mechanism could be of relevance for stress and anxiety disorders (Swanson et al., 2005; Chiu et al., 2013). Interestingly, the formation of dendritic spines with GABAergic synapses has been reported (Knott et al., 2002; Chen et al., 2012; van Versendaal et al., 2012); therefore, this could represent a different type of spine that PKCδ^{+} (*tPA*+) neurons could target and whereby tPA/plasmin could develop their activity. Additionally, spill-over from adjacent GABAergic synapses could be affecting the shape of spines (Isaacson et al., 1993). To sum up, there are various mechanisms for interneurons to transform spine morphology of glutamatergic synapses that would be relevant for the current work and GABAergic spines could represent a new plasticity hub that would be worth exploring.

Dendritic spines are known to exhibit variations in volume, length and shape, which are accompanied by fluctuations in the content of organelles and proteins, such as ribosomes, endosomal systems or cytoskeleton proteins (Heck and Benavides-Piccione, 2015). These variable morphological changes are believed to be a reflection of different functional properties. Some parameters linked to functional properties can be measured, i.e. the size of the postsynaptic density, the number of postsynaptic receptors in the postsynaptic density, the strength of the synapse, its developmental stage or even its stability over time. In this sense, extended consideration has been given to the correlation of spine morphology with functional parameters (Bourne and Harris, 2008).

Our experiments reflect that spine density does not significantly change among the different variants of EphA4 (Figure 23). There is a sizeable amount of speculative literature about the functions of spine number and density. The newest *in vivo* time-series imaging studies that characterise spine dynamics

usually focus onto two parameters: overall changes in spine density and the location of the spine formation/retraction within the dendrite. Spine density is considered an estimator of the total number of excitatory synapses onto the postsynaptic neuron, and it is a parameter used to assess the health and development of a neuronal circuit (Rochefort and Konnerth, 2012). The location relative to the overall neuron structure, on the other hand, influences the relative contribution of specific spines to the transmitted electrical and chemical signals that are later integrated at the soma (Nevian et al., 2007; Spruston, 2008; Berry and Nedivi, 2017).

With regards to spine morphology, full-length EphA4 (in both wild-type and cleavage-resistant mutant forms) induces wider spine heads when compared to vector-transfected control cultures and a slightly longer shape in the case of wild-type EphA4 when comparing it with its cleavage resistant form (Figure 22). Large-head spines (also termed mushroom type of spines) are commonly interpreted as more mature and stable synapses that have been strengthened through a process of activity- or plasticity-mediated enlargement. Several *in vitro* and *in vivo* studies have shown a high direct correlation between the size of the spine head, the size of the postsynaptic density, the size of glutamate-evoked responses and the stability of the spine (Kasai et al., 2003; Matsuzaki et al., 2004). In contrast to full-length forms of EphA4, the increase in spine width is not observed in the neurons expressing the truncated EphA4 variant; in fact, these spines also develop a more elongated shape compared to controls (Figure 22). Elongated and thin spines with small heads are usually associated with dynamic, young, newly formed synaptic structures which are in general more unstable and susceptible to disappearing over time (Kasai et al., 2003, 2010; Bourne and Harris, 2007; Heck and Benavides-Piccione, 2015), which would suggest that truncated EphA4 promotes destabilised or immature synapses. Therefore, full-length forms of EphA4 would help to promote the interaction with postsynaptic proteins and simultaneously help to stabilise the spine structure, whilst cleavage of EphA4 at R516 by plasmin would favour the formation of “thin” dynamic dendritic spines.

These results are in agreement with the literature about EphA4. In particular, the

presence of the receptor induces more mature spines in terms of morphology (i.e. a clear head-like structure and short neck), indicating the involvement of EphA4 in the process of dendritic spine maturation (Tada and Sheng, 2006). For example, cortical neurons with higher expression of EphA4 induce an increased number of mature spines (Clifford et al., 2011), whereas EphA4$^{-/-}$ mice present elongated spines irregular in shape and disorganised (Murai et al., 2003). This is reflected in spine functionality since Eph4-deficient hippocampal CA1 region shows impairment of LTP and LTD (Grunwald et al., 2004).

The importance of EphA4 in spine morphology through its interactions and signalling is highlighted by a number of works referenced in the introduction of this chapter. For instance, the administration of ephrin-A ligands (as known ligands of EphA receptors) induces remodelling of the spines by F-actin reorganisation in spines (Zhou, Jones and Murai, 2012); however, treatment of EphA4$^{-/-}$ hippocampal slices with ephrin-A ligands does not evoke the same kind of morphological changes in spines (Murai and Pasquale, 2002). This fact suggests that only EphA4 activation (and not other EphA receptors) may play an important role in spine plasticity. However, challenging this presumption, EphA5 or EphA6 have been reported to regulate spine morphology. Therefore, although EphA4 seems to be of great importance in regulating spine morphology, further experiments are needed to confirm the implications of other EphA receptors.

Curiously, a previous study shows that stimulation of hippocampal slices with EphA4-Fc induces width reduction in spine heads and spine disorganisation that resembles that produced in EphA4$^{-/-}$ (Murai and Pasquale, 2002) and tEphA4, which would suggest inhibition of EphA4 as a common signalling event in all these cases. The particular signalling pathways involved in the regulation of spine shape by EphA4 are not yet known; however, the activation of EphA4 and the integrity of EphA4's kinase activity are required to keep normal spine parameters. Some candidates have been proposed, such as the Rho family GTPases, but different interactions, like the one with gephyrin described in further chapters of this work, must be explored in order to fully understand this process.

These pathways possibly involve the cleavage of EphA4 by proteases. In this line, a previous work by Inoue et al. (2009) argues that the cleavage of EphA4 by

metalloprotease (MP) is able to modify dendritic spine morphology. The cleavage results in an intracellular domain (ICD) that conserves the integrity of the kinase domain and produces a C-terminal fragment (CTF) similar in size to the one produced by tPA/plasmin in our work (i.e. tEphA4). However, that MP-generated ICD produces contrasting effects when comparing to tPA/plasmin-generated CTF. MP-induced ICD does not produce any change in spine length or width but it contributes to changes in spine density, an effect that is not observed in any other work, including ours, when ligands are not used. This suggests that not only the presence of the intracellular domain generates changes in spine morphology but different cleavage points within the extracellular structure (such as the MP-generated or the plasmin-generated ones) also exert different signalling resulting in different effects in morphologies of spines and dendrites. Curiously, although the mentioned ICD does not change spine morphology, it increases the formation of lamellipodia-like structures in NIH3T3 cells (a fibroblast-derived cell line) similar to the filopodia structures produced by tEphA4 in neurons. It would be interesting to investigate whether these similar phenomena produced by EphA4's CTFs could have any similarities in terms of mechanisms generating cell protrusions.

In line with the role of proteases in processes affecting synaptic plasticity at the functional and morphological level, a work from Wang et al. (2008) describes how activity of the metalloprotease, MMP-9, induces concomitant enlargement of spines and synaptic potentiation. However, the blockade of protein synthesis, while applying MMP-9, cancels these effects. Although these experiments were carried out with a two-photon microscope (in comparison to a less-precise confocal microscope used in my thesis), the volume of the spine seems to increase with MMP-9 cleavage (Wang et al., 2008), whereas the cleavage of EphA4 (tEphA4) does not seem to modify this parameter. Instead, in the case of EphA4, the lack of cleavage would be the event increasing the volume of dendritic spines (Figure 22). As reviewed in the introduction of the present chapter, tPA/plasmin disrupt dendritic growth and spine maturation, which would suggest that tPA/plasmin or EphA4 cleavage produce opposite effects to those of MMP-9. Despite of this difference, EphA4 and MMP-9 mechanism modifying spine morphology have something in common, which is the mediation of β1-integrin.

Future experiments would be needed to observe whether tPA/plasmin cleavage of EphA4 and MMP9 belong to a bidirectional mechanism modulating morphology and LTP via β1-integrin.

In relation to how these spine changes could affect fear and anxiety, the predominant hypotheses related to the role of dendritic spines in these conditions vary over the periods of history and with the different authors. Hence, from the literature, there is not a clear opinion about whether spine morphology can influence the development of stress and anxiety. However, modulating the general inhibitory tone of the CeA by affecting the synaptic strength or the number of receptors could potentially produce unconditioned aversive responses (Ciocchi et al., 2010). Inversely, there is a general acceptance of data related to the effects of stress and anxiety in spine morphology. For example, various studies are showing that stress hippocampus and mPFC, whereas it shows an increase in the amygdala and the nucleus accumbens (Leuner and Shors, 2013). In the amygdala, morphological changes in arborisation are combined with morphological changes in spines and total spine number or density. In particular, chronic stress seems to increase dendritic length, branch points, spine number, and spine length on BLA neurons but not in the CeA (Vyas et al., 2002; Pawlak et al., 2003; Vyas, Bernal and Chattarji, 2003; Mitra et al., 2005; Vyas, Jadhav and Chattarji, 2006; Qin et al., 2011). Similar dendritic changes were observed when applying a single prolonged stressor (Cui et al., 2008). Since these effects are not detected in the tPA/plasmin-related EphA4 variants, it is unlikely that these proteases affect chronic stress through EphA4 cleavage. By contrast, acute form of stress does not seem to be characterised by dendritic elongation, but it does induce formation of new spines over time (Mitra et al., 2005), which would be in association with thinner, and presumably more versatile spines, similar to the ones produced by the truncated EphA4 variant (Leuner and Shors, 2013). Therefore, tPA/plasmin/EphA4 cascade could be associated with short forms of stress that produce more plastic spines.

CONCLUSIONS

tPA, plasmin, EphA4 and its downstream effectors regulate all forms of neuronal plasticity, including the morphology of dendritic spines that form postsynaptic terminals. This observation raises the question of whether CeL-associated *tPA*+ ($PKC\delta^{+}$) interneuronal efferents can modify this morphological correlate of plasticity through downstream tPA/plasmin-associated cleavage of EphA4 receptor.

In the current work, I show that spines of amygdalar neurons overexpressing full-length EphA4 variants (wild-type and cleavage-resistant mutant) induce wider spine heads (normally interpreted as more mature, stable and more active synapses) when compared to vector-transfected control cultures. Oppositely, neurons expressing the plasmin-associated truncated form of EphA4 at R516 produce thinner and longer spines compared to controls and full-length forms, which commonly correlates with young, newly formed synaptic structures, more unstable and susceptible to disappearing over time. This analysis suggests that tP5 promotes destabilised or immature synapses. Therefore, full-length forms of EphA4 would help promote stabilisation of the spine structure, whilst cleavage of EphA4 at R516 by plasmin favours the formation of “thin” and pliable dendritic spines.

These morphological changes in spines, with EphA4 as a molecular underpinning, are associated with the content of glutamatergic receptors in the synapses and, therefore, they could be likely affecting the synaptic strength and the transmission in the centrolateral amygdala and vicinity areas that would lead to modified aversive behaviours. Importantly the mechanisms explained here would be relevant for heterosynaptic connections with a GABAergic presynaptic terminal and a glutamatergic postsynaptic terminal.

Experiments performed in neuronal primary cultures from amygdalae P0 murine tissue do not show differences in dendritic arborisation or spine density when they are transfected with either wild-type EphA4, plasmin-associated cleavage-resistant EphA4 or truncated EphA4 at R516, which are changes associated with chronic stress paradigms and ligand-bound Eph receptors.

FUTURE WORKS

Since the morphology of spines is differentially affected in the hippocampus, mPFC and amygdala, an obvious set of experiments would be to repeat all these experiments in areas presenting tPA/plasmin/EphA4 cascade and known to be affected by stress and anxiety, such as the hippocampus, the BNST or the hypothalamus. Furthermore, a thorough analysis of the microcircuits and types of synapses and cells where tPA/plasmin/EphA4 are expressed and localised in CeL or other areas will allow for more precise dissection of the functions that these proteins may produce through their proteolytic cascade of events.

Moreover, *in vivo* imaging and time series scans could shed some light on the precise dynamics of synaptic morphology. Infusion or conditioned expression of tPA/plasmin in these areas or expression of different EphA4 variants would be necessary to address this question. Although CeA is a deep structure that could be challenging to reach with *in vivo* imaging techniques, other shallow structures in which tPA/plasmin/EphA4 (e.g. hippocampus) could be an interesting target to observe changes in spine morphology *in vivo*.

Importantly, there is also a need to confirm whether morphological changes produced by EphA4 truncated form in CeA neurons have a functional correlate in neuronal plasticity. It would be expected that this cleavage induces an effect in electrophysiological correlates of synaptic plasticity or in neuronal activity. Since CeA neurons are mainly GABAergic and EphA4 is highly expressed in postsynaptic GABAergic synapses of CeA, mechanism related to this type of synapsis should be carefully explored. Conditions of the experiments would aim to explore GABAergic receptor currents. To this end, *in vivo* and *in vitro* extracellular recordings and patch clamp should be able to give a clear idea of how these processes are affected by the cleavage of EphA4. Spontaneous inhibitory and excitatory postsynaptic currents (IPSCs and EPSCs) and $GABA_A$ receptor-mediated postsynaptic currents ($GABA_A$-PSCs) would be recorded as well. Because spines are produced mainly in glutamatergic synapses, excitatory currents in glutamatergic cells would need to be assessed as well.

As argued in the discussion of this chapter, different cleavage points in the structure of EphA4 may produce diverse effects in neuronal morphology. All experiments performed in this work were focusing on the main cleavage product of plasmin (at R516); however, other sub-products were also detected with mass spectrometry techniques that have not received equal attention. Future experiments will resolve whether these fragments have similar effects and functions when comparing to R516 cleavage. Additionally, extracellular fragments of EphA4 produced as a by-product of the cleavage by plasmin may have specific functions and their properties would need to be tested. The hypothesis behind this idea is that the cleaved N-term end of EphA4 is not a mere waste product of the proteolysis reaction but a metabolite with its own functions. In this line of events, neuropeptides, such as the substance P, are able to influence anxiety-like behaviours through the neurokinin (NK) 1 receptor. Agonists of this receptor have a clear anxiogenic effect in the plus-maze when microinfused into the periaqueductal gray while the N-terminal fragment has an anxiolytic effect. Additionally, many other receptors, such as cytokines receptors, are also activated by soluble peptides which gives further support to this hypothesis.

CHAPTER 5: DOWNSTREAM MECHANISMS AND SIGNALLING

INTRODUCTION

In this chapter, I will address the interactions of EphA4 that may allow for changes in biochemistry, behaviour and plasticity.

A previous microarray-based analysis from our lab highlighted the GABAergic synapse as the main functional unit regulated by the tPA in amygdala upon restraint stress. This is evidenced by the differential mRNA levels of seven GABA receptor subunits on the microarray upon deletion of the tPA gene.

Chemical-mediated synaptic transmission requires mechanisms to couple pre and postsynaptic interacting molecules. The trans-synaptic bidirectional communication and the functional changes in spine proteic composition are vital factors for synapse formation and maintenance. At the proteomic level, scaffolding proteins are an important component of the synapse that behaves as anchors to maintain the stability of synapses and enables the specific subcellular location of complementary proteins. There are various proteins described to act as scaffolds in glutamatergic synapses (Sheng and Sala, 2001; Collins et al., 2006; Choii and Ko, 2015); however, much less is known about the GABAergic synapse where tPA/plasmin/EphA4 cascade seems to be taking place. Nonetheless, a notable component of this latter type of synapses, called gephyrin, has emerged as a key protein of that kind (Alvarez, 2017).

Gephyrin

Gephyrin is a multidomain protein that was originally purified as a 93 kDa protein associated with glycine receptors (GlyRs). It lacks PDZ domains, yet it forms what has been described as “clusters”, “aggregates”, “patches” or “spots” by auto-oligomerisation and these acquire different conformations in proximal and distal synapses. The concentration of these self-assembled gephyrin molecules is evidenced as puncta (0.05–2 μm^2) in immunostaining experiments that were selectively located at GABAergic and glycinergic postsynaptic sites *in vivo* (Alvarez, 2017). Furthermore, the size of the postsynaptic density, which

correlates with the size of the gephyrin cluster, is directly proportional to postsynaptic receptor content and the amplitude of the postsynaptic currents (mIPSPs) in both glycinergic and GABAergic synapses (Alvarez, 2017).

Multimeric conformations of gephyrin that form the mentioned puncta have been proposed but the actual structure for full-length gephyrin protein is still unknown (Choii and Ko, 2015). This is, in part, because gephyrin structure depends on alternative splicing that, in turn, depends on the tissue- and species-specific expression patterns (for instance, the mouse gephyrin gene contains 30 exons, of which, 10 are subject to alternative splicing). These splice variants present different gephyrin oligomerisation properties and affinities for postsynaptic receptors. Therefore, this variety of multimers potentially provides adaptability for the anchoring to the cytoskeleton and the scaffolding for multiple postsynaptic proteins. However, the *raison d'être* of gephyrin isoforms and whether alternative splicing mechanisms are regulated by activity has not been investigated so far (Fritschy, Harvey and Schwarz, 2008). One of the few works on this topic indicates that glutamate-mediated seizure-induced alkalosis affects gephyrin splicing and generates isoforms that lack a gephyrin domain (the G-domain) necessary for gephyrin's aggregation and scaffold formation at synapses, the $GABA_AR$ clustering and synaptic function.

In vertebrates, gephyrin is composed of three different domains: G, E and C domains. G and E domains took their names from homologous *Escherichia coli*'s enzymes, MogA and MoeA, which are essential proteins to synthesise a cofactor required for the activity of invertebrate molybdoenzymes (Alvarez, 2017). However, this could not be the case in vertebrates, since an initial analysis of primary neuron and astrocyte cultures indicate that molybdenum cofactor synthesis in the CNS takes place only in astrocytes (Smolinsky et al.,2008). The intermediate C domain of gephyrin contains several residues for post-translational modifications and binding sites to other synaptic proteins, such as $GABA_A$ receptor-associated protein (GABARAP) and dynein light chains 1 and 2. Therefore, C domain is believed to have some function rather than being only a connection sequence. Additionally, other individual domains have specific functions, such as the phosphorylation of the E domain, which controls the

binding affinity to GlyR β-subunit or the E domain ability to regulate cytosolic aggregation and postsynaptic receptor clustering.

P1 gephyrin is a common variant at inhibitory synapses that is composed of three domains: an amino-terminal G domain, a carboxy-terminal E domain and a linker C domain. C-linker includes many phosphorylation sites, proteolytic sites and binding sites for interacting proteins. It is targeted by a number of kinases including CaMKII, ERK, Cdk5 and GSK3 and phosphorylation-isomerisation effectors (Pin1), which modulate gephyrin´s aggregation and density. As for G and E domains, they participate in gephyrin's clustering at the membrane by trimerising and dimerising. The E dimerisation interface binds with high affinity to the cytoplasmic loop of GlyR.

Gephyrin interactome

With reference to gephyrin interactome, initial works of co-purification with polymerised tubulin positioned it as a scaffolding protein. Furthermore, the wide array of interactors of various functional classes of proteins also point towards that thesis (Tyagarajan and Fritschy, 2014).

Gephyrin has been demonstrated to interreact with various proteins that can anchor to the postsynaptic actin cytoskeleton and modulate F-actin elongation and stabilisation. One example is the Mena/VASP (mammalian enabled/vasodilator-stimulated phosphoprotein) complex, which links gephyrin and F-actin. Also, it directly interacts with profilins which bind G-actin (involved in actin filament elongation). More recently, elongating factors eEF1A1 and eEF1A2 (involved in the bundle and stabilisation of actin) were shown to co-localise with gephyrin postsynaptically, and their overexpression produces increased gephyrin cluster size and density. These interactions provide gephyrin with the ability to introduce changes in structural plasticity (Alvarez, 2017).

Gephyrin also interacts with molecules of the inhibitory synapse structure, namely neuroligins and collybistin. These interactions are thought to affect the formation

and maturation of inhibitory synapses of different types.

Neuroligins (NL) are postsynaptic density transmembrane proteins that form adhesion connections with presynaptic neurexins and bind to gephyrin. Adhesion favours synaptogenesis and synapse stabilisation, maturation and function (Alvarez, 2017). Four NLs have been identified in rodents that further differentiate through splice variants. NL-2 and NL-4 are predominantly associated with inhibitory synapses, to which gephyrin directly binds. NL-2 knock-down decreases gephyrin clustering and reduces GABAergic and glycinergic neurotransmission. Oppositely, diminishing gephyrin and receptor clustering does not reduce NL-2 recruitment to the synapse; however, gephyrin scaffold disruption (in CA1 pyramidal $GABA_A$ α2-containing synapses) prevents NL-2 accumulation on the initial segment of the axon, but not in cell body's synapses (that present different $GABA_A$ receptors). Therefore, NL-2 has been proposed to mediate the recruitment of gephyrin to the postsynaptic GABAergic synapses uninfluenced by nearby $GABA_A$ receptor activation. Moreover, NL-2 overexpression can induce presynaptic differentiation in passing axons. Furthermore, NLs knockdown in the hippocampus selectively decreases inhibitory synaptic transmission from parvalbumin-positive interneurons but not from somatostatin-positive interneurons (Alvarez, 2017).

Gephyrin binding to $GABA_A$ receptor subunits seems to be of lower affinity than GlyR binding, thus less likely to stabilise postsynaptic gephyrin scaffolds. In $GABA_A$ synapses gephyrin binding to the postsynaptic membrane is improved by its interaction with collybistin. This protein directly binds gephyrin and it contains a domain that binds to PIP3 (phosphatidylinositol-trisphosphate) membrane anchor, which bridges gephyrin to the membrane. The deletion of collybistin impairs GABAergic synapses in the hippocampus (but not glycinergic ones). In this interaction, NLs are necessary for collybistin to recruit gephyrin to the membrane. Curiously, like Eph receptors, collybistin binds to Rho-like GTPase which also acts in favour of collybistin-gephyrin membrane clustering. Complex neuroligin-collybistin interactions and phosphorylation events on gephyrin and NL-2 regulate the NL-2 binding to gephyrin (Alvarez, 2017).

EphA4 interactome

The interactome of EphA4 has been partially investigated (for review: Cramer and Miko, 2016; Ferluga and Debinski, 2014; Kania and Klein, 2016). In the field of neurobiology, these include the proto-oncogene tyrosine-protein kinase Fyn (phosphorylated at position Tyr-602), the signal-induced proliferation-associated 1-like protein 1 (SIPA1L1) (which interacts via PDZ domain) and various GTPases, like RAP1 (RAP1A or RAP1B) and RAP2 (RAP2A, RAP2B or RAP2C). It also interacts with the Cyclin-dependent kinase 5 (CDK5), CDK5R1, and the neuronal guanine nucleotide exchange factor (NGEF). Furthermore, EphA4 has been shown to interact with chimerin 1 (CHN1), which links EphA4 activation to RAC1 in axon guidance regulation (Ellis et al., 1996; Shamah et al., 2001; Beg et al., 2007; Iwasato et al., 2007).

Interestingly, various EphA4-related downstream signalling cascades promote the remodelling of the actin cytoskeleton and modify the properties of receptors, adhesion molecules and scaffolding proteins. These are events that underlie the morphogenesis of spines and homeostatic plasticity (Fiala, Spacek and Harris, 2002; Yamaguchi and Pasquale, 2004; Bourne and Harris, 2008). Spine morphology is modified by various signalling pathways, which include mediator proteins such as Cdk5, APC-Cdh1 complex, SPAR, integrins and phospholipase Cγ1 (Murai et al., 2003; Pak and Sheng, 2003; Fu et al., 2007, 2011; Richter et al., 2007).

Cdk5 and spine-associated RapGAP (SPAR) are signalling components that mediate both the decrease of synaptic strength and affect spine morphogenesis (Pak and Sheng, 2003; Fu et al., 2007; Richter et al., 2007), which makes them good candidates for encompassing morphological and functional changes in spine plasticity. Particularly, in the case of Cdk5, ephrin-A1-activation of EphA4 triggers the recruitment of Cdk5 to EphA4, which results in the tyrosine phosphorylation and activation of Cdk5. Blocking Cdk5 activity inhibits ligand-triggered spine retraction and decrease of mEPSC frequency at hippocampal synapses. Cdk5 increases the association EphA4 and Cdk5 increases ephexin1 activation, which in turn modulates activation of RhoA (a small Rho GTPase). The

association between EphA4 and ephexin1 was reduced in Cdk5$^{-/-}$ mice brains and Cdk5-dependent phosphorylation of ephexin1 is required for ephrin-A1-mediated regulation of spine density (Fu et al., 2007).

Synaptic activity seems to increase tyrosine phosphorylation of EphA4 in a ligand-dependent manner. This event is associated with an increase, in neurons, of the interaction of EphA4 with the ubiquitin ligase anaphase-promoting complex (APC) and its activator Cdh1. This interaction seems to promote GluR1 proteasome degradation in vitro, and accordingly, the deficiency of Cdh1 in neurons abolished the down regulation of GluR1 and a concomitant decrease in excitatory currents (Fu et al., 2011).

A different research group identified an interaction between EphA4 receptor and the SPAR, a GTPase-activating protein. This interaction regulates the inactivation of the related GTPases, Rap1 and Rap2, in neuronal cells, which has been shown to be affecting dendritic spine morphology and synaptic plasticity. These researchers demonstrated that SPAR-mediated inactivation of Rap1, but not Rap2, is essential for ephrin-A-dependent growth cone collapse and integrin-mediated adhesion. A different EphA4 interaction with β1-integrin signalling pathways also influences dendritic spine morphology (Bourgin et al., 2007; Fu et al., 2011).

Ephrin interaction with EphA4 leads to the recruitment and activation of the phospholipase Cγ1 (PLCγ1). Interestingly, EphA4 and PLC are able to disrupt the association of cofilin (an actin depolymerising/severing factor) with the plasma membrane. This signalling pathway may enable cofilin to change structural plasticity of spines by depolymerisation of actin filaments and restructure spines at sites of ephrin-EphA4 contact (Zhou et al., 2007; Fu et al., 2011).

RESULTS

Immunostainings in Figures 10 and 11 revealed that EphA4 and the GABAergic synapse marker, gephyrin, co-localise or are situated in each other's direct vicinity in the CeA. To verify whether those two proteins directly interact, I set to immunoprecipitate EphA4 from the mouse CeA and analyse its interactome as described in *Materials and Methods*.

Western blot revealed evident direct interaction between EphA4 and gephyrin in the CeA (Figure 24 A). This blot indicates that EphA4 interacts with a gephyrin variant of about 50 KDa, which is lower in molecular weight than the typical 93 KDa form. Gephyrin (93 KDa) was also overexpressed and immunoprecipitated from Neuro-2A cell line lysates to test the *bona fide* interaction between EphA4 and gephyrin, and the pulled-down proteins were analysed by Western blot (Figure 24 B). The 93 KDa gephyrin shows interaction with the wild type form of EphA4 (~110 KDa) (Figure 24 B). However, it also has a high affinity for a lower molecular weight variant of EphA4 (~50KDa) of unknown identity (Figure 24 B).

In order to investigate whether the plasmin-induced cleavage of EphA4 affects the EphA4/gephyrin interaction, EphA4 variants (i.e. wtEphA4, crEphA4 or tEphA4) were co-expressed with gephyrin in the Neuro-2A cell line followed by the immunoprecipitation of co-expressed gephyrin and the subsequent Western blot analysis of the different EphA4 variants co-immunoprecipitated. Quantification of the results confirmed a strong interaction of gephyrin with wtEphA4 and, strikingly, EphA4/gephyrin binding was enhanced when crEphA4 was expressed instead of wtEphA4, and disrupted upon the expression of tEphA4 (Figure 24 C and D, n = 4. ANOVA, $F(2,10) = 8.443$, $p<0.01$; Unpaired t-test: wtEphA4 control: 0.000 ± 0.010 a.u. vs. crEphA4: 1.484 ± 0.566 a.u., $p = 0.019$; Welch´s t-test: wtEphA4 control: 1.000 ± 0.010 a.u. vs tEphA4: -0.288 ± 0.032 a.u., $p = 0.003$).

FIGURES

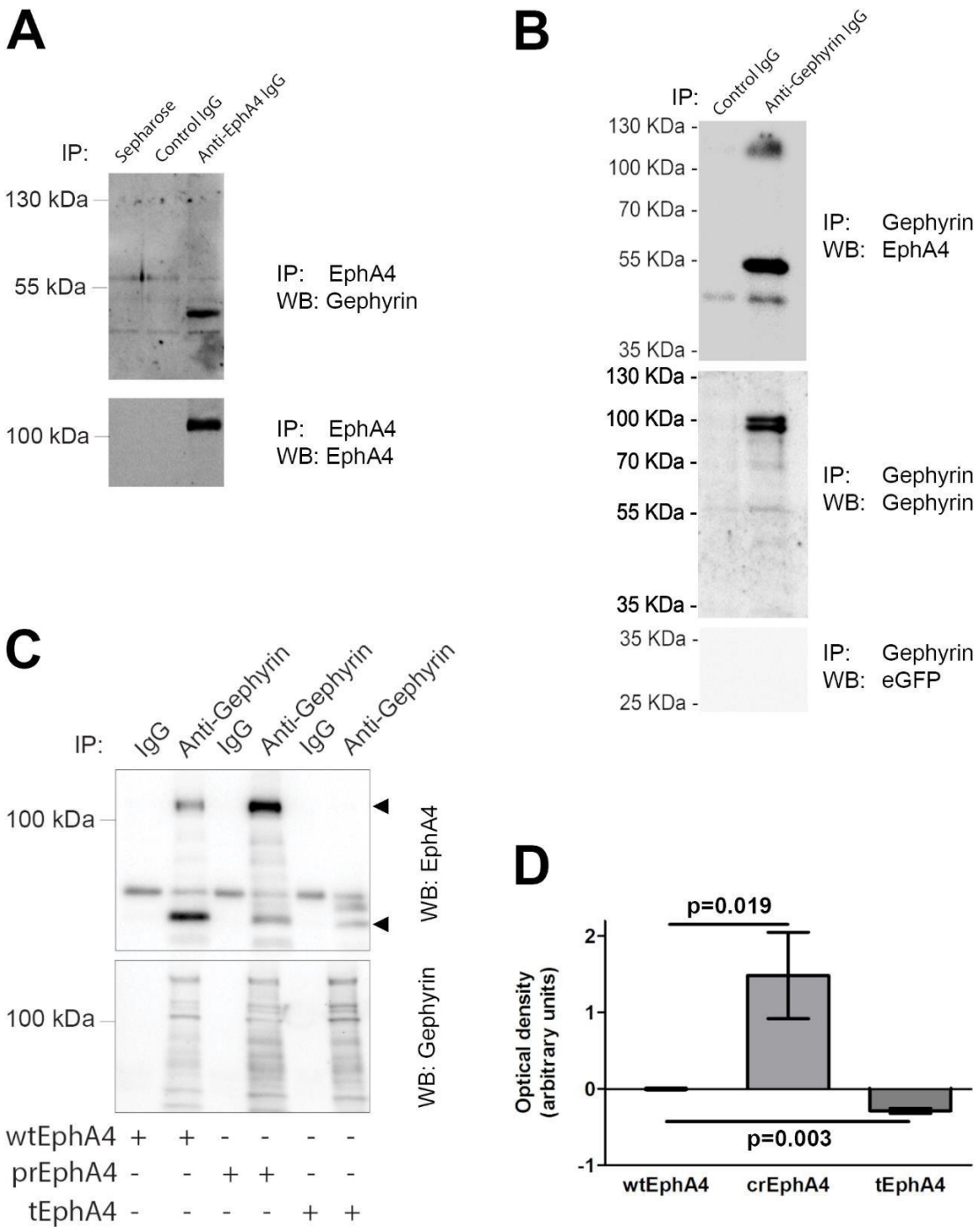


Figure 24. EphA4 variants related to plasmin cleavage differentially interact with gephyrin. IP = immunoprecipitation; WB = western blot; wtEphA4 = wild type EphA4; crEphA4 = cleavage-resistant EphA4; tEphA4 = truncated EphA4. The figure shows representative blots, generated by Western blot technique, of the products from the co-immunoprecipitation (co-IP) of gephyrin and EphA4. A) Co-IP of gephyrin using EphA4 from amygdalar tissue lysates as IP bait. A blot for EphA4 using a different antibody is included as an IP control. B) Co-IP of overexpressed wtEphA4 from N2A cell line lysates using gephyrin as IP bait. A blot for gephyrin using a different antibody is included as IP control. eGFP control blot shows the specificity of EphA4-gephyrin interaction. C) Co-IP of different variants of EphA4 overexpressed in N2A cell line (arrowheads) using gephyrin as IP bait. A blot for gephyrin using a different antibody is included as IP control. D) Quantification for EphA4 different variants on C panel (arrowheads). Total gephyrin was used as a normalising control for the interaction. Gephyrin seems to interact more with the non-cleavable variant and less with tEphA4. All data are presented as optical density (O.D.) mean ± SEM (n = 4). p values are included over the corresponding chart's bars.

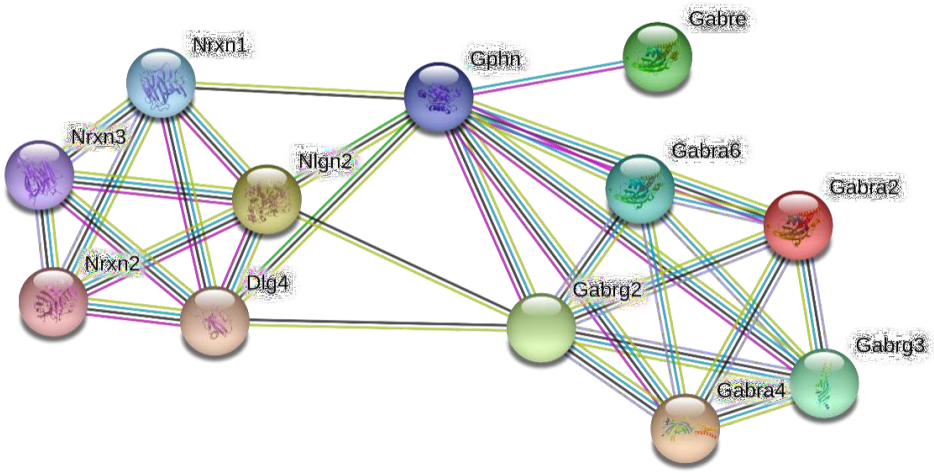
DISCUSSION

A previous mRNA microarray study from our lab compared specific transcripts in the amygdalae of tPA and $tPA^{-/-}$ mice. These data showed differences in a number of genes. In particular, pathway analysis of these data revealed that various $GABA_A$ receptor ($GABA_AR$) subunits were affected by the lack of tPA gene. This fact poses the question of whether the GABAergic signalling pathway could be influenced by the tPA/plasmin/EphA4 cascade.

I first examined whether the direct protein-protein interaction between EphA4 and each of the different tPA-affected $GABA_AR$ subunits could influence the GABAergic synapse composition and the transcriptome of these subunits. I focused on those $GABA_AR$ subunits whose protein levels are increased in CeA area using the Allen Brain Atlas databases (http://www.brain-map.org) as a reference for the protein expression levels (i.e. GABAα2, GABAγ1, GABAε). To this aim, I tried to co-immunoprecipitate any of the three subunits tested from either hippocampus or amygdala tissue homogenates by using EphA4 as the bait protein. However, none of the subunits showed any specific interaction with EphA4 (data not shown). This possibility will need to be reviewed in the future due to methodological concerns about the quality of the antibodies available. Although I could not confirm the direct interaction of EphA4 with these subunits, this idea cannot be rejected either. Further optimisation of the co-IP technique or a different protein-protein interaction analysis may be able to overcome this issue.

These results led to experiments based on possible non-direct interactions of EphA4 and the $GABA_AR$ subunits through a common protein node. For this purpose, I used STRING, which is an on-line software that compiles information of the protein-protein associations derived from high-throughput experimental data, from the mining of databases extracted from literature and from predictions based on genomic context analysis (Szklarczyk et al. Nucleic Acids Res. 2015 43 (Database issue):D447-52). This database failed to produce any relevant result for any known interaction between EphA4 and the different $GABA_AR$ subunits of less than four nodes in between them. However, the software revealed a very high probability of interaction between all the $GABA_AR$ subunits and gephyrin (a protein demonstrated to be co-localising with EphA4 in Chapter 3 of this thesis)

and with various members of the neurexin/neuroligin family (which are also synaptic proteins) (Image 10). Since gephyrin has shown the ability to interact with several other specific trans-synaptic adhesion molecules, such as neuroligins (Tyagarajan and Fritschy, 2014) and this protein co-localises with EphA4 in the GABAergic synapses of the CeA, I decided to investigate whether tPA could be affecting the GABAergic synapse through the cleavage of EphA4 and its interaction with gephyrin.



node1	node2	score
Gephyrin	Gamma-aminobutyric acid (GABA) A receptor, subunit gamma 2	0.986
Gephyrin	Gamma-aminobutyric acid (GABA) A receptor, subunit alpha 6	0.934
Gephyrin	Gamma-aminobutyric acid (GABA) A receptor, subunit alpha 2	0.929
Gephyrin	Gamma-aminobutyric acid (GABA) A receptor, subunit gamma 3	0.924
Gephyrin	Gamma-aminobutyric acid (GABA) A receptor, subunit alpha 4	0.914
Gephyrin	Gamma-aminobutyric acid (GABA) A receptor, subunit epsilon	0.895

Image 10. Scheme of STRING association network of GABAAR subunits and table showing the confidence score of each predicted association. The scores are produced by benchmarking the performance of the predictions against a common reference set of trusted, genuine associations. The benchmarked confidence scores in STRING normally correspond to the probability of finding the linked proteins within the same molecular pathway. Authors consider numbers >0.7 a high confidence range (Von Mering et al., 2005).

Indeed, immunoprecipitation of EphA4 using homogenised tissue from the mouse CeA revealed a direct interaction between EphA4 and gephyrin (Figure 24 A). Conversely, this interaction is also produced when gephyrin is the bait protein used for the immunoprecipitation and both gephyrin and EphA4 are co-expressed in the N2A cell line (Figure 24 B). The co-localisation of these two proteins detected in experiments using fluorescent immunohistochemistry and confocal microscopy indicates that this interaction is taking place in CeA (Figures 10 and 11).

Interestingly, EphA4 is interacting with a form of gephyrin that is not corresponding to any of the classically-described splice variants found at ~92 KDa. Instead, EphA4 interacts with a lower molecular weight variant, of ~50 KDa, detected with an antibody directed against the gephyrin's C-terminal E-domain (Figure 24 A). Its molecular weight indicates that this lower-molecular-weight form could be part of the E-domain. Moreover, it could correspond with a gephyrin variant that has been previously found to be notably present in the rat's brain and liver (Hermann, Kneussel and Betz, 2001). Other laboratories have also found these type of lower molecular bands in patients with Alzheimer's disease (Hales et al., 2014) and patients with temporal lobe epilepsy (Förstera et al., 2010), and they also seem to be part of to the C-terminal E-domain of the protein. These ~50 KDa forms of gephyrin have been neither identified nor proposed to exert any specific function, but it could be possibly playing a role in the maintenance of the GABAergic postsynaptic terminal's morphology and function. Further analysis will be needed to address the identity of this fragment, its relation with similar EphA4 fragments in humans and its functions.

Moreover, I performed further experiments to address the interaction between gephyrin and the different variants of EphA4. Co-immunoprecipitation studies confirmed positive interaction of wtEphA4 with gephyrin, but it also revealed that cleavage-resistant form of EphA4 (crEphA4) presents a higher interaction with gephyrin (when compared with wtEphA4) whereas truncated EphA4 variant presents a lower interaction (Figure 24 C and D). The consequences of this variability in the interactome of gephyrin could be diverse since gephyrin has been observed to have different functions.

A plausible possibility is that it affects the molecular scaffold properties that link gephyrin to receptors and the cytoskeleton. Interaction between gephyrin subunits reshapes postsynaptic gephyrin clusters aggregation and could reduce postsynaptic gephyrin (Förstera et al., 2010). This possibility would likely change dynamic processes underlying synapse formation, maintenance and plasticity at inhibitory synapses (Fritschy, Harvey and Schwarz, 2008).

GABAARs dynamics allow these receptors to be transported into the plasma membrane from cytoplasmic pools or diffuse laterally in and out of synapses within the membrane to regulate the concentration of receptors in this structure, and hence its synaptic strength. At postsynaptic sites, transient interactions receptor-scaffold control the “diffusion trapping” of the receptors and therefore impact the synaptic strength (Pizzarelli et al., 2019). In the case of GABAAR and gephyrin, this thesis gets reflected in the fact that in gephyrin-containing GABAergic synapses (where GABAARs and gephyrin correlate in a nanoscale level [Crosby et al., 2019]), the magnitude of retention of GABAARs is proportional to the gephyrin content. Furthermore, gephyrin blockade or reduction in gephyrin mRNA expression results in significant slowdown of the receptors kinetics, a decrease in the number of synaptic γ2-subunit-containing GABAARs clusters, and a reduction of amplitude and frequency of inhibitory postsynaptic currents (IPSCs), although the precise mechanism whereby gephyrin regulates the properties of GABAARs is still unknown (Pizzarelli et al., 2019). In sum, control over the diffusion dynamics of GABAARs is an important mechanism to regulate inhibitory plasticity where EphA4 cleavage could be participating in via gephyrin interaction.

Data from preliminary experiments I did not present in this thesis suggest that there are differences in the (auto)phosphorylation of the different EphA4 variants (wt-, cr- or tEphA4) and possibly in the phosphorylation of other proteins, such as gephyrin. Furthermore, for some interactors, phosphorylation controls their ability to be recruited by gephyrin (Pizzarelli et al., 2019) and make changes in the kinase activity of EphA4 worth to be explored in the future. Gephyrin is a major target for kinases (Alvarez, 2017) and phosphatases have been shown to modify gephyrin clusters (Bausen et al., 2006). Previous works have evidenced that

kinases can control gephyrin by producing reduced (Tyagarajan et al., 2013) or enhanced gephyrin clustering (Flores et al., 2015); and protein manipulations producing phosphomimetic gephyrin mutants indicate that these variants produce smaller and higher-molecule-density clusters as compared to wild types, which suggest gephyrin compaction. Moreover, gephyrin phosphorylation can modify synaptic function. ERK1/2-dependent phosphorylation of gephyrin at serine 268 reduces the scaffold area and miniature IPSC amplitude while blocking GSK3β-mediated phosphorylation at serine 270 increases mIPSC amplitude and frequency (Pizzarelli et al., 2019). Consequently, phosphorylation events, although they regulate the $GABA_AR$ diffusion similarly, they promote opposite effects on synaptic events. Interestingly, in the BLA, the expression of a palmitoylation-deficient gephyrin mutant causes reduced GABAergic transmission that leads to marked anxiety-like behaviours in rats (Shen et al., 2019). Palmitoyl group facilitates the attachment of gephyrin to the membrane, which results in enhanced surface accumulation (Pizzarelli et al., 2019) and helps to stabilise GABAergic synapses (Tyagarajan and Fritschy 2014). Therefore, posttranslational mechanisms affecting gephyrin and its interactors could influence their protein interaction, synaptic function and even anxiety-like behaviours.

More examples in the literature argue in favour of the close relationship between the GABAergic system and EphA4. For instance, works using GABAR and interacting small molecules during the development have shown that the application of the $GABA_A$ antagonist, picrotoxin, produces a decrease in the rhythmic bursting activity of motoneurons and in the levels of EphA4, which are rescued by restoring the normal busting frequency (Kastanenka and Landmesser, 2010). Different *in vitro* analyses using the GABAR antagonist, bicuculline, triggers an EphA4-dependant reduction in GABA-mediated inhibition. It diminished the amplitude of miniature EPSCs in cortical neurons through a decrease in AMPA receptors (Fu et al., 2011; North, Clifford and Donoghue, 2013). The same compound induces tyrosine phosphorylation of EphA4, which might be a necessary mechanism for the reduction of miniature-EPSC amplitude (Fu et al., 2011) and adds to the justification for the study of the kinase domain of EphA4.

Additionally, during development, the absence of EphA4 (EphA4$^{-/-}$ mice) in the ventral spinal cord provokes an abnormal increase in the number and proportions of the glutamate and glycine presynaptic transporters but not the GABA transporter. This suggests that the potential changes in GABAergic synapses provoked by gephyrin may be postsynaptic, which would also support the postsynaptic nature of the changes produced by the interaction between EphA4 and gephyrin (Restrepo et al., 2011).

Therefore, in our hypothesised model, interactions of gephyrin with EphA4 helping to maintain GABAergic spine morphology could be disrupted upon plasmin-mediated EphA4 cleavage and modify synaptic transmission. At the circuitry level, EphA4 cleavage (and the subsequently reduced interaction with gephyrin), would hypothetically produce a dysfunctional CeL tone. This defective inhibitory GABAergic circuit would likely produce an increased CeL-to-CeM tone, which is known to increase anxiety-like behaviours (Davis, 2000).

CONCLUSIONS

Previous mRNA microarray data from our lab comparing mouse amygdalae from tPA and tPA$^{-/-}$ indicate that this protease affects the expression of $GABA_A$ receptor ($GABA_AR$) subunits, which leads to the question of whether EphA4 cleavage by the tPA/plasmin cascade could affect protein-protein interactions and destabilise GABAergic inhibitory neurotransmission.

EphA4 does not show direct interaction with any of the different $GABA_AR$ subunits affected by the lack of tPA (detected by the mRNA microarray), at least with the methodology used in this work. However, co-immunoprecipitation experiments using CeA homogenised tissue revealed a direct interaction between EphA4 and gephyrin, a protein anchoring the GABA receptor subunits. Further experiments show that plasmin-induced shedding of EphA4 weakens the interaction of EphA4 with gephyrin, whereas plasmin cleavage-resistant form strengthens this interaction. The consequences of this effect are still unknown, but a lessened

interaction with the membrane receptors (e.g. EphA4) can lead to an increase in gephyrin trafficking and thus clustering. These changes have been observed to produce destabilisation of GABAergic synapses and to potential changes in their morphology and functional plasticity. The results presented here would then be in line with a destabilising effect of the tPA/plasmin cascade that would raise the excitatory tone in the CeL amygdala. The excitatory tone in CeL would thus increase output signals from CeM amygdala that would produce the expression of anxiety-like behaviours.

FUTURE WORKS

In order to translate these findings into human research, there is an undeniable need to know what downstream processes could be affected upon cleavage of EphA4. In this work, we have demonstrated that the cleavage of EphA4 affects its interaction with gephyrin, a scaffolding protein that harbours postsynaptic GABAergic synapses. The central role of GABAergic synapses in CeA poses GABA receptors as the main target for changes in plasticity and downstream processes related to the EphA4 receptor. The possibility of direct interaction between GABARs and EphA4 could not be ruled out in this work. Therefore, the importance of the GABAergic system in CeA makes these interactions worth to be explored. In addition, gephyrin interaction with GABARs can change upon EphA4 cleavage. Special attention will be paid to $GABA_A$ α2, α5 and γ1 subunits since they are the most highly expressed subunits in the central nucleus of the amygdala or have been previously affected by EphA4 or gephyrin.

Interaction of EphA4 and gephyrin may be of importance for the structure and function of GABAergic synapses. To define the functionality of this interaction further experiments are required. Mass spectrometry techniques would be of use to confirm the identity of the ~50 KDa gephyrin variant interacting with EphA4. Then, specific genetic/proteomic manipulations would need to be performed to assess the role of this gephyrin isoform in brain processes and how it is generated. Moreover, to address the influence of the kinase domain in the

interaction with gephyrin, loss and gain of function experiments in which phosphorylation sites are constitutively activated or deactivated could address the specific meaning of posttranslational phosphorylation in this receptor-scaffold interaction and whether it has any influence in stress-related behaviours. Furthermore, synaptic activity increases tyrosine phosphorylation of EphA4, which through interaction with APC-Cdh1 complex promotes GluR1 proteasome degradation and a concomitant decrease in excitatory currents (Fu et al., 2011). Hence, phosphorylation of EphA4 upon cleavage and other events should also be checked in order to understand downstream interactions of the receptor.

Moreover, there are other receptors in the brain that are directly involved in the maintenance of anxiety over time and have not been tested for their interaction with EphA4. A clear example is CRFR2 in the CeA, which is involved in recovery from stress. Consequently, interactions of EphA4 with glucocorticoid and mineralocorticoid receptors could shed some light on how anxiety is maintained over time. Another example is glutamatergic receptors. GluR1 glutamatergic receptor, which has been shown to be required for synaptic plasticity, is downregulated upon changes in EphA4 phosphorylation. Other glutamatergic receptors could be equally affected by the cleavage of EphA4 and its phosphorylation and have consequences for synaptic plasticity and the CeA functions. Therefore, glutamatergic receptors are another sensible option to explore in terms of EphA4 interaction.

As mentioned in this thesis, EphA4 has also been linked with molecules that form the cytoskeleton of dendritic spines and neuronal bodies. These interactions could be directly related to the capacity of the receptor to modify functional plasticity. In this line, Rho family GTPases have been shown to play a central role. These include proteins such as Rap1 GAP, Rab5-GEF Rin1, RhoA-specific GEF ephexin1 or RhoA. Other molecules, such as serine/threonine kinase Cdk5, the APC-Cdh1 complex, SPAR, β1-integrin or phospholipase Cγ1 are also related to these processes and shown to be necessary for neuronal plasticity. Therefore, it would be interesting to explore how EphA4 cleavage can affect these pathways and others that could modify cellular functionality.

CHAPTER 6: ANXIETY-LIKE BEHAVIOUR

INTRODUCTION

The PKCδ^{+} interneurons located in the CeL and the tPA/plasmin system have been shown to be critical components of the neural circuit of anxiety in this work and previous references in the literature. This chapter investigates whether the tPA/plasmin/EphA4/effectors signalling pathway in CeA can influence the expression of anxiety-like behaviours.

The characterisation of the expression of emotions in humans and other mammals as phylogenetically conserved phenomena set the foundations for experimental research in behavioural neuroscience. It has also contributed to the understanding of the neurobiology of psychiatric diseases and the discovery of new drugs. Animal models of emotional states endeavour to reproduce specific aspects of human nervous system processes and disorders. Theoretically, a model should reproduce all features of the investigated phenomenon; however, this ideal situation is not common due to the complex physiology and pathology of brain processes and the, sometimes notable, differences between humans and other species used as animal models (e.g. rodents). Therefore, animal models of anxiety have not aimed to replicate all features and symptoms of a specific anxiety disorder but to generate an emotional state similar to anxiety (anxiety-like) that could be related to these disorders. Particularly, these models try to reproduce the physiological and behavioural changes associated with specific emotional states (which would cover the “face validity” of the model), the aetiology of diseases (“construct validity”), and responses to pharmacological treatments (“predictive validity”) (Lister, 1990; Bourin et al., 2007; Campos et al., 2013).

Animal models used to study stress-related disorders

A combination of behaviours, neurophysiology, cognitive changes and somatic responses are produced in several animal species to respond to the potential presence of a threat (Gordon and Hen, 2004). The alternative strategies chosen will depend on a mix of psychobiological profile (e.g. whether the subject of study is a dominant individual in a group), previous experience, appraisal of the

situation and environmental factors. In the case of behavioural responses in anxiety disorders, they are usually characterised by the avoidance of threatening situations. For various types of rodents (the most commonly used animals in neurobiology preclinical research), motivational conflicts can be a principal source of anxiety. Motivational conflicts involve an individual being exposed to reinforcements of opposite valence (one positive and one [opposed] negative reinforcement). Wrong choices can potentially lead to negative outcomes, such as unpleasant situations, lower social status or even life-threatening situations. Therefore, decisions with unpredictable consequences are a source of stress. In rodents, conflict situations can be achieved by presenting a simultaneous combination of approach- and avoidance-inducing situations that trigger opposite motivational states. For instance, approach situations are induced by unconditioned exploratory motivations (such as the innate attraction to new environments) or in seeking responses that have been previously conditioned (e.g. giving the animals a nice food). Conversely, avoidance situations can also be induced by unpleasant stimuli. For example, unconditioned environments (brightly lit, open and elevated places) or learnt environments (aversive stimuli such as electric shocks or a restraint position) (Lister, 1990; Bourin et al., 2007; Campos et al., 2013).

Animal models based on untrained (unconditioned) aversive behaviours, in which there is a measurement of conflicts, are defined as “ethological” (e.g. elevated plus-maze test [EPM], shock-probe burying test [SPB], light/dark exploration test [LD], social interaction test [SI], and the separation-or shock-induced ultrasonic vocalisation test [USV]). On the contrary, models that make use of trained responses are defined as “conditioned operant conflict” tests (e.g. Geller-Seifter conflict test [GSCT] or Vogel conflict test [VCT]). Finally, models that mainly involve classical conditioning are discussed as “classic-conditioning” tests (e.g. Pavlov’s conditioning) (Campos et al., 2013).

Ethologically-based animal models and tests of anxiety try to recreate the natural conditions in which such emotional states are evoked, and therefore they are thought to minimise possible confounding effects of conditioned states. However, there still exist individual differences and variable behavioural baseline levels in

these models that could be potentially considered as confounding factors (Bourin et al., 2007).

I will only describe in detail the tests used in this research; i.e. the EPM, LDB and OFT. These models have been sufficiently challenged and extensively tested, including pharmacological (predictive) validity, which means that all of the index-measures of anxiety are inhibited by peripheral administration of commonly used anxiolytic drugs (e.g. intra-peritoneal diazepam). Lack of training, along with their convenience (in terms of space, maintenance, literature about them or similarities with human biology), are the main strengths of these models (Carola et al., 2002; Bourin et al., 2007; Engin and Treit, 2008).

The elevated plus maze is the most used, challenged, characterised and pharmacologically validated animal tests of anxiety to date (Finn, Rutledge-Gorman and Crabbe, 2003). The apparatus used in this paradigm consists of an elevated platform in the shape of a plus sign. It is built with a central square from which four arms (identical in length and width) extend. Two opposing arms are enclosed by high walls that make this area dark; whereas the other two opposing arms are opened and often illuminated, consequently making it a brightly lit area. The maze bases its adequacy on the conflict between the instinctive positive physiological impulse of rodents to explore a new environment and the natural aversion to opened spaces, elevation and a context not familiar to the animal (Borsini, Podhorna and Marazziti, 2002). In this test, anxiety is measured in terms of the percentage of open arms entries or time spent in the open arms. Hence, increased levels of anxiety will be displayed as a decrease in open arm entries or in time spent in the open arms. Other parameters that correlate with anxiety-like behaviours can be used, such as crossings of the animal into the light area or number of total crossings between areas. The EPM has been shown to be a robust method to establish anxiety-like states (such as in anxiety-modulating drugs or mouse genotypes), although changes in test conditions or parameters (age, gender, animal handling, test timing, illumination, size of the maze and method of scoring) can influence inter- or intra-experiment results (Bourin et al., 2007).

The light-dark box is another commonly-used test to measure anxiety. It is also

based on the concept of the conflict between innate exploratory behaviours provoked by a new environment versus the aversive stimulus produced by lit and open areas (Finn, Rutledge-Gorman and Crabbe, 2003). The apparatus used for the test consists of two compartments. One of them is intensely illuminated and may be slightly smaller than the other one. The second compartment is either entirely closed or enclosed by a black-dyed transparent wall. The animals are allowed to freely move between both compartments. The most commonly measured parameters to assess anxiety in this test are the time spent in the illuminated chamber, but also the latency to exit the dark chamber and the number of transitions to the light chamber. Therefore, when an animal shows a decreased time spent in the light compartment, more latency to exit the dark compartment or a reduced number of transitions to the light compartment, it would be interpreted as having an increased level of anxiety.

Another of the most commonly used tests to measure anxiety-like behaviours is the open-field test. It consists of a chamber (completely unknown for the animal) with an open space arena that is surrounded by high walls and its dimensions depend on the type of animal used. The animals are allowed to freely move within the chamber. Although it might be considered a simple task, there is no consensus in the scientific community on which would be the most appropriate features of the test. For instance, some apparatus for this test are square-shaped whereas others are circular. They can be clear or opaque, bright or dark, with tops or totally open, with the presence of objects within the arena or not, with a different placement of the animal in the open field or a different recording period. And even the events measured or recorded are also variable in the literature. The only agreement on this last point is that the test should measure activities other than spontaneous exploratory locomotion and the trend of the animals to move to the periphery (as opposed to crossing the centre) of the arena (Wu, Kim and Zhuo, 2008). Pharmacological validation for this task is controversial as well because although it is sensitive to the majority of benzodiazepines and 5-HT_{1A} receptor agonists used to treat anxiety clinically, other molecules used do not have any effect on this task. These facts indicate that it may not be an accurate and robust method to model all features of anxiety disorders (Prut and Belzung, 2003).

The influence of stressful situations in anxiety-like behaviour: models of stress

As mentioned in Chapter 1, there is increasing evidence of stressful experiences occurring throughout life that may be critical to the development and pathogenesis of several psychiatric disorders, such as anxiety disorders (McEwen, 2003a, 2003b; McEwen, Gray and Nasca, 2015). Additionally, those psychological symptoms of anxiety disorders are accompanied by changes in the biochemistry of the individual, such as the activation of the hypothalamic-pituitary-adrenal (HPA) axis and changes in hormones, glucocorticoids and cytokines. Numerous studies in rodents have also shown the association between exposure to stressful situations and episodes of anxiety-related behaviours (Campos et al., 2013). Studies in models related to anxiety disorders, such as rodents subjected to chronic stress, describe differences in behaviour that can be described as anxiety-like behaviours (Magariños, Deslandes and McEwen, 1999).

The neurobiology of the relationship between somatic and psychological consequences provoked by extreme stressors has started to be better understood, thanks to the development of models of stress. These models focus on evaluating the changes induced by acute or chronic exposure to stressors (e.g. movement restriction, predator odour, electric shocks). The variation among these models is established by the duration (acute or chronic) and the nature of stressor exposure. Depending on the scope of the research, animals can be exposed to psychological (neonatal isolation, noise stress, circadian rhythm changes, predator stress) or physical challenges (restraint stress, immobilisation stress, temperature variation stress, electric footshock stress) (Campos et al., 2013).

Restraint stress (RS) and chronic immobilisation stress (CIS) are two of the most used ways to induce effective stress-related responses in terms of behaviour and biochemical reactions (Kvetňanský and Mikulaj, 1970). The restraint of the animals is usually achieved by restricting animal movements in a cylinder, a flexible wire mesh restrainers (rats) or conical tubes (mice) with ventilation holes for 120-180 min (Padovan and Guimarães, 2000). In the case of immobilisation

stress protocols, restriction of movements is limited to upper and lower limbs, typically by gently wrapping them with adhesive tape for 120 min and head movement is restricted by a metal loop wound around the neck (Hill, Hunter and McEwen, 2009; Shansky et al., 2009). These are considered acute paradigms for stress. The procedure can be repeated for several days in a row to induce chronic stress (7-21 days). After restraint or immobilisation stress, animals exhibit higher levels of anxiety-like behaviours in the EPM, OFT, LD and other tests of anxiety. Biochemical parameters are also changed (Hill, Hunter and McEwen, 2009; Shansky et al., 2009).

A detailed review of all the different stress paradigms used as a preclinical model of anxiety is out of the scope of this review. However, I am including a brief description of the paradigms that are used in this type of modelling apart from RS and CIS:

- Chronic unpredictable stress (CUS) is produced by daily exposure to a single different stressor such as food/water deprivation, immobilisation stress, cold stress, swim stress or cage movement. The sequence of the stressors is randomised and rodents are subjected to these stressors for 'chronic' periods ranging from 10 days to several weeks.
- Repeated/Chronic restraint stress (R/CRS): consists of restraint stress repeated for 7-21 days.
- Platform stress: involves brief exposure to an elevated platform in a brightly lit arena.

Various social stress paradigms are also included in this classification:

- Social isolation stress: consists of housing animals individually as opposed to control rodents, which are group-housed.
- Single-prolonged stress: involves exposure to a variety of different stressors (restraint stress, swim stress or exposure to ether) which is usually followed by a recovery period.
- Social instability stress (SIS): is based on the replacement of cage mates. This paradigm is sometimes carried out in combination with other stressors.

Apart from these stress paradigms, other works have explored the use of acute and chronic corticosterone exposure in rodents as a method to mimic the outcomes of activation of the HPA axis (Wilson et al., 2015).

The influence of the tPA / plasmin / EphA4 axis in anxiety-like behaviours

The importance of tPA and plasmin in anxiety-like behaviour has been reviewed in Chapter 1 and, as discussed in that chapter, several Eph receptors (such as EphA4 and EphB2) and ephrins have been shown in numerous studies to play roles in different behavioural paradigms of rodents. Here, I will only focus on the subject of our study, EphA4.

EphA4 receptor and behaviour

As there is no literature on the effects of EphA4 in anxiety, in order to understand EphA4's possible roles in anxiety-like behaviours, they need to be hypothesised and extrapolated from paradigms related to stress-induced behaviours from unconnected experiments. For example, contextual fear conditioning memory seems to be intact in mice with targeted kinase-inactive EphA4, meaning that EphA4 kinase-mediated forward signalling is not necessary for contextual fear memory formation and this could be the case for anxiety-like behaviours as well. Furthermore, different evidence shows that when EphA4 is abolished in pyramidal neurons of the forebrain in the CaMKII-cre:EphA4$^{lx/-}$ mice strain, their long-term contextual fear conditioning memory is only attenuated (Dines and Lamprecht, 2014, 2016).

A different group has suggested that the increase of LTP observed in the amygdala is inhibited after single prolonged stress (SPS, a model of PTSD) in rats due to the influence of Rin1 in the endocytosis of EphA4. Furthermore, they claim that the protein expression of EphA4, among others, is increased in amygdala and hippocampus after immobilisation-stress (Han et al., 2017).

A third group has also found a relation between EphA4 and stress-related disorders. In their work, they demonstrate that the chronic unpredictable mild stress (CUMS) paradigm downregulates the expression of EphA4 and upregulates ephrin-A3 in the hippocampus. Furthermore, they show that this tendency is inversed when the antidepressant, fluoxetine, is administered (Li et al., 2014).

Although the current knowledge about the role of EphA4 in stress response is limited to these studies, other types of behaviours are also affected by this receptor. EphA4 knockout mice (EphA4$^{-/-}$) are impaired in their ability to perform different tasks. For instance, they show impairment in a short-term spatial recognition memory test when tested by a spatial novelty preference task in the Y-maze (Vuillermot et al., 2011; Willi et al., 2012). EphA4$^{-/-}$ mice are also impaired in spontaneous alternation in the T-maze test (Deacon and Rawlins, 2006). Similarly, these animals show impaired hippocampus-dependent spatial memory when clustered EphA4 was administered before induced transient global ischemia (Yang et al., 2014).

Another set of experiments demonstrated that the kinase activity is not the only mechanism by which EphA4 works. Egea et al. used an EphA4 variant with a constitutively active kinase showing that this knock-in EphA4 receptor was able to normally regulate functions like midline axon guidance, hindlimb locomotion, *in vitro* growth cone collapse and phosphorylation of ephexin1. However, these animals had deficiencies in the development of thalamocortical projections and abnormalities in the spinal central pattern generator neurons, a group of neurons that, when overexcited, produce a hopping gait pattern in rodents (Egea et al., 2005).

In conclusion, tPA and plasmin are closely related to the development of stress-induced anxiety-like behaviours (e.g. Pawlak et al., 2003; Matys et al., 2004,2005) and some avoiding behaviours (e.g. Calabresi et al., 2000, Pawlak et al., 2002) among other behaviours; however, the precise mechanisms of these conducts are still elusive. Given the involvement of EphA4 in similar stress-induced behaviours (Li et al., 2014; Han et al., 2017), I tried to address the question of whether the cleavage of EphA4 would be a possible mechanism able

to produce any significant change in the expression of this type of behaviours in mice. In order to achieve this goal, signalling generated by either EphA4 or cleaved EphA4 (induced by tPA/plasmin) was mimicked by the overexpression of wild-type EphA4, a truncated form of EphA4 or an uncleavable variant of the receptor. These three variants of the receptor represent three ways of signalling that were then tested in conditions emotionally challenging for the animals. I decided to express these receptors in the brain area where tPA is known to be most active in anxiety-like behaviours of rodents, the CeA (Pawlak et al., 2003). As there are not experiments in which anxiety-like behaviours are evaluated when tPA is exclusively expressed in CeA, it would be difficult to predict any result from extrapolation. However, since anxiolytic phenotypes are produced, in general, by the lack of tPA, an anxiolytic phenotype would be expected with full-length forms of EphA4 (mimics the lack of cleavage by tPA/plasmin) and the opposite (anxiogenic phenotypes) would be expected with the presence of cleaved forms of EphA4. To evaluate these behaviours, I employed two of the most used and robust methods to test anxiety-like behaviours, the elevated plus maze (EPM) and the dark-light box. Normally, these two tests are meant to reflect similar changes in anxiety-related behaviours, however, as it will be further discussed, the reaction of the animals in these paradigms becomes divergent with different types or duration of stress applied, drug treatments or the schedule of the tests. Therefore, although similar results would be expected from these two tests, differences between them would not be strange.

RESULTS

EphA4 protein cleavage following stress

Previous works from our lab and others indicated that tPA and Eph proteins may be critically involved in the stress response (Melchor and Strickland, 2005; Attwood et al., 2011; Tovote, Fadok and Lüthi, 2015). To investigate whether psychological stress triggers changes in the levels of EphA4 receptor or its proteolytic cleavage, I performed an experiment in which a group of wild-type mice underwent restraint stress (RS) protocol while a control group remained unstressed as described in *Materials and Methods*. Then, protein levels of the EphA4 C-terminal end were measured by Western blot.

Analysis of the amygdalae of these groups did not yield any observable effect in EphA4 levels or EphA4 cleavage when looking at extracts comprising the whole amygdala (data not shown). However, the extraction of the central amygdala separately after 1 h of restraint stress (RS) produced an increase in the intensity of the main cleavage product of EphA4 without significantly affecting the total levels of the main EphA4 band (Figure 25. ANOVA, $F(2,10) = 8.671$, $p = 0.006$; Tuckey´s test: 0 min of RS: 1.000±0.156 a.u. vs. 60 min of RS: 2.973±0.552 a.u., $p = 0.012$). This main cleavage band is also produced by plasmin and tPA cleavage, which suggests that these enzymes could be affecting the cleavage of EphA4 during the RS paradigm.

Importantly, this intensification of the band corresponding to the main cleaved form of EphA4 in the CeA was not observable in $tPA^{-/-}$ or $plasminogen^{-/-}$ mice subjected to restraint stress (Figure 26 A, ANOVA, $F(2,11)= 0.576$, $p>0.05$; Figure 26 B, ANOVA, $F(2,6)= 1.944$, $p>0.05$), meaning that tPA and plasmin would be necessary for the elevated levels of this variant.

This is a table that serves as an overview of the experiments shown so far in relation to the cleavage of EphA4 by tPA/plasmin in conditions of restraint stress:

Time of RS / Breed	No RS	15 min	60 min
WT	No effect	No effect	Increased cleavage of EphA4
tPA Knockout (tPA KO)	No effect	No effect	No effect
Plasminogen Knockout (Plg KO)	Reduced cleavage of EphA4	No effect	No effect

Cleavage of EphA4 fosters anxiety-like behaviours

To investigate the effect in mice of the cleavage of EphA4 in the stress and anxiety-like behaviours, wild-type EphA4 (wtEphA4), cleavage-resistant EphA4 (crEphA4) and truncated EphA4 (tEphA4) were expressed in mouse central amygdala using a lentiviral delivery system as described in *Materials and Methods*.

After the injection, animals were allowed to recover and overexpress EphA4 variants for at least 21 days. After this period, animals were restraint-stressed and tested in the elevated plus maze (EPM) and the light-dark box (LDB) to quantify anxiety-like behaviours (schematic representation in Figure 27). Additionally, motility parameters were assessed with the open-field test.

EPM results revealed that the time spent in the light arms did not present any difference between any of the non-restrained (naive) groups of animals (Figure 28 A. ANOVA, $F(7, 65) = 6.796$; Bonferroni: $p>0.05$). Interestingly, animals subjected to restraint stress and expressing wtEphA4 and crEphA4 did not show any decrease in the time spent in the light when compared with the stress-naive corresponding controls (Figure 28 A. ANOVA, $F(7,65) = 6.796$, $p>0.05$). However, animals infected with the control vector or the tEphA4 variant presented a notable decrease in the time spent in the light (Figure 28 A. ANOVA, $F(7,65) = 6.796$; $p<0.001$. Bonferroni's comparison test: Vector-infected naive:

54.640±3.280 s vs. Vector-infected RS: 33.560±4.792 s, p<0.01); tP5: 59.800±8.245 s vs. tP5 RS 9.043±1.875 s, p<0.001). Additionally, the tEphA4-infected animals showed a marked decrease in the time spent in the light arms compared to vector-infected controls (Figure 28 A. ANOVA, F (7,65) = 6.796; p<0.0001. WT RS: 45.660±7.688 s vs. tP5 RS 9.043±1.875 s, p<0.05). In fact, restraint-stressed tEphA4-infected animals presented a reduction in the time spent in the light compared with all of the studied groups (Figure 28 A. ANOVA, F (3,65) = 6.796; p<0.0001. Vector-infected naive: 54.640±3.280 s vs. tP5 RS 9.043±1.875 s, p<0.001; Vector-infected RS: 33.560±4.792 s vs. WT: 58.110±13.490 s, p<0.05; Vector-infected RS: 33.560±4.792 s vs. tP5: 59.800±8.245 s, p<0.05; Vector-infected RS: 33.560±4.792 s vs. tP5 RS 9.043±1.875 s, p<0.01; WT: 58.110±13.490 s vs. tP5 RS 9.043±1.875 s, p<0.001; P5: 52.930±8.542 s vs. tP5 RS 9.043±1.875 s, p<0.01; P5 RS: 47.960±8.321 s vs. tP5 RS 9.043±1.875 s, p<0.05).

Other parameters related with the immobility of the animals were detected to be different in the restrained animals injected with tEphA4 virus. In particular speed was significantly reduced (Figure 30. ANOVA, F(8,50) = 5.7906; p ≤ 0.00.1. tP5 RS: 0.001±0.00001 m/s vs. Vector naive: 0.0022±0.0004 m/s p<0.001; vs. Vector RS: 0.0024±0.0005 m/s, p<0.001; vs. WT: 0.0021±0.0004 m/s, p<0.001; vs. WT RS: 0.0027±0.0005 m/s, p<0.001; vs. P5: 0.0021±0.0007 m/s, p<0.001; vs. P5 RS: 0.0024±0.0008 m/s, p<0.001; vs. tP5: 0.0017±0.0005 m/s, p<0.001) and immobility significantly augmented (ANOVA: F(8,50) = 10.6455; p < 0.001. tP5 RS: 247.46±10.68 s vs. Vector naive: 159.4±15.53 s p<0.001; vs. Vector RS: 147.69 ± 19.50 s, p<0.001; vs. WT: 161.19 ± 24.44 s, p<0.001; vs. WT RS: 135.57 ± 15.80 s, p<0.001; vs. P5: 162.97 ± 33.42 s, p<0.001; vs. P5 RS: 145.87 ± 25.68 s, p<0.001; vs. tP5: 168.94 ± 34.13 s, p<0.001).

No significant differences were detected in other parameters of the EPM, such as the number of entries into the light or the total number of entries in both arms (Figure 28 B and C. ANOVA, F(3,65) = p>0.05); however, a tendency towards anxiety-related behaviours was observed in stressed tEphA4 mice (Figure 28 B).

Light-dark box results did not show any significant difference between any of the groups studied in terms of anxiety-related parameters (Figure 32 A: ANOVA,

F(7,50) = 1.097, p>0.05. Figure 32 B: ANOVA, F(7,50) = 2.395, p>0.05).

In the open field test, there were not any significant differences in any of the motility parameters compared to unstressed vector-infected controls (Figure 33), which indicates that all groups would be within control parameters marked by unstressed controls expressing none of the EphA4 variants. However, stressed animals, except for the stressed tEphA4, exhibited a common tendency to travel longer distances at higher average speeds compared to unstressed vector-infected controls. Differences among stressed and unstressed groups were overall not significant, with the exception of the average speed between unstressed WT and the unstressed crEphA4 mutant (Figure 33 B). Although this result could indicate an anxiolytic effect in crEphA4 mice it overall gives information about the lack of motility impairments in all these groups.

FIGURES

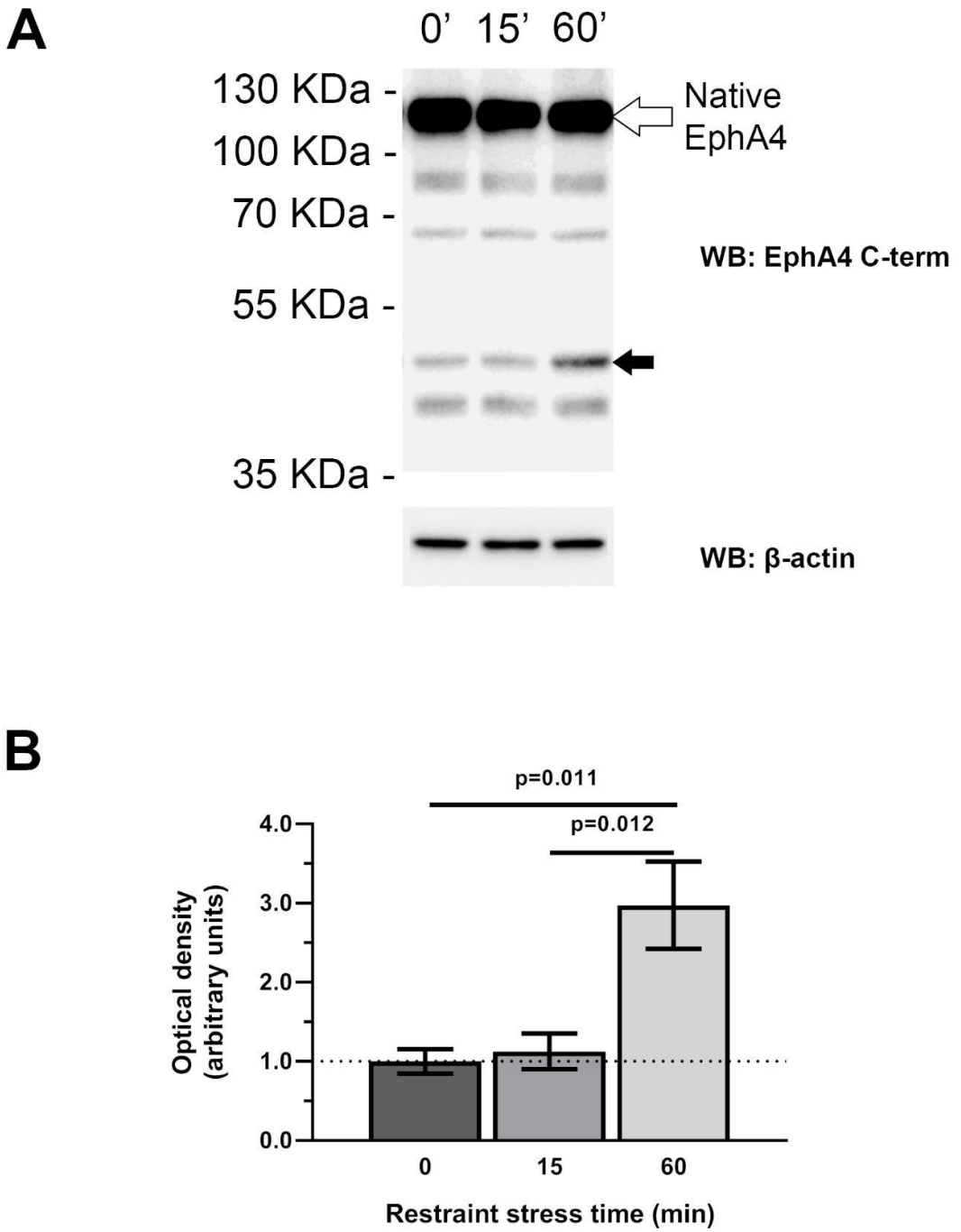


Figure 25. EphA4 cleavage is increased in the mouse central amygdala after 60 min of restraint stress. WB = Western blot. The figure shows a blot, produced by Western blot technique, of the protein levels of the C-terminal EphA4 fragments in the central amygdala after mice underwent restraint stress for increasing time intervals (0, 15 and 60 min). A) A representative blot and B) the quantification for the main cleavage product (black arrow) are shown. Restraint stress resulted in increased levels of the main (tPA and plasmin) cleavage product of EphA4, but unaffected native EphA4 levels, which indicates that central amygdala is of particular importance for this cleavage. All data are presented as optical density (O.D.) mean ± SEM (n = 3-5). p values are included over the corresponding chart bars.

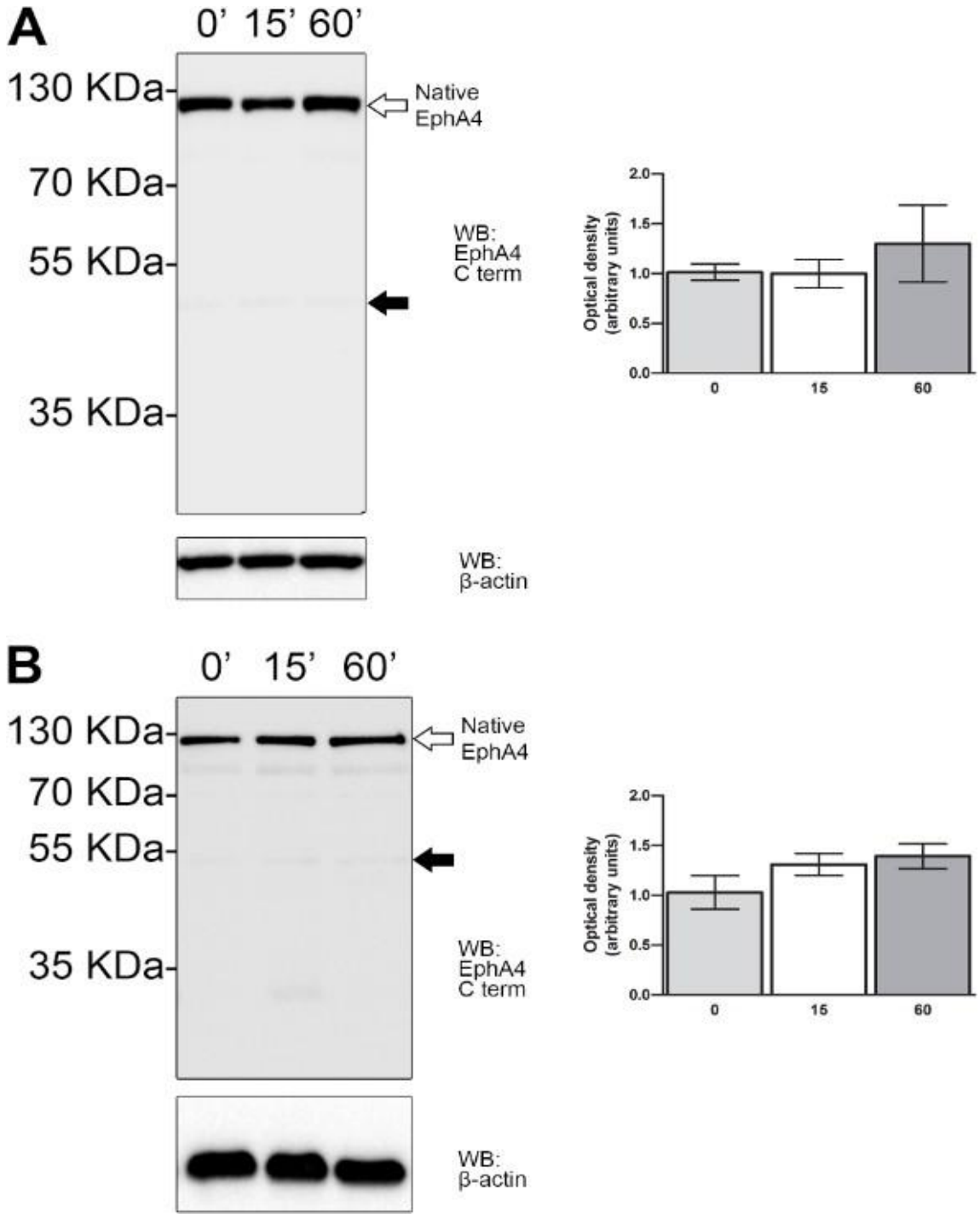


Figure 25. EphA4 cleavage does not increase in the mouse central amygdala of tPA knockout or plasminogen knockout animals after 60 min of restraint stress. The figure shows representative blots and quantification of the main cleavage products (black arrow) for protein levels of EphA4, measured by Western blot technique, after restraint stress performed for different periods of time (0, 15 and 60 min) in the mouse amygdala of A) tPA knockout animals and B) plasminogen knockout animals. Restraint stress did not affect the main cleavage product of EphA4 (produced by tPA and plasmin). All data are presented as optical density (O.D.) mean ± SEM (n = 3-5). p values are included over the corresponding bar charts.

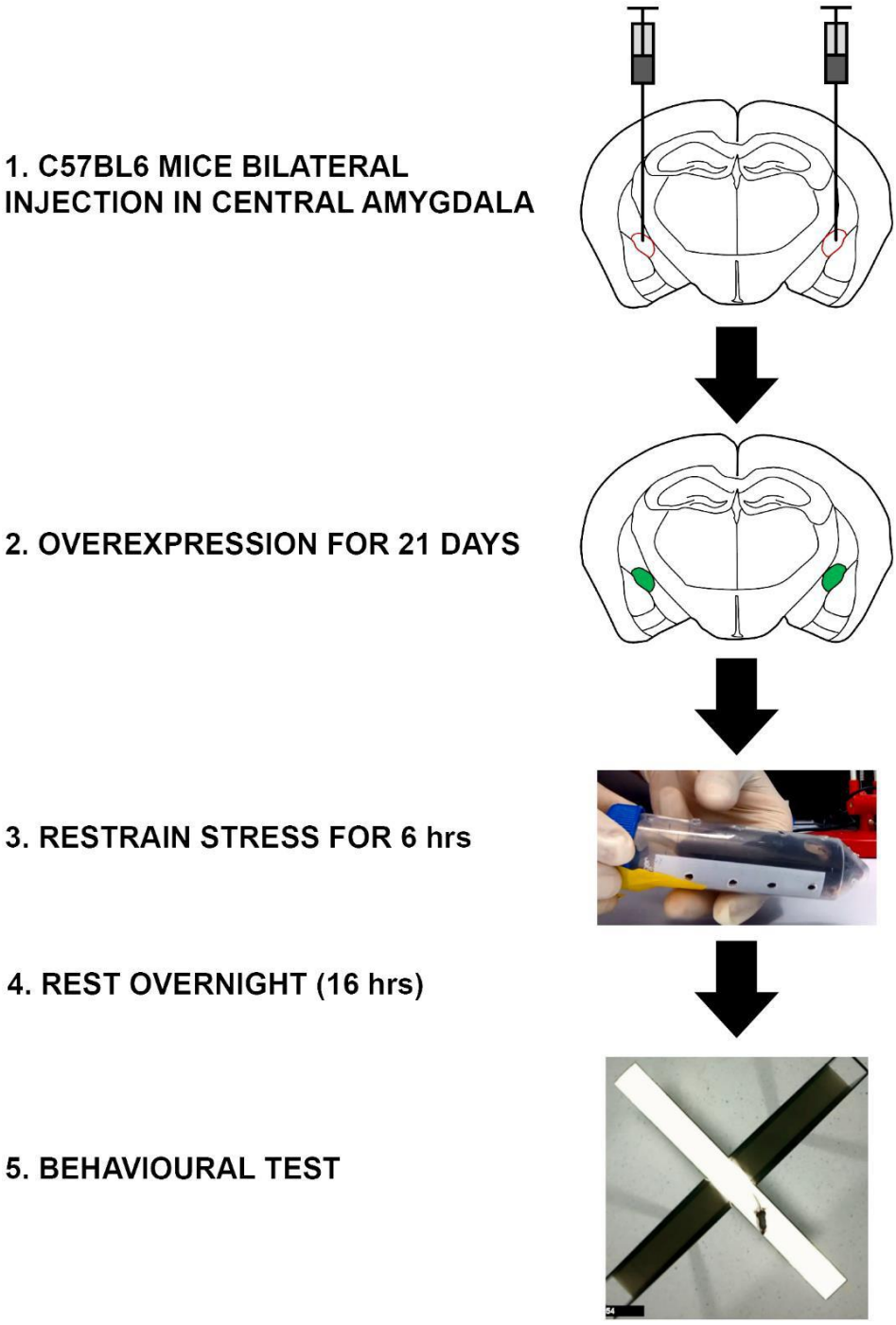


Figure 26. Experimental design for the overexpression in the central amygdala of the mouse wild-type EphA4 receptor, the cleavage resistant EphA4 P5 (R516Q) variant and the EphA4 truncated at P5 (R516) variant. First, lentiviral particles containing plasmids for the overexpression of wild-type EphA4 and its variants were injected bilaterally in the central amygdala of C57BL6 mice using stereotaxic surgery. The viral particles were allowed to infect and overexpress the respective proteins for at 21 days. Then, the animals were stressed by restraining them in their home cage for 6 h in 50 mL falcon tubes with sufficient ventilation and a bright light. After that, they were released and allowed to rest overnight. Next day, behavioural tests were performed in a time-consistent manner between 10:00-16:00.

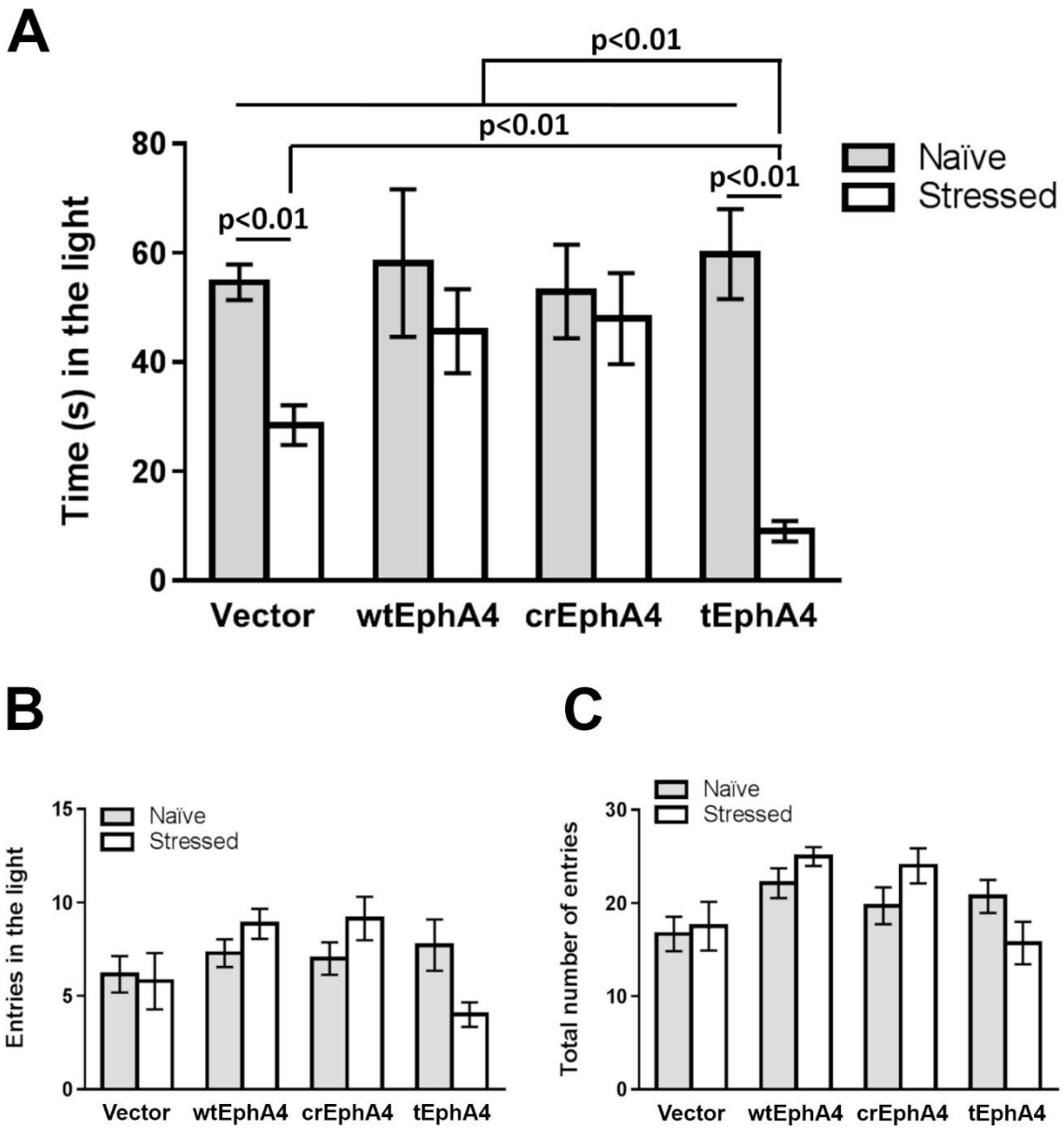


Figure 27. Elevated plus maze produces decreased anxiety-like behaviours in mice overexpressing wtEphA4 or crEphA4 in the CeA, and increased anxiety-like behaviours in mice overexpressing tEphA4 after 6 h of restraint stress. This figure shows measurements of anxiety-related parameters. Mice were subjected to restraint stress for 6 h. On the next day, anxiety-related parameters were tested and compared to their respective unstressed controls. A) Time spent in the open arms (one of the most robust anxiety-related parameters) is significantly reduced after stress in mice overexpressing tEphA4. This effect suggests that tEphA4 can increase anxiety-like behaviours only when animals experience a stressful situation. This result is supported by other parameters related to anxiety, such as B) a tendency to enter less number of times into the open arms, although C) the total number of entries into any space was unaltered. All data are presented as mean ± SEM (n = 6-8). p values are included over the corresponding chart bars.

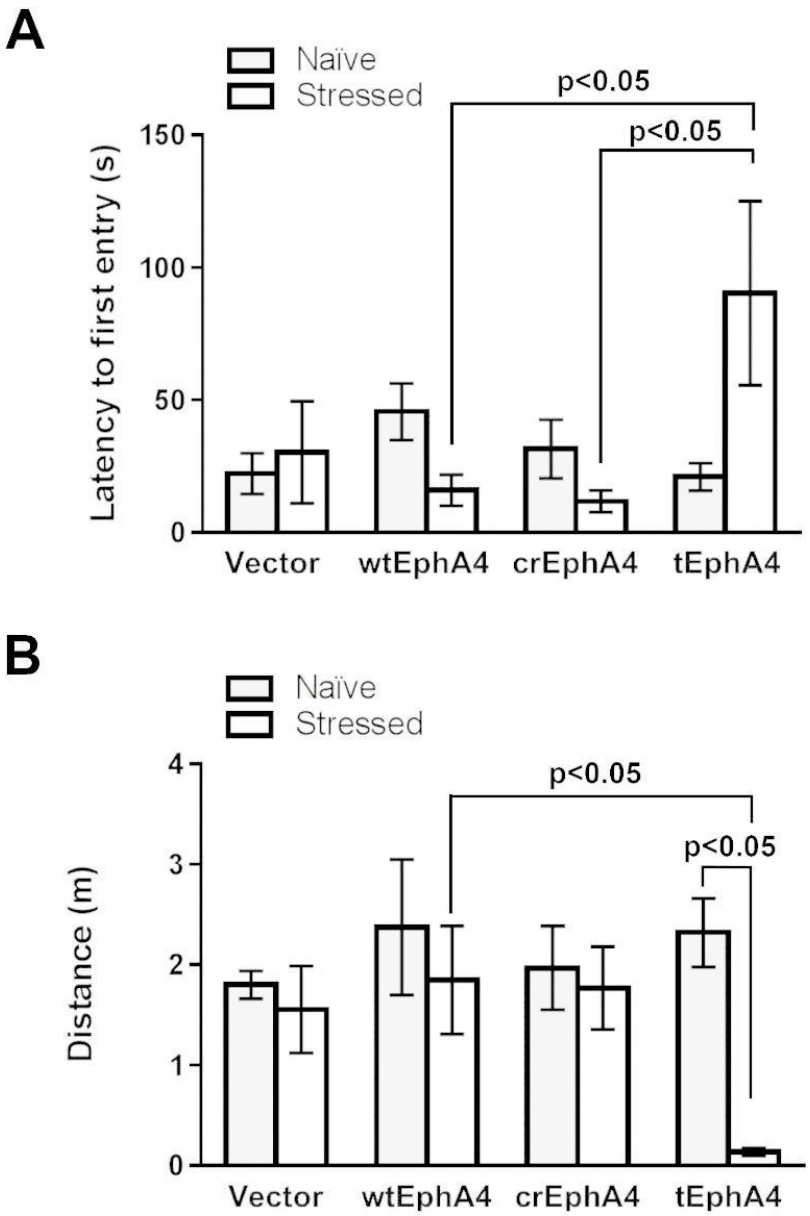


Figure 28. **Elevated plus maze (EPM) produces decreased anxiety-like behaviours in mice overexpressing wtEphA4 or crEphA4 in the CeA and increased anxiety-like behaviours in mice overexpressing tEphA4 after 6 h of restraint stress.** This figure shows the results of general parameters in the EPM after the overexpression in the CeA of wtEphA4 and its variants (crEphA4 and tEphA4). Mice were subjected to restraint stress for 6 h. These parameters were not significantly modified in unstressed mice expressing any of the variants when comparing them to the mice infected only with the "vector" controls, which only overexpress eGFP. However, the relatively lower speed and distance covered by stressed tEphA4 mice could be explained by the immobility caused by the freezing behaviour of these animals when they were performing the test. All data are presented as mean ± SEM (n = 6-8). p values are included over the corresponding chart bars.

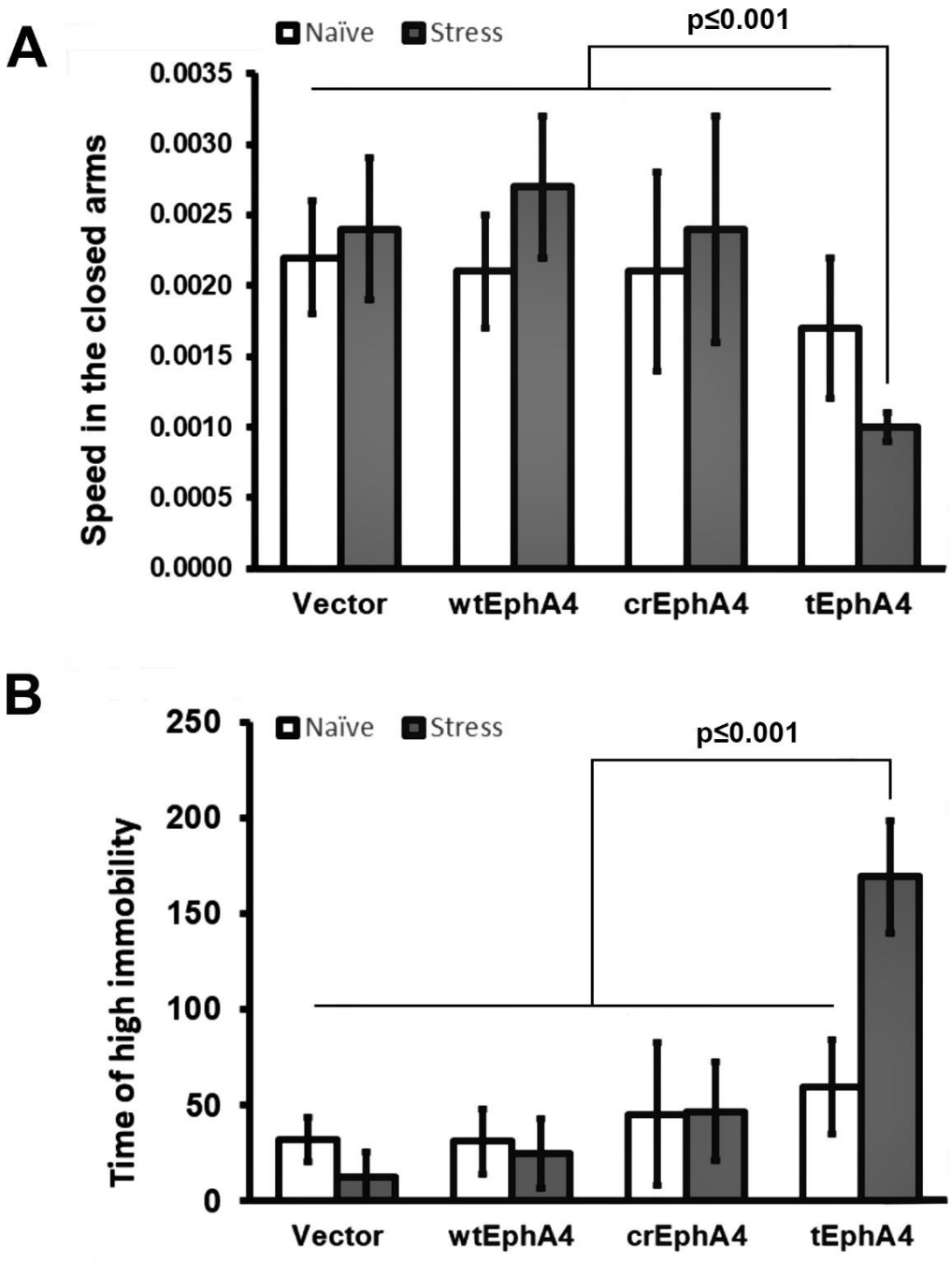


Figure 29. **Elevated plus maze (EPM) produces decreased anxiety-like behaviours in mice overexpressing wtEphA4 or crEphA4 in the CeA and increased anxiety-like behaviours in mice overexpressing tEphA4 after 6 h of restraint stress.** This figure shows parameters of mobility in the EPM after the overexpression in the CeA of wtEphA4 and its variants (crEphA4 and tEphA4). Mice were subjected to restraint stress for 6 h. Speed was significantly reduced and immobility highly increased in the closed arms. All data are presented as mean ± SEM (n = 6-8). p values are included over the corresponding chart bars.

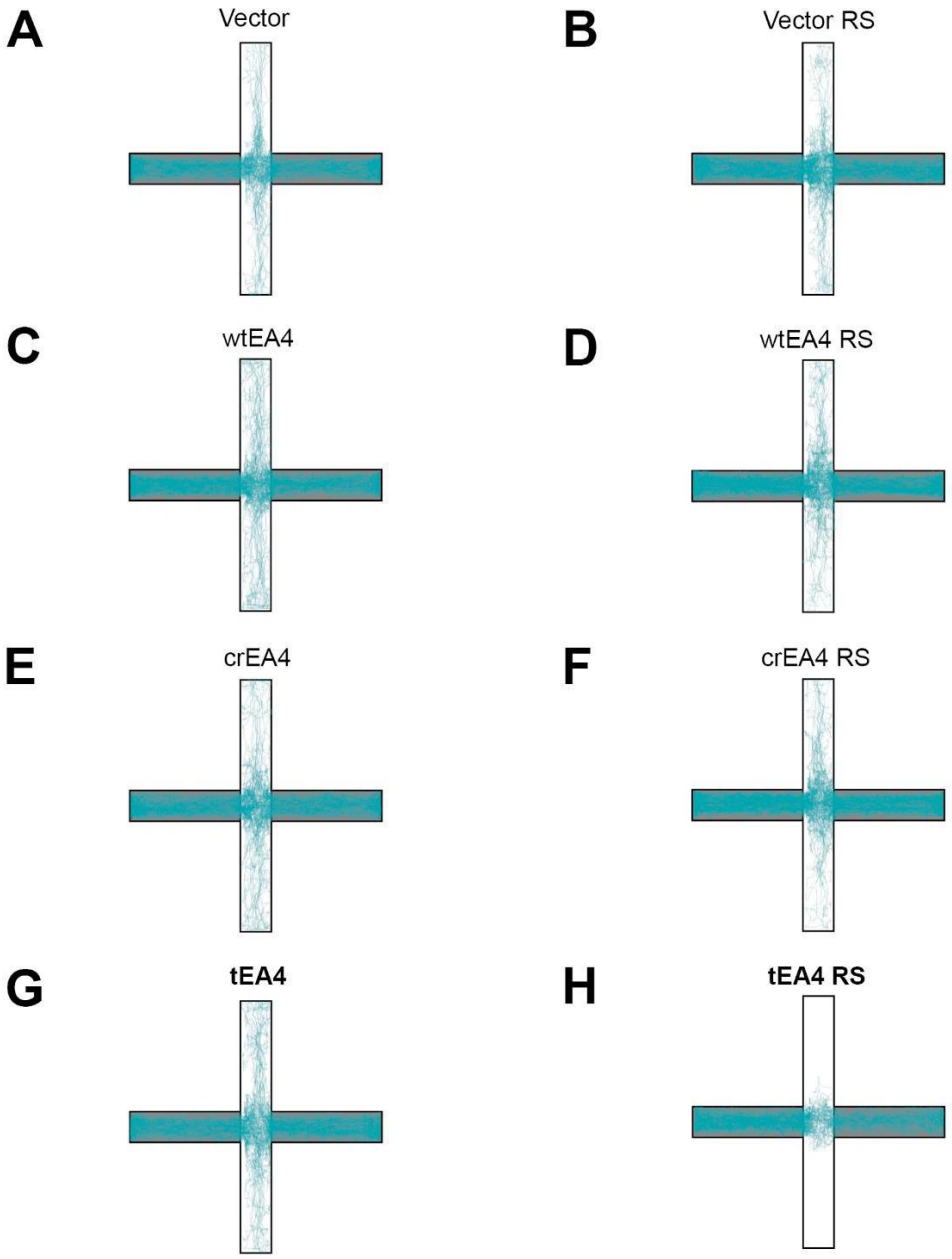


Figure 30. Mice overexpressing the EphA4 receptor variant truncated at P5 (R516) cleavage site (tEphA4) present more anxiety-like behaviours after 6 h of restraint stress. RS = restraint stress. This figure shows the outline of the elevated plus maze apparatus (open arms in white and close arms in grey) with an overlay of the combination of tracks followed by the centre of the body of the mice (turquoise) overexpressing wtEphA4 (C, D) and its variants (crEphA4 [E, F] and tEphA4 [G, H]), or a control vector [A, B] in the CeA. Mice that were subjected to 6 h of RS are presented on the right (B, D, F, H) and non-restrained on the left (A, C, E, G). Of note, the track followed by restraint-stressed mice overexpressing tEphA4 (H, tEphA4 RS) barely crosses into the open arms, which is indicative of increased anxiety-like behaviours. This result is supported by other parameters related to anxiety, such as the time spent in the opened arms, the number of entries into the open arms and a tendency towards a higher latency to enter into the opened arms (n = 6-8).

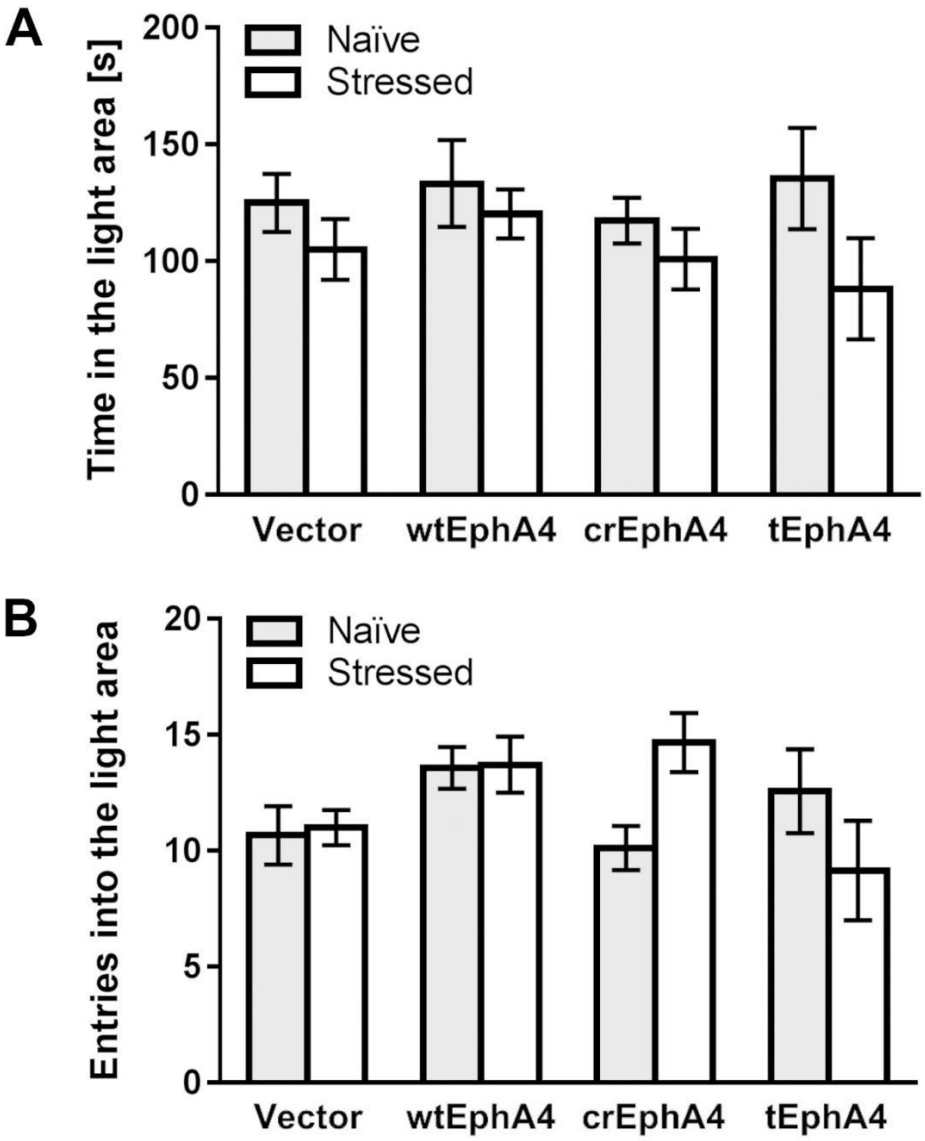


Figure 31. Mice overexpressing wild type EphA4 (wtEphA4) and its cleavage-resistant and truncated variants (crEA4 and tEphA4, respectively) in the central amygdala (CeA) are not significantly more anxious than the vector-transfected controls after restraint stress when measured by dark-light box test. This figure shows the results of the measurement of parameters related to anxiety. Mice were subjected to restraint stress for 6 h. A) The time spent into the dark box and B) the number of exits from the dark box do not differ between the groups. However, tEphA4 stressed animals present a not-significant tendency to spend more time in the darkness when comparing to the rest of the groups and a lower number of entries into the light, which suggests that the technique or the experimental design used might not be adequate to measure these anxiety-like parameters. All data are presented as mean ± SEM (n = 6-8). p values are included over the corresponding chart bars.

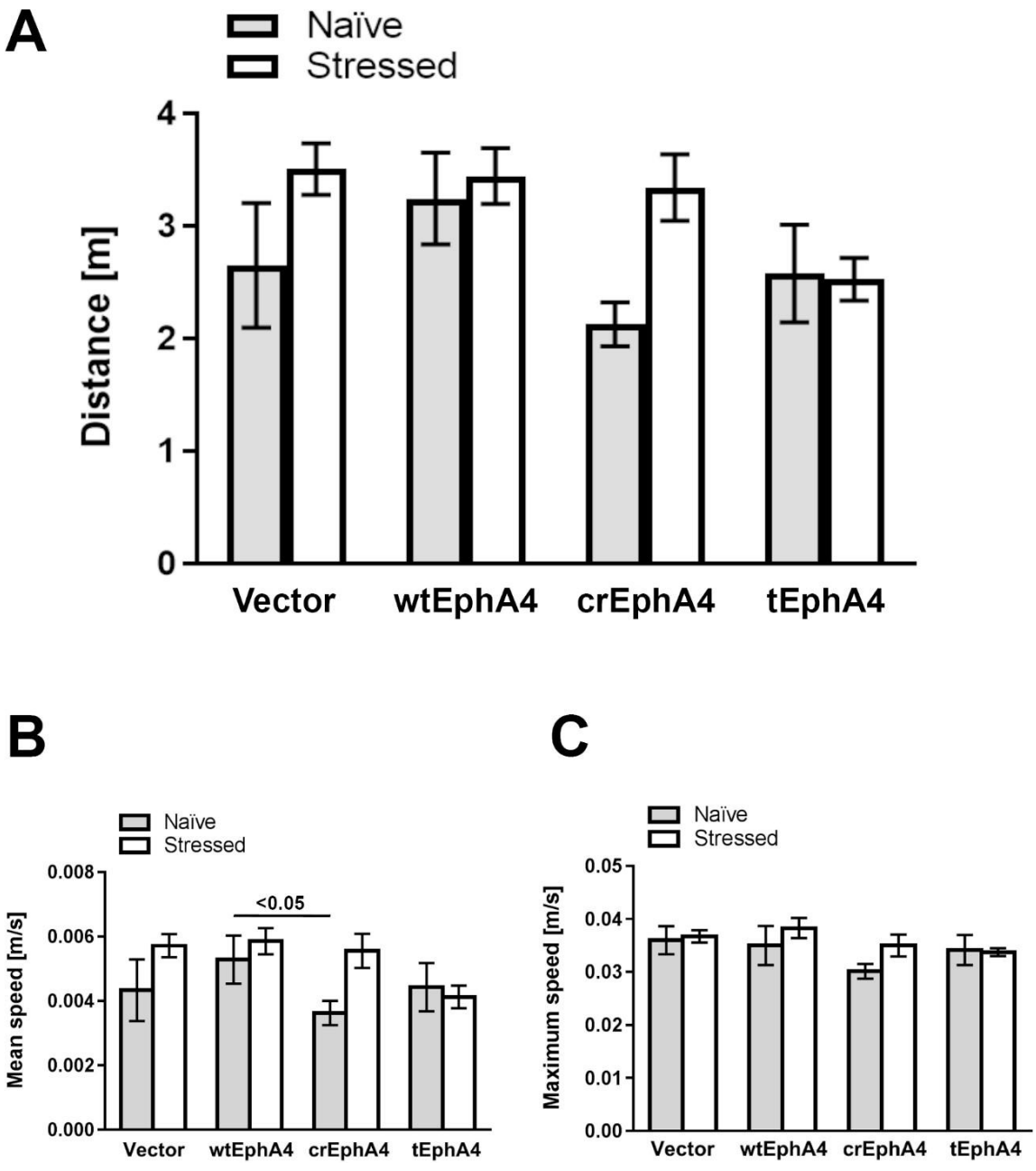


Figure 32. Locomotion is unaltered in mice overexpressing EphA4 (wtEphA4) or its cleavage-resistant and truncated variants (crEA4 and tEphA4, respectively) in the central amygdala. This figure shows the results in the open-field test for parameters measuring the locomotion of mice. Half of each group of animals were subjected to restraint stress for 6 h. A) Distance B) average speed C) and maximum speed are not significantly different in any of the groups when comparing them to the unstressed control mice injected with the vector. This means that the effects observed in these animals are not due to changes in locomotion. All data are presented as mean ± SEM (n = 6-8). p values are included over the corresponding chart bars.

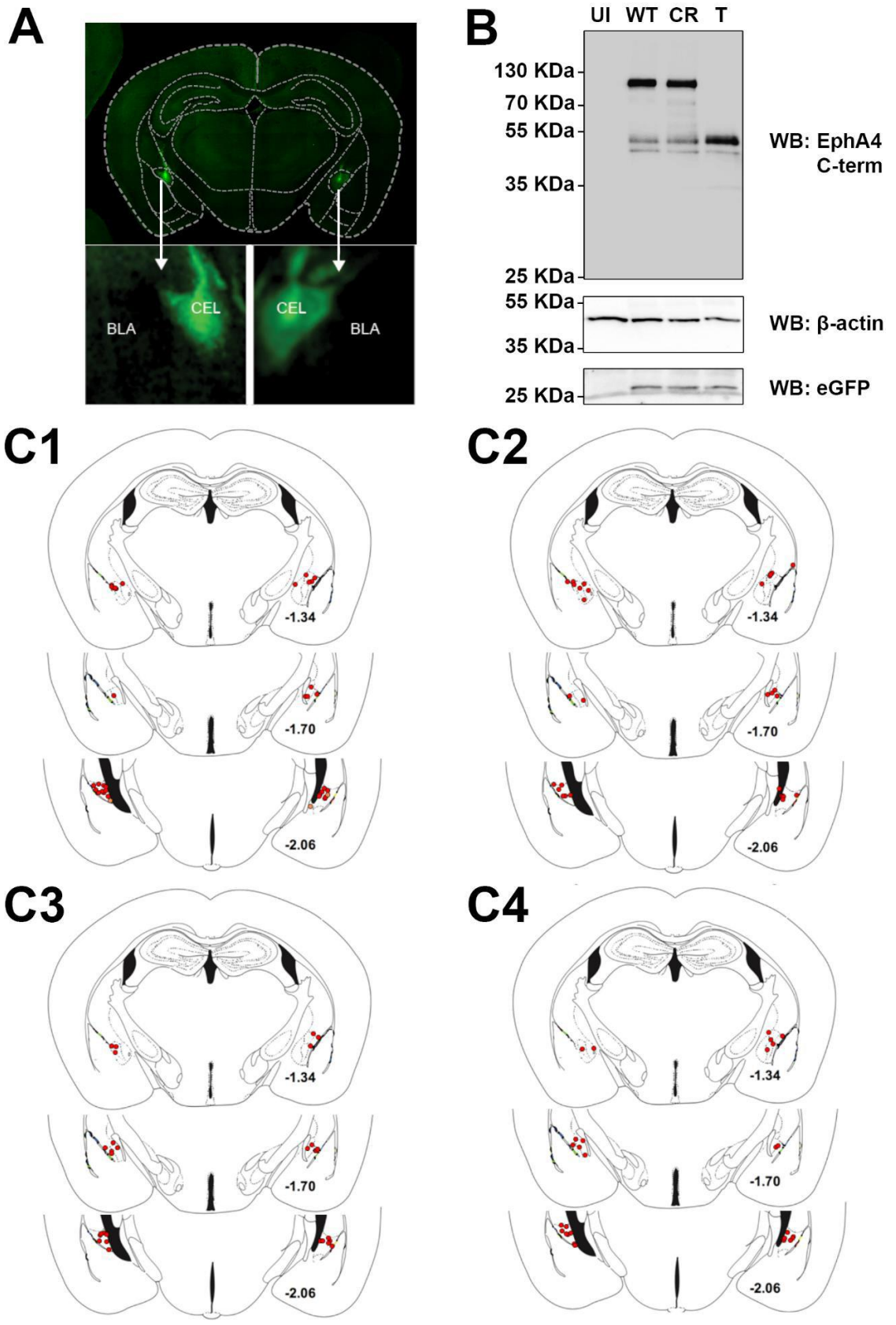


Figure 33. Injection of lentiviral particles containing EphA4 constructs. (UI = uninfected, WT = wild-type EphA4, CR = cleavage resistant EphA4 at P5, T = truncated EphA4 at P5. A) Representative fluorescent immunohistochemistry after bilateral injection in the central amygdala and overexpression for 21 days of the lentiviral constructs. B) Overexpression of EphA4 lentiviral particles with the different constructs in N2A cells C) Injection site verification of C1) Vector-transfected control. C2) Wild-type EphA4. C3) Cleavage resistant EphA4 at P5. C4) Truncated EphA4 at P5.

DISCUSSION

As discussed in previous chapters, EphA4 is a possible target for tPA/plasmin cascade in the central amygdala of the mouse brain. As this brain area is a key node in the expression of anxiety-like and stress-related behaviours, I set to study the cleavage of EphA4 in the CeL in response to an anxiogenic stimulus. To this aim, wild-type C57BL/6J mice were subjected to acute restraint stress (RS) for 60 min. Then, I dissected their amygdalae and examined the levels of the native and cleaved variants of EphA4 through Western blot technique. Results proved that the density of the band corresponding to the main plasmin-cleaved form of EphA4 is increased after 60 min of RS (Figure 25); however, this effect would be expected to be observed after longer times, since the activity of tPA has been observed to be increased up to 6 h of RS (Pawlak et al., 2003).

Consistent with an active role of tPA/plasmin cascade in EphA4 cleavage after stress, the density of this band remained unchanged in $tPA^{-/-}$ or $plasminogen^{-/-}$ mice (Figure 26). This indicates that, in response to an anxiogenic stimulus (restraint stress), plasmin cascade would be at least partly responsible for the cleave of EphA4 in the CeA, and the hub for this process, as discussed in previous chapters, would be eminently formed by GABAergic synapses downstream of $PKC\delta^{+}$ interneurons. Interestingly, tPA seems to be relevant for the cleavage of EphA4 upon restraint stress (Figure 26), but does not seem to be essential for the shedding of EphA4 in baseline conditions (Figure 20).

To explore the effects of tPA/plasmin/EphA4/effector signalling pathway in anxiety behaviours, in a different experiment I made use of lentiviral particles containing plasmids aiming the overexpression in mammalian cells of the wild-type EphA4 (wtEphA4), the cleavage-resistant form of EphA4 (crEphA4) or the truncated form of EphA4 (tEphA4) or the corresponding empty vector backbone. tEphA4 and crEphA4 are designed to mimic plasmin cleavage or resistance to it, respectively; and the empty vector acts as a negative control for the expression of EphA4 variants. I performed a bilateral injection in the central amygdalae, as it was the area with the most marked increase in EphA4 cleavage after restraint stress (RS) paradigm and the area where most active tPA ($PKC\delta^{+}$) is located.

Then, in order to test different types of anxiety, half of the animals of each group were subjected to RS and the other half was left unstressed before being tested in a battery of behavioural paradigms. The use of RS before performing anxiety-related behavioural tests is a type of enhanced model of state anxiety (i.e. it occurs as a reaction to an anxiogenic stimulus; e.g. RS) that has been used in the past and has shown robust results in generating anxiety-like behaviours (Mechiel Korte and De Boer, 2003). Whereas this method allows the evaluation of state anxiety, the group not subjected to RS enables the analysis of anxiety-like behaviours in baseline conditions (which is more ethological, i.e. not conditioned by any previous stress-related experience). These animals were restrained for 6 h, since shorter times were observed to not have any effect on the EPM phenotype in previous experiments of former members of our laboratory (data not shown). Although the longest time point regarding tPA/plasmin cleavage of EphA4 shown here was set at 1 h (to observe the minimum time necessary for the cleavage of EphA4), cleavage by tPA increases until 6 h of RS in the CeA (Pawlak 2003), which indicates that the cleavage of EphA4 could be occurring at this time point. Therefore, although a specific experiment was not performed to assess cleavage of EphA4 at a 6 h time point, this point is compatible with both, a correct EPM phenotype, and a cascade initiated by tPA, such as the EphA4 one.

I tested the animals using two broadly-used and well-validated tests, namely the elevated plus maze (EPM) and the light-dark box (LDB) test (Carola et al., 2002). In general, the various tests designed to measure anxiety-like behaviours in rodents are based on the principles that behavioural test are experimental preparations developed in one species to study a phenomenon in another species and that whenever a relation holds between elements of the animal model this same relation should hold in the organism of study. These principles are translated into three basic validity assumptions that endow the particular model with the necessary legitimacy to model anxiety behaviours. These are: face validity, construct validity and predictive validity (Belzung and Griebel, 2001). The reasons to choose the EPM and the LDB as my tests to measure anxiety-like behaviours are manifold and will be defined in the following paragraphs.

In first place, they are founded on these validity fundamentals. Face validity refers to the similar behavioural response of animal models and humans. Although anxiety is not a unitary process, in terms of aetiologies and symptoms (some of them are really difficult to model [e.g. low self-esteem, suicidal ideations]), various behavioural phenotypes are common to most of the conditions and can be modelled in behavioural tests, including the EPM and LDB. For instance, excessive avoidance common to increased anxiety is reproduced in the ethogram of the rodent models by staying away from aversive areas of the maze. In the case of EPM and LDB, these aversive areas are exposed spaces represented by open and well-lit spaces (open arms and open arena, respectively). Therefore, the measurement of time in the open arms and areas are two fundamental parameters in my experiments. Also, hypervigilance observed in anxiety disorders can be identified in rodents as stretched postures, immobilisation periods, upright positions and body positioning in relation to the stimulus. Thus, times of immobility were also measured but the interpretation of these is more complex than approach/avoidance locomotion as it will be explained in further sections. Additionally, generalisability (a common feature of emotions) can be stress-induced before subjecting them to the test of choice. This induction leads to a transfer of higher levels of anxiety-like behaviours in anxiety-related tests, i.e. higher avoidance of aversive areas in EPM and LDB. In my experiments, stress was achieved by restraining mice as explained before.

These two tests also fulfil the requirements for predictive and construct validity. Predictive validity relates to the sensitivity of the model to detect pharmacological anxiogenic/anxiolytic treatments (e.g. both respond to diazepam [Rodgers et al., 1992; Chaouloff et al., 1997] and have been validated and used in drug discovery experiments of about 4,000 anxiolytic compounds [Griebel and Holmes, 2013]). Construct validity involves similar theoretical rationale behind the similar behavioural responses in the modelled and the studied species, meaning that similar circuits and areas are activated during high anxiety and fear states, such as the BLA-CeA connection [Janak and Tye, 2015]).

Second, the chosen tests are ethological, meaning that the response to them is unconditioned (they do not rely on the presentation of a noxious stimulus, e.g.

electric shock, food/water deprivation, loud noise, predator odour]) and therefore, they are closer to natural conditions. Plus, as the animals do not need to be trained, it avoids adding more confounding factors to the test. The EPM and LDB are amongst the most popular ones (Carola et al., 2002). These exploration tests based on approach-avoidance locomotion take advantage of the natural aversion of mice for open, lit and high areas and the natural tendency to explore as a foraging species. These type of tests are also really attractive, since they do not need any previous training and the devices needed are relatively simple. However, they have the inconvenient of needing intact motor functions and hence the requirement to have additional measures of these skills in a non-anxiogenic environment. In my experiments, although the OF test can reveal anxiety-like behaviours, I mainly used this test as a measure of the normal motor skills of our animals.

A third reason to choose various tests is that, although all these tests are all associated to a general concept of “anxiety-like behaviours”, there are variations in behaviour among tests within the same pharmacological studies that indicate construct differences between these tests. According to some authors, this is due to the multidimensional nature of anxiety-like behaviours, which underlie different psychobiological phenomena that would influence differential features of the tests and would be only accessible to knowledge through the use of a series of tests involving diverse stressful stimuli (e.g. brightness, novelty, openness and punishment). This rationale would argue in favour of performing different tests in the same animal. Additionally, according to some authors, an anxious trait would be not qualitatively different from state anxiety. In other words, an individual would not be always anxious or differently anxious, but would be anxious more often than others. Consequently, the only way of measuring this trait anxiety would be to assess how often (or how intensely) the individual experiences anxious states in different situations. In rodents, an increased trait anxiety would describe a tendency to react anxiously in different anxiety-related tests. This way of thinking also advocates for the use of different tests to measure anxiety-like behaviours (Ramos et al., 2008).

Regarding the order in which these tests needed to be carried out, there is mixed

information in the literature and no methodical studies have been carried out in this regard. I chose to use the EPM in first place and make it my method of reference, so no other previous test would act as a confounding factor for it. EPM is a well-established paradigm and a standard method of election when measuring anxiety-like behaviours due to its renowned face, construct and predictive validity and hence the accuracy when translating results to humans (Walf and Frye, 2007). In general, pre-exposure to a different novel environment or a behavioural test (such as the OFT or the hole-board test) before EPM increases the motor activity in this latter test, and therefore, the likelihood of entering the open arms of the maze (Pellow et al., 1985; File et al., 1975a; File et al., 1975b; Voikar et al., 2004; Paylor et al., 2006), which would be a confounding factor for the interpretation of anxiety-like behaviours. Also, when pre-exposing animals to different tests (including EPM) in previous days, the results of the EPM seem to be modified, whereas the results in the LDB and OFT seem to be more stable over time (Onaivi and Martin, 1989; Lad et al., 2010; McIlwain et al., 2001; Heredia et al., 2014; You et al., 2019; Flandreau et al., 2012). Due to time constrains, the tests needed to be performed on the same day, so the resting time in between tests in my experiments was of about 1 h. Unfortunately, information about shorter periods of rest like this is missing in the literature, especially if, like in my case, stress-induced anxiety protocols are used. Although the time in between tests we used was shorter than previous assays, such as the ones in Paylor et al., 2006 (1 h vs days), I took the data in the literature as precautionary information to set an order among the tests, so less-likely-to-change tests (LDB and OFT) would go later in the sequence of tests.

Since not equal or highly similar experimental designs have been used in the past, it was futile to anticipate any behavioural results in test posterior to EPM. However, there is evidence to suggest similarities between these tests despite their multidimensional nature. For instance, almost three-quarters of the mouse strains with manipulated genes regarded as relevant to anxiety display anxiety-related behaviours in at least two different tests, including the EPM, OFT and LDB. Moreover, evidence for resilience of LDB and OFT results to change when a previous test is performed (Lad et al., 2010; McIlwain et al., 2001; Flandreau et al., 2012) should be also considered. Therefore, although discrepancies between

tests would not be odd due to the mentioned multidimensionality of tests and shorter resting times (Ramos et al., 2008), I expected to observe similar results in the EPM and LDB tests.

I tested both groups of mice 16 h after the RS. A lack of behavioural changes in the EPM has been shown in the past when rodents were tested within 2 h after immobilisation in RS, so longer times allow them to develop an adequate stress phenotype (Padovan and Guimarães, 2000). Additionally, mice have been tested in EPM after resting periods after stress of 24 h with successful results in sensitive phenotypes (Jakovcevski et al., 2008), which would argue in favour of the 16 h time frame choice. I used a single session of RS since there is evidence of the disappearance of the behavioural changes (due to habituation) induced by RS on the EPM performance when the animals are subjected to repeated daily 2 h immobilisation periods (Padovan and Guimarães, 2000).

Truncated EphA4 variant and cleavage-resistant EphA4 variant have opposite consequences for anxiety-like behaviours in the elevated plus maze

Regarding anxiety-related parameters in the EPM, naive (unrestrained) animals did not show any different phenotype among groups when assessed by the time spent in the light, which indicates that the expression of different EphA4 variants does not affect baseline anxiety. On the contrary, the time spent in the light showed some differences among groups of restraint-stressed animals. Only tEphA4-injected mice previously exposed to RS (tEphA4-RS) displayed signs of increased anxiety-like behaviours in the EPM when compared to vector-infected controls, whereas the full-length EphA4, either in its native form (wtEphA4-RS) or the cleavage resistant form (crEphA4-RS) made the animals irresponsive to the restraint stress stimulus. The anxiogenic effect of that previous negative-valence experience is evidenced by a dramatic decrease in the time spent in the open arms and the latency to the first entry into the open arms when compared to unstressed controls (Figure 28 and 29). This effect is significantly more pronounced in the tEphA4-infected mice than in the vector-infected ones,

meaning that tEphA4-infected animals present a higher degree of anxiety-like behaviours.

Other anxiety-related parameters in EPM, such as the entries into the open arms and the total number of crossings between areas were not significantly affected, although the pattern observed in these parameters resembles the one of the time spent in the light arms (Figure 28 B and C). Interestingly, treatment with anxiolytic drugs can increase the number of crossings between the two compartments, without modifying the time in the dark compartment (Bailey and Crawley, 2009). This is interpreted as an increase in exploratory activity, not as an anxiety sign. In the case of the experiments carried out for this thesis, the combination of modified preference of restrained animal for the light areas combined with the similar number of crossings between areas should be considered as a clear indication of a change in anxiety-like behaviours.

Immobility response in tEpha4-RS animals

Interestingly, the behaviour produced in wtEphA4 and crEphA4 animals is not qualitatively different from the vector-infected controls (in terms of distances travelled [Figure 29] speed [Figure 30] or areas of the maze covered [Figure 31]), although it is quantitatively so. However, when the restraint-stressed mice (tEphA4-RS) were positioned into the EPM, they developed conducts of immobility, qualitatively different from the rest of the groups. Most of the tEphA4-RS mice stayed highly immobile for a considerable period of their five-minutes test [Figure 30], they did not engage in investigating distant areas of the aversive open, brightly-lit and elevated area of the EPM (Figure 31), and it is even reflected in the distance travelled (Figure 29). Nonetheless, exploratory activity was not completely abolished in these restraint-stressed animals as it is evidenced by the movement within the confinement of the walled part of the maze (Figure 31 D). Furthermore, in relation to the characterisation of the mouse exploratory behaviours, it was not possible to accurately verify the vertical motion of the animal from the zenithal view used to film these animals. This would be relevant because vertical movements of the head and rearing up on their hind limbs would

be two parameters commonly associated with exploration and opposite to anxiety-like behaviours (Thompson, Berkowitz and Clark, 2018). Although these vertical movements could not be assessed, lateral head movements (in the horizontal plane) were often observable in these immobile animals (Figure 30), meaning that not all head movements were abolished and therefore, exploratory behaviours could not be discarded during these immobility periods.

Freezing is a type of behavioural response regarded as complete immobility except as required for breathing (Campos et al., 2013) and it is typically in relation with some time-limited triggering stimulus (that the investigator normally controls and monitors), so as there is a close timely relationship between the two events (i.e. freezing and the stimulus). In my experiments, total immobility of could not be guaranteed due to the lack assessment of the vertical movement and resolution of the image. Additionally, lateral head movements present during periods of immobility argue against a canonical freezing response in the animals studied in my experiments. Moreover, I did not use any time-defined event that would define the limits of a freezing reaction. By virtue of these reasons, I decided that this reaction could not be called freezing and I defined this type of motion immobility behaviour.

In line with this observation, C57BL/6J mice (the strain used in our research) have been previously shown to exhibit immobile high protected stretched postures in the EPM (Ducottet and Belzung, 2005). That reaction resembles the defensive and increased-attention behaviours observed in various animal anxiety states (Grupe and Nitschke, 2013) and fear-related states (Blanchard et al., 1989).

This intricate pattern of behaviours may arise from the modified activity of a single area of the brain, which then projects to a variety of target areas critical for the expression of defensive behaviours. As mentioned in introductory chapters, lesions of the whole amygdala are known to impede several reactions to negative-valence stimuli in various species (such as the Kluver-Bucy syndrome). However, the results of our experiments would be more in agreement with a selective electrical or chemical stimulation of the CeA, which elicits a reduction of the ongoing behaviour that is critical for the expression of aversive behaviours, such as freezing. These behavioural reactions are mainly mediated by neurons

in the centromedial amygdala (CeM). These nerve cells send projections to various brain regions that directly regulate responses intrinsic to anxiety and fear (reviewed in J. E. LeDoux, 2000; J. E. LeDoux, Iwata, Cicchetti, and Reis, 1988; Sah et al., 2003; Tovote et al., 2015). In particular, the CeM abundantly projects to the periaqueductal gray, which mediates freezing responses. Additionally, CeM projects to the pontine reticular formation involved in fear-potentiated startle, as well as to the pedunculopontine, dorsal motor vagal, and solitary tract nuclei, which are also related to fear-processing (Rosen et al. 1991). Electrical stimulation of CeM also produces activation of the jaw and the facial motorneurons mediating facial expressions of aversive reactions and other brainstem reflexes (such as the masseteric, the baroreceptor nictitating membrane, the eye-blink and the startle reflex). Therefore, the activation of CeM could elicit this kind of defensive behaviours. In the same manner, inhibition of areas controlling the regulation of CeM, such as the CeL, would result in the activation of CeM and the subsequent defensive behaviours (e.g. Ciocchi et al., 2010). This latter thesis would be in agreement with an excitatory effect of the injection of tEphA4 in the CeL.

In physiological conditions, there are certain characteristics of anxiety responses that could be potentially causing the observed immobility behaviours, like the malfunction of risk-assessment response (in this case to an ambiguous threatening cue, such as bright lights, a predator or an odour). Consequences of this malfunction include (but are not restricted to) increased arousal, levels of alertness and vigilance and increased sensitivity to threatening stimuli (Grupe and Nitschke, 2013). These cognitive processes are often expressed as cautious scanning of the environment in a hunched position and the use of stretched body positions to attempt the approach to the threatening stimulus, which is normally interpreted as an increase in anxiety-like behaviours (Blanchard et al., 1989). This description establishes a further parallelism between the behaviours presented by tEphA4-RS mice in this thesis and anxiety-like conducts. Also, from a clinical perspective, these animal model's states are analogous to the states found in patients with anxiety disorders (LeDoux, 2015). A detailed review about these disorders can be found in "Anxious" by LeDoux (2015), but the responses in patients from different types of anxiety disorders range from increased attention

towards threats (hypervigilance) to impaired ability to discriminate threats and safety, increased avoidance, heightened reactivity to threat uncertainty, overvaluation of threat significance or a maladaptive cognitive control in the presence of threats. In line with these statements, observations in the literature show that downregulation of the general tone of CeL projection neurons seems to favour general arousal and risk assessment (Ciocchi et al., 2010)

Previous research on the evaluation of these characteristics through rodent anxiety-like behaviour tests (as opposed to fear-like tests involving a CS) shows experiments that allowed the detection of some of the neurobiological substrates of these behaviours. In the performed tests, animals were challenged in situations where there was unpredictability about whether a real threat was occurring, or in situations where a threat (CS) had an uncertain beginning and end. This type of experiments has raised the importance of the BNST and its connectivity with the amygdala (LeDoux, 2015) and their differential contributions to behaviours in certain vs uncertain threat. Similar to the CeA, the BNST connects to hypothalamic and brainstem circuits that control defensive behaviours such as freezing, as well as the autonomic nervous system, the endocrine function, and brain arousal. Additionally, a considerable part of the afferents to the BNST comes from the amygdala, allowing BA and CeA to communicate bidirectionally with the BNST (LeDoux, 2015). Particularly, CeA sends dense projections to the lateral BSNT (lBNST). Consequently, electrical or chemical stimulation of the CeA activates cells that project to the BNST. Similarly, chemical lesions of the CeA that spare neuronal fibres can also block the transmission between CeA and the BNST. Thus, manipulations at the level of CeA have potential effects on both the CeA and the BNST, as well as BLA-BNST connections that pass through the CeA (Davis and Whalen, 2001; Xu et al., 2011).

It could not be entirely excluded that the appearance of the immobility behaviour triggered by changes in the CeA in tEphA4-RS animals could be also produced by a state of increased nonspecific attention or arousal through mechanisms likely overlapping with the risk-assessment response (Pessoa, 2010). The projections related to arousal include efferents from the lateral extended amygdala to the central gray, which are related to a general defence system in

conditioned fear (including freezing, sonic and ultrasonic vocalisation or stress-induced hypoalgesia).

Since anxiety and fear circuits significantly overlap, fear mechanisms cannot be ruled out when describing these immobility behaviours. These mechanisms are related to paradigmatic fear-related CS experiments that involve the CeA and the subsequent defence responses. In particular, efferents from the CeM to the ventrolateral periaqueductal grey (vlPAG) are important for suppressing ongoing motivated behaviours, enhancing freezing and producing vocalisation and analgesia. Excitatory or inhibitory stimulation of CeM produces these effects, probably through GABAergic projection from the CeM to the ventrolateral PAG (vlPAG) (LeDoux, 2000; Tovote, Fadok and Lüthi, 2015). These data are in conjunction with the fact that lesions in the PAG block fear responses to a predator; from flight and freezing to risk assessment (Sukikara et al., 2010). Therefore, changes in CeA led by EphA4 could be a potential modulator of the intensity of the fear response (Cezario et al., 2008) and possibly for anxiety responses.

In summary, the immobility behaviours observed in tEphA4-RS animals of our study have been observed in the past to be closely related to CeA and the pathways connecting this area (LeDoux et al., 1988; Roozendaal, Koolhaas, Bohus, 1990; Roozendaal, Koolhaas, Bohus, 1991; Möller et al., 1997; Wilensky et al., 2006; Zimmerman et al., 2007; Ciocchi et al., 2010; Haubensak et al., 2010; Li et al., 2013; Ventura-Silva et al., 2013). These connections are known to vastly affect the behaviour developed during stress-related responses, such as conditioned fear and anxiety-like behaviours. Therefore, due to this relation between this brain area and the behaviour observed it is likely that the manipulation of the EphA4 signalling at the level of CeA circuit could be directly affecting these circuits and hence, the activity of the tPA/plasmin system in this area would be an important conditioning factor for the development of stress-related behaviours.

Motor skills argument

It could be argued that the tendency for the reduced motor skills measured in the EPM (distance travelled and speed) is due to locomotion impairments in tEphA4 RS animals. In fact, previous works suggest that the parameters that measure anxiety-like behaviours in the EPM cannot be simply dissociated from changes in locomotion, which may confound the interpretation of the results obtained using this paradigm, especially with the use of some psychostimulants (Weiss et al., 1998).

However, subsequent supplementary tests show that this low locomotion is likely due to the lack of movement associated with the described immobility response and not a simple motor impairment. In particular, motility parameters of tEphA4 and tEphA4-RS animals assessed by the open-field test are comparable to the reference values displayed by vector-infected controls and wtEphA4 (Figure 33). Furthermore, daily visual assessment on the animals' health and movements and reflexes by the staff from the university's animal facilities and the laboratory staff evidenced intact motor skills.

Additionally, changes in the locomotion of mice in the open field test are affected by the variation of some other parameters apart from stress, such as lighting (Valentinuzzi et al., 2000). However, keeping conditions under control (lightning, environment, odours or noises) in our experiments ensures consistent results across trials.

Anxiety-like parameters in the light-dark box and the open field tests are not affected by EphA4 after the elevated plus maze test

Significant differences in anxiety-like behaviours were found in the EPM (Figure 28), but these could not be reproduced in the LDB test (Figures 30). As noted at the beginning of the discussion section of the present chapter, although behavioural tendencies were difficult to predict in the LDB due to the lack of data regarding batteries of tests for anxiety-like behaviours, I expected to observe

similar results in the EPM and LDB tests. However, as observed in my data, the six-hours stress paradigm, plus the EPM test followed by a one-hour resting time may be not sufficient to produce an anxiogenic effect in the LDB.

Different reasons can explain these discrepancies between tests. One of them is that the results of these behavioural assessments are often modified if the test is to be conducted as part of a test battery (as opposed to a single-test performance) (Bailey and Crawley, 2009; Schneider et al., 2011), perhaps due to habituation to stressful events. These type of changes in the outcome of a test due to previous exposure to a stressor or a different test has been frequently observed in the past (Grey et al. 2013). For instance, there is a dampening effect in the anxiolytic response to diazepam in the LDB when there is a prior plus-maze exposure (Rodgers 1993). Furthermore, a study suggests that acute stress immediately before testing mice in a LDB can enhance the anxiolytic-like response (Hascoët, Bourin and Dhonnchadha, 2001), so EPM (considered as a stressing experience) may contribute to this effect. Therefore, anxiety-related results achieved in the LDB and the OFT (performed after the EPM) must be interpreted with caution.

Another feasible reason for test-related differences is that LDB and OF are not as sensitive as EPM to show particular anxiety-related behaviours. For instance, Chotiwat and Harris showed that restraint stress resulted in anxiety-like behaviours in the EPM and LDB, but not in the marble burying paradigm. Thus different tests likely affect diverse neural pathways that regulate anxiety-like behaviours differently (Chotiwat and Harris, 2006). Therefore, EphA4 truncation may play a role in the CeA during anxiety-related states in the EPM, but not during states induced by other tests. Furthermore, in the literature, there are examples of drugs whose effectivity has been largely demonstrated in humans and in different preclinical behavioural paradigms but fail to show that outcome in some other specific paradigms. Additionally, there are various stress levels associated with these paradigms. For instance, the EPM seems to be more threatening than the OF free exploratory test. Therefore, only more aversive types of paradigms would trigger anxiety-like responses (Ducottet and Belzung, 2005).

The paradigm-specific result may also be secondary to the type of stress used.

In the case of this study, I used a mild RS paradigm of single exposure for 6 h, which might decrease the capacity of LDB and OF tests to detect signs of anxiety when comparing it with longer and more animal-disturbing kinds of paradigms, such as the chronic unpredictable stress or repeated/chronic RS. In fact, LDB results have been shown to be inconsistent between studies following predator exposure (Wilson et al., 2015). Therefore, a more thorough study on the type of stress and timing studied will address which type is best to observe suitable effects in the LDB.

The results presented in this thesis emphasise the importance of the utility of multiple behavioural tests to analyse stress-related behaviours and locomotion. Plus, they highlight the importance of choosing the right paradigm to evaluate behaviours, since results may vary when using two or more tests, such as in the case of EPM and LDB in the present study. In my experiments, the EPM has been the only anxiety-related test not performed before any other test, so the results of this test should stand over the subsequent when assessing anxiety-like behaviours as the only one without additional confounding factors. The EPM has been, for decades, the test to measure anxiety-like behaviours in rodents that has shown more consistent and robust results and, until better or irrefutable methods are developed, this is the most accurate method to assess changes in anxiety-like behaviours in rodents.

Altogether, these data reveal that EphA4 cleavage significantly affects mice's state anxiety (after RS) but not trait anxiety (non-previously-stressed mice). The fact that the anxiogenic effect is only perceptible after RS indicates the existence of an underlying plasticity process produced during RS that is strongly blocked or enhanced depending on the EphA4 variant overexpressed. This plastic change results in a stress-related behaviour (pleiotropic effect) manifested when mice are challenged in the ethological approach/avoidance conflict of the EPM. It is then reasonable to think that these changes could be associated with the capacity of tEphA4 to modify morphological plasticity of dendritic spines in amygdala cultures (shown in previous chapters), which ultimately would affect synaptic transmission driving these behavioural fluctuations.

Plasticity effects in the CeA and consequences in the affected areas

Alteration of anxiety circuits produced by EphA4 during restraint stress at the level of the CeA would explain the modified expression of anxiety-like behaviours in the EPM observed in our experiments; which, in the case of EphA4 variants, are represented by altered times in the bright part of the EPM accompanied in cases by immobility responses.

Because these animals were not exposed to EPM before and no conditioning or prior learning paradigm was used in these animals, it must be assumed that connections for the expression of anxiety-like behaviours in the EPM are already formed in the adult brain. Therefore, at least part of the behaviour developed in the EPM is likely due to changes in these connections that have already been "hard-wired" during evolution; and the activity of the CeA (and its outputs and inputs) would produce these defence responses in the absence of prior aversive stimuli. Hence, in the experiments presented in this thesis, changes produced by plasticity (triggered by RS) would result from a change in synaptic inputs of a prior-existent pathway present in the CeA connectome. In Chapter 4, I detailed how the morphological plasticity of spines can be affected by stress and its consequences. However, in the current discussion, the focus will be in the circuits that can be primarily influenced by plastic changes in the CeA.

Until recently, research on plasticity in the amygdala has focused on the BLA. This is because different types of fear conditioning paradigms produce Hebbian plasticity changes in the BLA and because synaptic plasticity in BLA neurons is critical for associative (conditioned) aversive learning (e.g. J. Kim, Kwon, Kim, Josselyn, and Han, 2014; McKernan and Shinnick-Gallagher, 1997; Pape and Pare, 2010; Quirk, Armony, and LeDoux, 1997; Michael T. Rogan, Stäubli, and LeDoux, 1997). Additionally, CS-induced plasticity produced by negative stimulus in the LA has been thought to have a leading role in conditioned fear behaviour because it anticipates other plasticity events produced in the cortex and thalamus, and plasticity events in the BLA develop faster than the conditioned behavioural response (Quirk, Armony and LeDoux, 1997; Repa et al., 2001).

Many groups have been able to produce plasticity-related events in the BLA, such as LTP (Pape and Pare, 2010; for review Bocchio, Nabavi and Capogna, 2017), and even potentiate or inhibit the sensitivity to the acquisition of fear-related stimuli (e.g. Rogan and LeDoux, 1995). However, these are just examples, because a diverse array of synaptic plasticity mechanisms has been implicated in aversive conditioning (Pape and Pare, 2010; for reviews, see Johansen et al., 2011; Orsini and Maren, 2012).

Although early research in amygdalar plasticity looks to the LA as the primary site for plasticity, new studies in fear conditioning and anxiety paradigms show that the CeA is also necessary for some types of aversive learning and that it is not just a passive node connecting the BLA with downstream structures. For instance, the CeA inactivation by infusion of the $GABA_A$ agonist, muscimol, before conditioning impairs fear memory retrieval (Wilensky et al., 2006), meaning that synaptic plasticity occurring in inhibitory neurons of the CeA may be necessary for fear learning. Also, blocking protein synthesis in the CeA impairs fear memory consolidation (Wilensky et al., 2006). Furthermore, a work done by Ciocchi and colleagues (2010) shows that pharmacological inactivation (through a GABA agonist) of the CeL (but not the CeM or the whole CeA) or optogenetic activation of the CeM induces unconditioned freezing, which poses this structure as a modulator of the fear responses and expression. It further suggests that CeM is under inhibitory control of the CeL and that the CeM is vital for the expression of fear. But this observation is not restricted to fear conditioning. Tye et al. (2011) proved that BLA–CeL–CeM circuits in mice could be also participating in anxiety-related behaviours (Tye et al., 2011). In this work, somatic activation of BLA neuronal somata projecting into the CeL produced increased anxiety-like behaviour, whereas the activation of excitatory BLA axonal fibres that project into the CeL was anxiolytic. The types of subpopulations in the BLA that elicit these differential responses have not been elucidated yet, and it is also conceivable that different CeL circuits leading to anxiety behaviours substantially overlap the ones that elicit fear behavioural responses.

There is a substantial probability that these circuits involved in anxiety response are GABAergic. The CeA consists of ~95% GABAergic neurons. While cells of

the CeL are medium spiny neurons (McDonald, 1982) that show considerable dendritic branching, GABAergic cells of the CeM cells have minimally branched dendrites and few spines (McDonald, 1982; Cassell and Gray, 1989; Sun and Cassell, 1993). Thus, GABAergic CeL neurons are in principle a more likely hub of plasticity affecting anxiety. Indeed, interference with GABAergic signalling in the amygdala has been shown to affect anxiety (Tovote, Fadok and Lüthi, 2015). Alternatively, genetic differences in GABA or CRF tone in the amygdala have been also shown to potentially contribute to excessive responses to stress or anxiety (Adamec, 1997; Rosen and Schulkin, 1998). And there is also accumulating evidence indicating that the neuronal activity and plasticity of the CeA afferent and efferent projection neurons are tightly controlled by GABAergic inhibition (Tovote, Fadok and Lüthi, 2015).

Therefore, disruption of the GABAergic synapses in the CeL by interaction with gephyrin or other means could potentially affect the behavioural outcomes related to fear learning or fear expression. There are many other examples for plasticity in the CeA but their molecular mechanisms are unclear. In the current work, I propose a mechanism operating through GABAergic synapses that may act as a modifier of this plasticity.

GABAergic subpopulations related to aversive stimuli in CeA

In the context of aversive stimuli and responses, three subpopulations of GABAergic inhibitory neurons within the CeL have been roughly characterised. One of them, that is not activated during fear conditioning paradigm (coined as CeL_{OFF}), mainly expresses protein kinase C-δ (PKCδ), but generally lacks somatostatin (SOM) (Ciocchi et al., 2010; Haubensak et al., 2010; Li et al., 2013). Interestingly, data from this thesis show that the main population of neurons expressing tPA in the CeA are $PKC\delta^{+}$. Therefore, changes that affect the depolarisation of these neurons could likely affect tPA/plasmin/EphA4 cascade. These CeL-located $PKC\delta^{+}$ neurons inhibit CeM neurons and are also actively suppressed by CeL $PKC\delta^{-}$ neurons during the exposure to CS (Ciocchi et al.,

2010; Haubensak et al., 2010). Therefore, the activation of CeL PKCδ⁻ cells or pharmacogenetic silencing of PKCδ⁺ would produce disinhibition of the CeM, and consequently, it would facilitate the expression of fear-like behaviours (i.e. freezing). In our model, because tPA is released upon depolarisation, activation of PKCδ⁺ neurons would release tPA in the local circuits of the CeL and the CeM; this would interfere with the GABAergic tone by reducing it, which would facilitate the disinhibition of the CeM and hence the expression of behaviours related to anxiety by downstream structures, for example, through their projection to the PAG (Ciocchi et al., 2010; Tovote, Fadok and Lüthi, 2015).

Interestingly, Ciocchi et al. showed the tonic activity of PKCδ⁺ cells is more intense after fear conditioning, for instance, during a second tone that is not paired with a footshock in mice (Ciocchi et al., 2010). Since this type of generalised aversive response is contemplated as a major hallmark of anxiety (Blanchard, 2008), Ciocchi's work links the changes in tonic activity within fear circuits of the CeA with overlapping circuits of anxiety-like behaviours. Moreover, in line with all of this argumentation, GABA release onto PKCδ⁺ neurons in the CeL protects against fear generalisation (Botta et al., 2015).

Among the three GABAergic CeL subpopulations characterised to date, tPA neuronal population mainly colocalises with PKCδ⁺ neurons and is expressed in fewer cells of the other two no-PKCδ⁺ subpopulations studied (Figure 9). Therefore, this suggests that changes in the activity of PKCδ⁻ populations would, in theory, minimally affect *tPA*+ neurons.

CeL-somatostatin-positive (CeL-SOM⁺) neurons form one of these no-PKCδ⁺ populations. Notably, PKCδ⁺ and SOM⁺ neurons are, to a great extent, non-overlapping (Li et al., 2013). Selective prevention of synaptic potentiation onto SOM⁺ neurons during the conditioning hampers fear memory formation and the conditioning-induced plasticity in the CeL but not in the CeM. Furthermore, activation of these neurons is necessary for fear memory recall and sufficient to drive fear responses (Li et al., 2013), but SOM⁺ cells include long-range projection neurons that circumvent the CeM and reach the PAG without any secondary connection (Li et al., 2013).

A third important GABAergic subpopulation is a group of inhibitory CRF–expressing neurons that are essentially different from previously-characterised SOM^+ and $PKC\delta^+$ (although a minimal part of them are also SOM^+). CRF neurons seem to be arranged in a local inhibitory network in the CeL. They experience plasticity following threat conditioning and are selectively activated by threat-predictive stimuli. Fluorescent reporter experiments measuring intracellular Ca^{2+} concentrations *in vivo* suggest that CRF neurons are more active following a CS paradigm than in resting conditions. The authors also propose that these cells are relevant for (aversive) cue-intensity discrimination through mechanisms dependent on GABA. This argument is based on the fact that local inhibitory electrical signals were sensitive to the $GABA_A$ receptor antagonist picrotoxin, but not to the glutamate receptor antagonist CNQX. However, that discrimination disappears when the stimulus is excessive (Sanford et al., 2017). In conclusion, SOM^+ and CRF^+ populations have been only observed to be affected in fear paradigms whereas $PKC\delta^+$ populations could be playing a dual role in fear or anxiety *per se* or by affecting tPA release.

CONCLUSIONS

Acute restraint stress for one hour (i.e. an anxiogenic stimulus) produces a significant increase in protein levels of lower-molecular-weight forms of EphA4 in wild-type mice, an event that is consistent with tissue plasminogen activator/plasmin cleavage of this receptor in the central amygdala. However, the same cleaved forms of EphA4 remained unchanged in tissue-plasminogen-activator-deficient or plasminogen-deficient mice.

The injection of lentiviral particles in the central amygdala containing plasmids for the overexpression of the wild-type EphA4, the cleavage-resistant EphA4 mutant (R516Q), the EphA4 variant truncated at R516 or the corresponding empty vector backbone resulted in different anxiety-like behaviours when measured with the elevated plus maze; but not when subsequently measured with the dark-light box or the open-field test. Mice expressing the truncated EphA4 variant and subjected

to restraint stress show a significant increase in anxiety-like behaviours when compared to the rest of stressed groups, as measured by the time spent in the open arms of the EPM. Conversely, restrained mice expressing full-length forms of EphA4 (wild-type or cleavage-resistant) do not show a reduction in the same parameters when compared with the vector-infected control or the truncated EphA4 group, which evidences anxiolytic effects. Nevertheless, no difference is observed among the non-stressed groups, which suggests that trait anxiety is not affected in these animals. Additionally, stressed animals expressing truncated EphA4 exhibit immobility behaviours that could be a reflection of the functions of the central amygdala as the main output area for the expression of anxiety-like behaviours.

Altogether, these results suggest that plastic modifications mediated by EphA4 cleavage and likely affecting the GABAergic neuronal populations of the mouse central amygdala can regulate defence mechanisms important for anxiety-like behaviours.

FUTURE WORKS

In this work, the measurement of anxiety-related processes has been established through behaviours associated with these emotions. Although they are relatively robust methods to observe changes produced by these internal states, further works could assess levels of anxiety processes that can be unnoticed in a behavioural paradigm. For instance, by measuring HPA axis parameters, like CRF, ACTH, glucocorticoids (corticosterone) levels or other criteria associated with stress and anxiety, such as cardiovascular parameters (high blood pressure, heart rate or increases in body temperature).

Despite the elevated plus maze is the most used and reliable behavioural paradigm to measure anxiety-like behaviours to date, it would be advisable to confirm these behaviour changes with other behavioural paradigms, such as the dark-light box, the open maze, the zero maze or the forced swimming test. And,

although some of these tests were carried out in the present work, interference of previous behavioural tests may have yielded biased results. Moreover, data suggest that the time mice spent in the lit area and behavioural activities such as locomotor and rearing behaviours may be more useful measures of the anxiolytic potential of a compound than transitions between the two compartments. In fact, the measurement found to be most consistent and useful for assessing anxiolytic-like action was the time mice spent in the lit area, this parameter providing the most consistent dose–effect results with the drugs (Young and Johnson, 1991).

Furthermore, manipulations, such as the lentiviral expression of EphA4 variants in this thesis (and others that would allow a better temporal and spatial resolution, such as optogenetics) could be performed in other areas in which tPA/plasmin/EphA4 system could be active, such as hippocampus, BNST or hypothalamus.

Although the activation of the tPA/plasmin system has been evidence here indirectly with the use of tPA- and plasminogen-deficient animals, the direct interaction *in vivo* of these proteases and EphA4 has not been assessed. Therefore, it would be interesting to visualize the cleavage of EphA4 and the tPA/plasmin activity or interaction at the same time to determine the localisation of this mechanism. To observe tPA's activity, zymographic gels could be used to observe the co-existence of tPA activity and EphA4 cleavage, although it would not evidence direct interaction. Unfortunately, no specific substrates have been identified for plasmin, hence zymographic assays are not possible for this protease to date. To assess the direct interaction between tPA/plasmin and EphA4 other techniques could be used. These include bioassays such as the Förster resonance energy transfer (FRET) assay, the bioluminescence resonance energy transfer (BRET) assay or the proximity ligation assay (PLA).

CHAPTER 7: MODELS OF DISEASE

INTRODUCTION

In this chapter, I will reference instances in which the tPA/plasmin/EphA4 proteolytic cascade might be relevant for a particular brain condition and they will be discussed on the basis of previous works related to the components of this cascade and the animal models related to each of them.

The main focus of the present dissertation are conditions in which tPA/plasmin/EphA4 cascade may affect anxiety-related behaviours; however, because of its relevance to our research, these types of conditions are discussed explicitly in a separate chapter (Chapter 6).

Research about proteolytic systems encompasses a wide array of conditions, but the most studied ones are related to neuronal degeneration and neurotoxicity. There are examples of tPA/plasmin regulating the neuronal damage of various conditions, such as excitotoxicity, Alzheimer's disease (AD), stroke, infarct formation and seizure spreading (Melchor and Strickland, 2005).

Alzheimer's disease

Alzheimer´s disease is one of the most common types of dementia. One of its main pathological hallmarks is the deposition of a peptide called β-amyloid (Aβ) in the brain parenchyma, which is able to activate inflammatory responses. tPA/plasmin system has been linked to the degradation of Aβ in both mice and humans, and Aβ aggregates can stimulate the expression of tPA and uPA (Zhao and Pei, 2008). Reduced tPA/plasmin activity (also proven experimentally with the use of plasmin inhibitor, PAI-1) contributes to the raise in Aβ levels. This event induces PAI-1 gene and elevates PAI-1 levels, which results in further depression of tPA activity, closing a vicious cycle of pathology. Also, decreased plasmin activity has been observed in disturbed lipid rafts of brains from AD patients (Melchor and Strickland, 2005). Additionally, PAI-1 is considerably elevated in the hippocampus of AD mouse models, and the PAI-1-mediated inhibition of plasmin-induced Aβ clearance can be eliminated by the specific PAI-1 inhibitor, PAZ-417,

restoring the tPA/plasmin system activity. Furthermore, PAZ-417 reduces memory deficiencies in mice (Zhao and Pei, 2008).

Interestingly, plasmin also produces the non-amyloidogenic α-cleavage of amyloid precursor protein (APP). However, mice deficient in plasminogen do not present increased Aβ levels, suggesting that plasmin does not regulate physiological levels of Aβ but may be involved in Aβ clearance after aggregation is initiated in AD (Zhao and Pei, 2008).

There is also evidence connecting EphA4 with AD (Cissé and Checler, 2015). For instance, Simón et al. (2009) showed that EphA4 is decreased in the hippocampus of the APP transgenic mouse model of AD and in humans with AD. A different analysis looking at the synapse shows that EphA4 mRNA levels in synaptoneurosomes are augmented in samples from AD patients and the protein levels are increased in the area circumscribing senile plaques in human hippocampi. Also, elevated levels of active EphA4 in AD brains have been reported. Therefore, EphA4 shows an altered expression and distribution in AD cases, which seems to be more evident near neuritic Aβ plaques and phosphorylated tau (Rosenberger et al., 2014).

Additionally, Aβ oligomers (AβOs) bind to various neuronal receptors, among which EphA4 and EphB2 can be found. They have been proposed as two of the AβOs-interacting receptors that cause synaptic damage (Vargas et al., 2018). Two groups have published data demonstrating that AβOs induce EphA4 activation (Fu et al., 2014; Vargas et al., 2014) and that the inhibition or absence of this receptor in hippocampal neurons prevents synaptic loss.

Although EphA4 phosphorylates Cdk5 and this kinase affect various aspects of AD (like tau phosphorylation), no investigation has provided evidence of any link between tauopathies and EphA4 (Vargas et al., 2018).

Pentylenetetrazol (PTZ) kindling model of epilepsy and other neurotoxicity models

Epilepsy is a CNS disorder characterised by recurrent seizures, which are the clinical expression of abnormal, excessive, hypersynchronous electrical impulses generated in certain populations of cortical neurons. Prolonged or repeated seizures are known as *status epilepticus*. Seizure phenotypes depend on the location and function of the neuronal network involved in the synchronous bursting and these impulses can spread to neighbouring brain regions creating severe phenotypes. Seizures (and therefore epilepsy) may have different aetiologies that result in the instability of the neuronal cell network, such as the disturbance of extracellular ion homeostasis, the altered energy metabolism or the malfunctioning of the reuptake of receptors and neurotransmitters. Despite these differences, seizures of similar characteristics are produced in a large proportion of the cases. Therefore, the study of models of seizures and neurotoxicity are useful in the study of epilepsy and other seizure-related pathologies (Bromfield, Cavazos and Sirven, 2006).

The term epileptogenesis refers to the sequence of events that transforms a healthy neuronal network into a pathological one in the context of epilepsy. Perhaps the most extensively studied animal model of epileptogenesis is kindling. Kindling consists of repeated subconvulsive stimulation (either electrical or chemical) of some brain regions (e.g. hippocampus or amygdala) resulting in electrical afterdischarges that eventually lead to stimulus-induced and spontaneous clinical seizures. The exact mechanisms underlying kindling and its relation with human epilepsy are still unknown. However, it is known that the changes produced in the animal's brain excitability are permanent and potentially involve biochemical and structural long-lasting changes in the CNS (Bromfield, Cavazos and Sirven, 2006).

Kindling can be chemically induced by the injection of compounds that lead to a decreased inhibition or an increased excitation of the brain networks. These treatments result in seizures, which, as stated before, is a main feature of epilepsy but also can end up producing excitotoxicity. One of the most used compounds creating hyper-excitation is kainic acid (KA), which is a very well-

known glutamate receptors agonist (Ben-Ari et al., 1979). Although it does not reproduce all the features of epilepsy, intra-amygdaloid injection of KA induces behavioural epileptic-like seizures and produce lesions that are similar to those occurring in patients with temporal lobe epilepsy (i.e. neuronal degeneration in the CA3 region of the dorsal hippocampus). Other glutamate receptor agonists, such as N-methyl-D-aspartate and cocaine, can be used to induce similar effects. Additionally, inhibitory circuits can also be altered to produce hyper-excitation. GABA antagonists have been used to create excitation through a decreased inhibition of the network (e.g. picrotoxin, bicuculline). Pentylenetetrazol (PTZ) is another broadly used $GABA_A$ receptor antagonist that has been shown to induce chemical kindling in mice and is therefore also used to model some aspects of epilepsy. The protocol to induce seizures consists of the administration of a subconvulsive dose of PTZ for several days in a row; although a higher single dose can produce involuntary movements as well. Then, a seizure score evaluates the magnitude and type of each seizure (Dhir, 2012).

Among other processes, seizures upregulate the activity of extracellular proteases, such as the tissue plasminogen activator (tPA) and the metalloproteinase-9 (MMP-9). First data linking tPA to epilepsy was provided by a study from Qian et al. (1993). They showed increased tPA mRNA expression in the rat cortex and hippocampus at 0.5-4 h following PTZ-induced seizures and in the hippocampus 1 h after single perforant path stimulation evoked an after-discharge. Since then, tPA has been shown to be involved in all kinds of models of epilepsy, such as kindling and other models capable of producing seizures (Qian et al., 1993; Tsirka et al., 1995; Pawlak and Strickland, 2002; Benarroch, 2007). Accordingly, observations from different works also show an increase in expression and mRNA levels of plasminogen activators (PAs) in various experimental models of epilepsy (Salles and Strickland, 2002; Lukasiuk, Kontula and Pitkänen, 2003; Lahtinen, Lukasiuk and Pitkänen, 2006; Gorter et al., 2007; Zurolo, 2013; Gorter, van Vliet and Aronica, 2015).

In experimental models of epilepsy, the expression of tPA mRNA seems to be increased, but also tPA protein levels and its enzymatic activity. A rise in proteolytic activity specific to tPA was detected in the mouse amygdala at 10-60

min following KA injection (Yepes et al., 2002) and in the CA3 area of the hippocampus at 7 h after the *status epilepticus* (SE) was induced by intraventricular KA injection (Endo et al., 1999). In contrast, tPA enzymatic activity is transiently decreased in the hippocampus and cortex at 1 day after the SE in the amygdala, as well as after intra-hippocampal injection of KA (Salles and Strickland, 2002; Lahtinen, Lukasiuk and Pitkänen, 2006). These data indicate dynamic temporal regulation of tPA activity. Interestingly, seizure spreading is attenuated in $tPA^{-/-}$ but not in $plasminogen^{-/-}$, demonstrating that seizure progress is probably a plasminogen-independent process. Accordingly, seizure's onset is delayed by neuroserpin (a selective inhibitor of tPA). Similarly, seizures after ethanol withdrawal in mice are attenuated in $tPA^{-/-}$ mice with mechanisms not dependent on plasminogen (Melchor and Strickland, 2005).

Little information is available about tPA expression in human epilepsy. Remarkably, Iyer et al. (2010) studied various focal epileptogenic conditions: hippocampal sclerosis (HS), focal cortical dysplasia (FCD), tuberous sclerosis complex (TSC) and gangliogliomas (GG). Their work showed an increase in tPA mRNA in several of these conditions and an increase of tPA expression in neurons of HS and in the affected tissue of individuals who experienced FCD, TSC and GG. Additionally, strong immunoreactivity was present in reactive astrocytes, microglia and the majority of blood vessels of these individuals.

Kainate excitotoxicity models also regulate the tPA effects in a proteolytic (through cleavage of plasmin) (Tsirka et al., 1997) and a non-proteolytic manner (Rogove and Tsirka, 1998; Rogove et al., 1999; Gravanis and Tsirka, 2008; Minassian, Striano and Avanzini, 2016). Tsirka et al. (1995) studied the effect of tPA deficiency in knock-out mice, particularly, in seizures induced by intraperitoneal injection of either KA or PTZ. tPA-deficient mice required higher doses of KA or PTZ than control mice to develop seizure phenotypes. In the case of KA injections, $tPA^{-/-}$ mice did not develop SE even with the highest dose tested (Tsirka et al., 1995). A similar effect has been shown by Yepes et al. (2002), who found a decrease in the rate of seizure progression and a lack of seizure generalisation in $tPA^{-/-}$ mice following intra-amygdalar KA injection. Additionally, in mice and rats, (2002) the application of the tPA inhibitor, neuroserpin,

attenuates the generalisation of seizures in a model of SE induced by intra-amygdalar injection of KA. This tPA-induced effect was not dependent on plasminogen activation, since spreading of seizures in plasminogen deficient mice did not differ from wild-type controls (Yepes et al., 2002).

tPA deficiency has been shown to be neuroprotective also in traumatic brain injury. tPA knock-out mice had significantly less lesion volume following a controlled cortical impact (a commonly-used model of brain trauma) (Mori et al., 2001). TPA has also been implicated in mossy fibre sprouting, a pathological form of axonal plasticity of granule cell neurons. Zhou et al. (2010) observed a decreased mossy fibre outgrowth following SE induced by intra-amygdalar KA injection in $tPA^{-/-}$ mice. This effect was independent of plasminogen and was not observed in plasminogen-deficient mice.

Furthermore, tPA may produce excitotoxicity due to intracellular calcium influx caused by cleavage of the GluN1 subunit of the NMDA receptor (Nicole et al., 2001; Samson et al., 2008)), although the validity of this cleavage has been controversial (Matys and Strickland, 2003). Also, non-cleaving interaction with the GluN2B receptor could affect the calcium influx mediated by this glutamate receptor (Pawlak et al., 2005; Gravanis and Tsirka, 2008). Other molecules comprising the ECM may also be involved in these mechanisms (Chen and Strickland, 1997; Tsirka et al., 1997; Samson et al., 2008).

To summarise all this literature, it is generally observable that when tPA inhibition is impeded, epilepsy and seizures become a main clinical feature, and seizure development is significantly delayed in the absence of tPA in mice (Yepes et al., 2002).

The relation of EphA4 with epilepsy or seizures has not been extensively investigated. Only separate, single studies give some insight in to the role of this protein. For instance, Filosa et al. (2009) showed that in the presence of dendritic EphA4, the number of glutamate transporters is increased and therefore, the glutamate toxicity is attenuated because the neurotransmitter is removed from the media, which in turn also provides some protection against PTZ.

In a model of epilepsy induced by pilocarpine (cholinergic agonist), EphA4 mRNA

and protein levels were gradually upregulated in the hippocampus in a period of 28 days post-*status epilepticus*. In this model, the interaction of ephrin-A5 and EphA4 seem to be upregulated as well. This research group also claimed that inhibition of EphA4 through ephrin-A5-Fc was associated with the decrease of p-Akt, p-ERK, and VEGF neuronal pathways (Yepes et al., 2002; Shu et al., 2016). Furthermore, EphA4 may also influence microvessel remodelling in the same model of disease (Feng et al., 2017).

Medial Cerebral Artery Occlusion and Photochemically Induced Thrombosis models of stroke

Stroke is one of the five main causes of death and acquired adult disability worldwide. Statistically speaking, 80% of strokes are the result of thromboembolic occlusion of a major cerebral artery or its branches, which produces ischemia or an ischemic cascade (deprivation of oxygen and energy, followed by the formation of reactive oxygen species, release of glutamate, accumulation of intracellular calcium and induction of inflammatory processes). The irreversible tissue injury is termed “infarction”. Currently, there are two main approaches to treat ischemic stroke based on reducing the area of ischemic brain tissue surrounding the infarcted core (ischemic penumbra); these are reperfusion and neuroprotection. Reperfusion can be achieved by mechanical devices or thrombolytic drugs that break the thrombus to restore blood flow in occluded vessels. Importantly, the only approved medical treatment for acute ischemic stroke to date is the intravenous injection of recombinant tissue plasminogen activator (rtPA) in a time window of up to 4.5 hours after stroke, which means that the treatment is usable in approximately 5% of all patients. Therefore, there is a real need for a more general treatment that can broaden the treatment options (Fluri, Schuhmann and Kleinschnitz, 2015).

MCA models of stroke

The middle cerebral artery (MCA) and its branches account for about 70% of

infarct cases in human ischemic stroke. Thus, techniques that occlude this artery are considered to be paramount in the study of human ischemic stroke. Several models have successfully helped to report many aspects of this pathophysiological process. A transient or permanent middle cerebral artery occlusion (MCAO) is one of the most used techniques to model ischemic stroke. This model is characterised by robust and reproducible infarcts in rodents and it is able to mimic many aspects of human stroke's heterogeneous and complex pathophysiology. Among the occlusive MCA stroke models, intra-arterial suture occlusion of the MCA (MCAO) is the most common method in rodents. It is one of the less invasive models and does not require craniotomy to be performed. It consists of a temporal occlusion of the common carotid artery (CCA). A suture directly introduced into the internal carotid artery (ICA) until it interrupts the blood supply to the MCA. This method enables permanent MCAO or transient ischemia with reperfusion. In mice, the area affected by the infarction includes an ample part of the hemisphere (including a large proportion of the cortex, striatum, thalamus, hippocampus and subventricular zone). The degree and location of the ischemia are highly dependent on the duration of the occlusion. Changing the occlusion time from 15 min to 30 min results in a fivefold increase in the infarct volume; however, no ischemic lesions are observed in mice subjected to MCAO for less than 10 min (Fluri, Schuhmann and Kleinschnitz, 2015).

Photothrombosis model of stroke

The second stroke model used in this project is based on a type of thrombosis produced by the natural adherence of platelets to an activated endothelium (i.e. a dysfunctional endothelium). In this case, the activated endothelium is achieved by the production of oxidative species induced by light. The procedure is called photochemically-induced thrombosis (PIT). In this technique, first, a photoactive dye (e.g. Rose bengal or erythrosin B) is injected intra-peritoneally in mice. Then, the intact skull is irradiated with a light beam at a specific wavelength that, in a matter of minutes, produces reactive oxygen species (ROS), which in turn generate free radicals derived from oxygen that cause damage on endothelial tissue, platelet activation and aggregation in vessels within the irradiated area.

The level of injury on endothelial cells is dependent on the intensity and timing of light radiation, the dose of dye and the type of the vessel studied. It allows for a precise and non-invasive way of producing thrombosis with high reproducibility of the lesion and low mortality during the procedure. Depending on the area irradiated this model may have different effects (Rauova, 2012; Fluri, Schuhmann and Kleinschnitz, 2015).

tPA and plasmin have a strong link with stroke research as they are part of the fibrinolytic cascade. So far, tPA analogues have been the only accepted treatment approved by FDA after ischemic strokes; specifically, alteplase IV (rtPA) is considered the gold standards for this kind of treatments (Gravanis and Tsirka, 2008). Also, plasmin has been assayed in clinical trials as a fibrinolytic drug but its success has been limited due to the poor thrombolytic effectiveness at the given doses (Mitchell et al., 2017).

Animal studies show contradictory results when it comes to proving tPA's neurotoxic properties. Several *in vitro* models of ischemia or, in general, neurotoxicity have been used to study this protease. In various studies, tPA was added to neuronal cultures from cerebrocortical or hippocampal areas and treated with oxygen-glucose deprivation, but also with haemoglobin toxicity, microglial conditioned medium or zinc. Different results have been attained from these works, with neuroprotective or neurotoxic effects depending on the study (Gravanis and Tsirka, 2008), so it is difficult to draw any conclusion about this topic.

These contradictory results were also observed in models that try to imitate ischemic stroke in laboratory animals. In the MCAO model, Wang et al. found a tPA-related increase in the damage caused by the occlusion whereas Tabrizi et al., in the same type of experiment, reported a protective role of tPA. An explanation for these opposite results could involve the model of ischemia used in each case. Wang et al. used a mechanical obstruction of the vessel whereas Tabrizi et al. used thrombi obstruction. The mechanical method is not modified by increased tPA, whereas in the thrombus obstruction, tPA could break the clot and hence have a beneficial effect. Other studies that used the recombinant form of tPA in rodent models of ischemic stroke yielded inconsistent effects. Again,

some of them resulted in beneficial effects, some in neutral effects and some in adverse effects (Gravanis and Tsirka, 2008).

In addition, different works confirm that the time and size of the infarction have an effect on its outcome and severity. This is similar to what has been reported from clinical research. The size of the infarction area and the time before tPA treatment administration is linked to the severity of the stroke effects. Particular experiments using MRI scans to determine the optimal timing of tPA administration revealed that tPA administered at 1 h after stroke induction was beneficial, but it is detrimental at a later time point (4 h). In relation to the size, Nagai et al. showed that smaller infarcts have better outcomes using tPA, but the effects are deleterious in larger ones (Gravanis and Tsirka, 2008).

Mechanisms for these contrasting outcomes have been suggested. Some of them are purely related to the effect of tPA on the circulatory system. tPA is thought to affect vessel tone, which in turn would affect perfusion of the ischemic area. tPA can also increase brain oedema and hence aggravate neurological outcomes in affections like brain trauma and intracerebral haemorrhage. This effect has been related to another extracellular protease, namely, MMP9, which has been shown to compromise the BBB integrity. tPA can directly activate MMP9 and or induce its expression. Thus, MMP9 has been also a target when treating thrombolysis-related pathologies (Gravanis and Tsirka, 2008).

Furthermore, tPA affects the brain parenchyma directly. tPA seems to interact with glutamate receptors, which are important mediators of excitotoxicity in the course of ischemic stroke. As mentioned in the previous section, tPA experiments with kainic acid show an important role of tPA in excitotoxicity. For instance, tPA can activate microglia in a non-proteolytic fashion and also trigger the production of monocytes for their accumulation into sites of inflammation. This is important because microglia has been implicated in cell death induced by kainate excitotoxicity (Gravanis and Tsirka, 2008; Minassian, Striano and Avanzini, 2016) and GluN2B subunits may be involved in this process (Nicole et al., 2001; Matys and Strickland, 2003; Pawlak et al., 2005).

tPA-mediated cleavage generation of plasmin can lead to subsequent

degradation of laminin and other components of the ECM which could result in anoikis (a kind of apoptosis produced by the loss of contact with the ECM) (Chen and Strickland, 1997). Plasmin could also have a chemotactic effect by activating MCP-1, which is a chemokine that mobilises the recruitment of monocytes and microglia to affected areas. Moreover, plasminogen-deficient mice present chemotaxis defects. Haemorrhage could aggravate the inflammation process by attracting more circulating monocytes from the bloodstream (Gravanis and Tsirka, 2008).

Interestingly, the main neuronal tPA inhibitor, neuroserpin, has been addressed to have neuroprotective properties when administered in ischemia and when administered in association with tPA in models of ischemic stroke. This would argue in favour of a neurotoxic effect of tPA, but no mechanism has been proposed for this effect yet. Additionally, some single nucleotide polymorphisms (SNP) in the neuroserpin gene play roles in the development of stroke and have been associated with a higher risk of suffering the disease (Cole et al., 2007; Gravanis and Tsirka, 2008).

The literature is not abundant on the relationship between EphA4 and stroke models, however, there is one study that directly addresses the presence/absence of EphA4 in a photothrombotic model of stroke (Lemmens et al., 2013). In this study EphA4 is shown to be up-regulated after stroke in the affected area, and the inactivation of the receptor increases functional recovery in models for these conditions. Specifically, in EphA4 conditional knock-out mice, although the time of recovery was reduced, the size of the infarction was not affected. The authors argue that the better recovery might be due to the activity of Rho-associated kinase (ROCK) downstream of EphA4. These and less relevant results led to the generation of nanobodies to achieve the inhibition of EphA4 signalling by targeting the ATP-binding pocket in the kinase domain or by blocking the interaction with ephrin ligands; but these tools have not been clinically tested yet (Schoonaert et al., 2017).

Treatment of brain pathologies by modulating Eph receptors

As referenced in this introduction, Ephs and ephrins have been implicated in various brain disorders, including anxiety (Attwood et al., 2011). Therefore, targeting this family of receptors and ligands may be interesting to develop new therapies and drugs that can tackle these diseases.

To date, various different approaches have been taken to target these receptors (Boyd, Bartlett and Lackmann, 2014). Most of the approaches focus on targeting the interaction between Eph receptors and ephrins by affecting their binding site.

An ideal drug for the treatment of brain affections should be able to be administered systemically and cross the BBB; plus, it should be specific enough to produce minimal side effects. This could be achievable through small molecules, which can be designed to fulfil those characteristics. Inhibition of EphA4 signalling can be achieved by targeting the ATP-binding site in the kinase domain or by blocking the interaction with ephrin ligands. Since the ATP-binding site is a conserved region, most of the molecules developed so far target the ligand-binging domain.

Attempts to develop such molecules have been made. For instance, inhibitors of ephrin, such as 2,5-dimethylpyrrolyl benzoate (Noberini et al., 2008; Noberini, Lamberto and Pasquale, 2012), disalicylic acid-furanyl derivative (Noberini et al., 2008; Noberini, Lamberto and Pasquale, 2012) and lithocholic acid derivatives (Giorgio et al., 2011) compete for ephrin-A binding with EphA receptors. Other different peptides and small molecules have been produced and demonstrated to block its interaction with ephrin ligands by binding the EphA4 LBD (Schoonaert et al., 2017). One of these EphA4 antagonists is the KYL peptide, which has been used for *in vitro* and *in vivo* experiments of spinal cord injury and ALS models, suggesting the potential of an EphA4-based therapeutic approach (Lemmens, Jaspers, Robberecht, and Thijs, 2013).

RESULTS

Cleavage of EphA4 in Alzheimer model of tauopathy is not affected

Alsheimer´s disease has been related to tPA/plasmin proteolytic system (Barker, Love and Kehoe, 2010; Barker, Kehoe and Love, 2012) and tPA treatment has been shown to improve the disease´s symptoms in familial Alzheimer´s model, APPswe/PS1 (ElAli et al., 2016). But no model of tauopathy has been linked to any kind of alteration in tPA/plasmin levels or activity.

The amount of EphA4 and its proteolytic cleavage in protein samples extracted from whole hippocampi of the rTg4510 mouse model of tauopahy was indifferent from the control samples (Figure 35).

Cleavage of EphA4 in the pentylenetetrazol model of epilepsy

C57BL/6J mice were injected intraperitoneally with a bolus (normally a single dose) of pentylenetetrazol (PTZ) until they developed epileptic seizures. Then, different times of seizures were picked to test EphA4, tPA and plasmin. Our results did not show a significant increase in tPA or plasmin(ogen) protein levels in C57BL/6J mice (data not shown), but an increase in the intensity of the main cleavage band of EphA4 is observable after 2 h of PTZ seizure induction when analysing hippocampi samples by Western blotting technique (Figure 36, ANOVA, $F(3,8) = 5.195$, $p = 0.278$. No seizure control (0'): 1.000±0.243 a.u. vs. 120 min of seizures (120'): 1.895±0.109 a.u.; $p = 0.029$). This result shows that EphA4 cleavage is affected by seizure induction using PTZ.

Cleavage of EphA4 in two models of ischemic stroke

During stroke, various proteases are known to be extravasated from the blood

vessels in humans and in mice (LapchakJohn and Zhang, 2018) triggering neurotoxicity and various other deleterious effects. After induction of an infarction area by MCAO or PIT methods in C57BL/6J mice, the animals were sacrificed, their hippocampi dissected and the relative protein levels were analysed by Western blot. A dramatic increase of the main cleavage band was detected in both models of ischemic stroke at ~50 KDa in the hemisphere of the brain affected by the ischemia (Figure 37). This band matches in size with the one produced by tPA/plasmin. However, in $Plg^{-/-}$ animals that underwent this same process, the increase in the ~50 KDa EphA4 cleavage form is also produced, indicating that plasmin is not the protease involved in this event (Figure 37). Interestingly, in the PIT model, the Western blot's barcode after ischemia also presents an increase in a second band under 35 KDa that is not present in MCAO model. As with the ~50 KDa, this PIT model's specific band is produced in $Plg^{-/-}$ animals, which, again, indicates that plasmin is not involved in EphA4 cleavage.

FIGURES

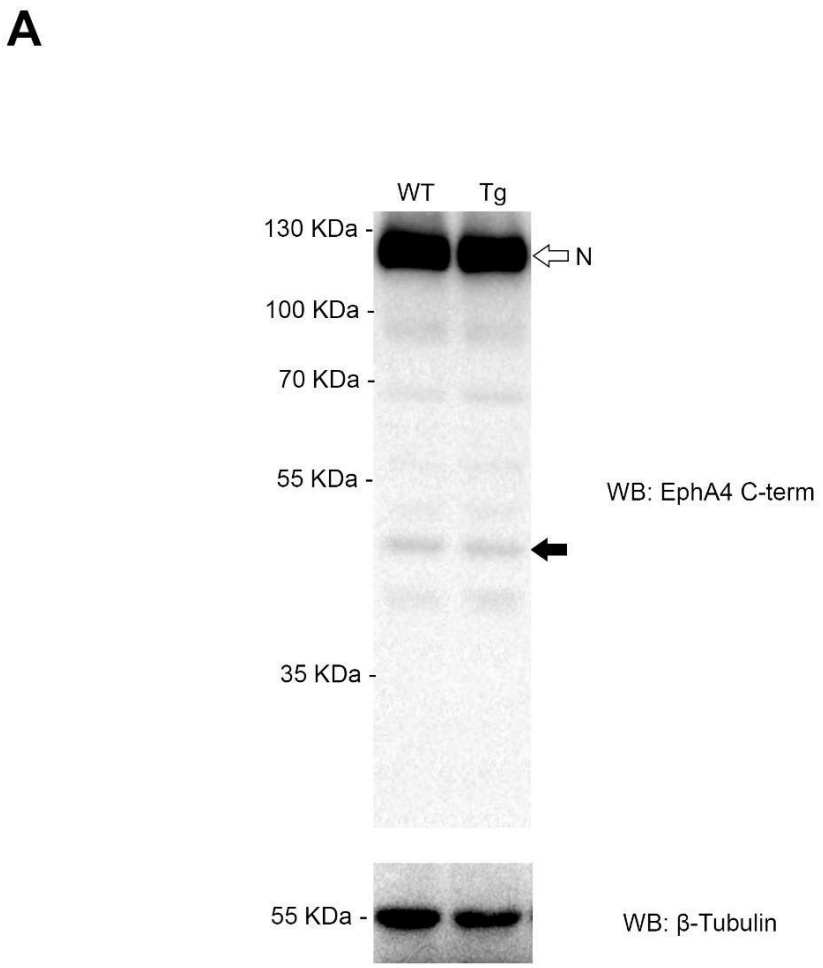


Figure 34. EphA4 cleavage is not increased in the Tg2010 tauopathy model of Alzheimer's disease. (WT = wild type; Tg = transgenic; N = native band; WB: Western blot). The figure shows protein levels of EphA4, measured by Western blot technique, of mouse hippocampus homogenates extracted from wild-type and transgenic mice. Main cleavage products are not affected by the transgene. This demonstrates that EphA4 does not change in this tauopathy model.

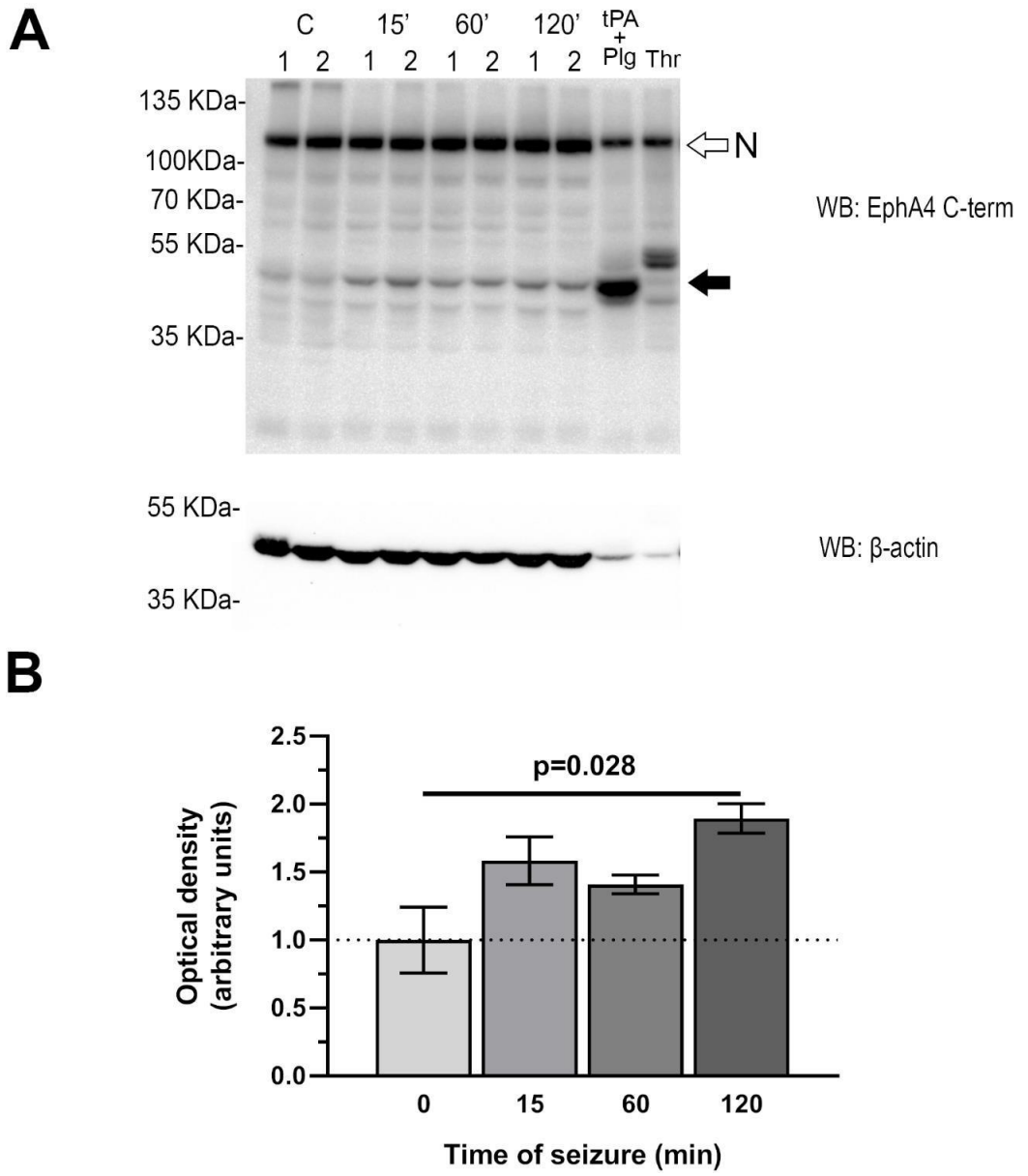


Figure 35. Pentylenetetrazol (PTZ) kindling model of epilepsy increases the cleavage of EphA4 after 120 min of epileptic seizures in the mouse hippocampus. (tPA = tissue plasminogen activator; Plg = plasminogen; Thr = thrombin; WB: Western blot). The figure shows protein levels of EphA4 from mouse hippocampus homogenates after a PTZ kindling protocol by using Western blot technique. Animals were treated at different time points with PTZ (6.4-8.6 mg/Kg) until convulsions were developed. A) Representative blot. It includes two lanes with induced cleavage with tPA + plasmin (tPA + Plg) and thrombin (Thr) as controls. B) Quantification of the main cleavage product of EphA4 (Black arrow on panel A). The treatment resulted in increased levels of the main cleavage product of EphA4, suggesting that the cleavage of EphA4 may be related to epileptic seizures and that this cleavage is likely to be plasmin-dependent. All data are presented as optical density (O.D.) mean ± SEM (n = 3). p values for Bonferroni's comparison test are included over the corresponding chart bars.

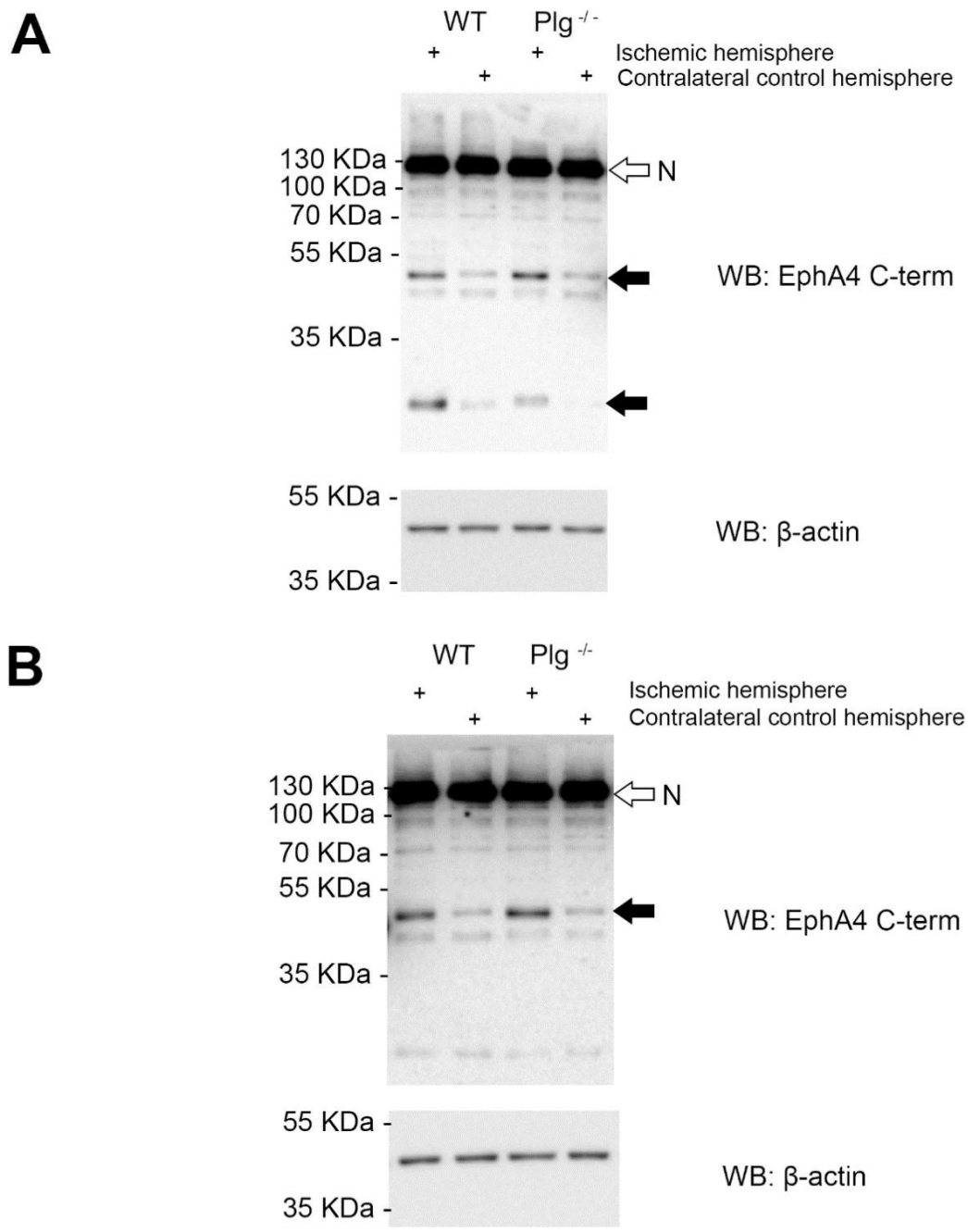


Figure 36. EphA4 cleavage is increased in the infarcted area of two mouse models of acute ischemic stroke. (WT = wild type; $Plg^{-/-}$ = plasminogen knockout; WB: Western blot). The figure shows representative protein blots of EphA4 from mouse hippocampus homogenates after the infarction provoked by a thromboembolic occlusion of a major cerebral artery. The occlusion was achieved by two methods:
A) a photochemically induced thrombosis (PIT) and
B) medial cerebral artery occlusion (MCAO). Different cleavage products are increased (black arrows) in the hemisphere affected by the ischemia in both models when compared with their contralateral controls while native EphA4 is unaffected. This demonstrates that EphA4 cleavage is increased in ischemic conditions, however, since plasminogen knockout animals also present an increased cleavage, this effect is not only attributable to plasmin cleavage.

DISCUSSION

Cleavage of EphA4 in Alzheimer model of tauopathy

Although tPA/plasmin cascade has been linked to AD in amyloidogenic models (Melchor and Strickland, 2005; Zhao and Pei, 2008), the particular studied model of tauopathy used in this research (rTg4510) did not show significant changes in EphA4 protein levels, protein cleavage or tPA/plasmin expression in hippocampal tissue samples analysed by Western blot (Figure 35). Different models of this disease and different stages of the development of the disease might elicit other outcomes.

Cleavage of EphA4 in epilepsy

Our results showed an increase in EphA4 cleavage after 2 h of the induction of PTZ seizures (Figure 36). This time frame is in agreement with previous works on similar conditions. Qian et al., (1993) demonstrated increased tPA mRNA levels in the rat hippocampus and cortex at 0.5-4 h following PTZ-induced seizures, as well as discharges evoked by single perforant path stimulation. Importantly, that study is the first one showing any relation between tPA/plasmin system and epilepsy; and, like in the current work, PTZ was used to induce seizures. After that, Salles and Strickland (2002) observed an increase in tPA mRNA and protein expression in the mouse hippocampus CA1 area following intra-hippocampal KA injection. This tPA induction was transient and the protein levels and activity were detectable after 2.5 h, peaking at 5-8 h. Some other experiments performed in rats set this time in as short as 1 h (Popa-Wagner et al., 2000). Therefore, the species, the type of compound that generates the seizures and its dose could influence the timing of tPA's expression and detection of its effect. Additional pieces of data show that tPA-deficient mice develop an excitotoxin-resistant phenotype. This means that higher doses of KA or PTZ are required to develop seizures when compared to control mice. Therefore, it would

be of interest to test the cleavage of EphA4 in tPA$^{-/-}$ upon KA and PTZ treatments to assess the role of these enzymes in these seizure models.

The observed increase in EphA4 cleavage (Figure 36) would suggest an increase in either plasmin(ogen) protein levels or plasmin activity. Plasminogen mRNA is found widespread in hippocampal neurons; however, the highest levels of this protein are typically found around the cell bodies of the pyramidal and granular layers of the hippocampus (Tsirka et al., 1997). Tsirka et al. performed intra-hippocampal injection of KA in plasminogen knockout animals to observe this enzyme's relation with seizures. This set of experiments were trying to reproduce the excitotoxin-resistant phenotype associated with tPA$^{-/-}$. KA injection produced an apparent growth of the protein levels in plasminogen-expressing hippocampal neurons (Tsirka et al., 1997), which would suggest that neuronal activity promotes the expression of the protease. Data obtained from the PTZ model in the current work does not show any increase in protein levels of plasmin(ogen) or tPA *per se* when measured by Western blot (data not shown). However, immunohistochemistry data reported in the literature shows disagreeing results (Tsirka et al., 1997; Salles and Strickland, 2002). A possible explanation for this phenomenon is that areas containing small changes in the amounts of plasmin(ogen) are constrained to specific subcellular spaces, and the relatively unspecific big volumes of tissue used in our experiments (homogenates of the whole hippocampus) would attenuate the detection of small increases in plasminogen protein expression. More precise dissection of the areas containing plasmin(ogen) could overcome this issue. Also, the intensification of bands correlating with EphA4 cleavage by plasmin (Figure 36) would suggest an increase in plasmin's activity or a decrease in the inhibition of the enzyme. And, because plasmin levels did not increase (data not shown), it would suggest that there is an increase in the protease activity rather than an increase in the protein levels. In addition, enzymes other than plasmin could be involved, so experiments using knockout models would be necessary to observe the overall role of tPA/plasmin in EphA4 cleavage in the context of PTZ-evoked seizures.

Interestingly, the overexpression of ephrin-A3 (a known EphA4 ligand) in astrocytes downregulates glutamate transporter levels, which is a known cause

of glutamate excitotoxicity and exacerbated PTZ-induced seizures. However, the necessity of EphA4 on this event was not assessed (Filosa et al., 2009). It would be interesting to study whether ephrin-A3 may have an influence in the development of PTZ-induced seizures through an EphA4-dependent mechanism.

Cleavage of EphA4 in models of ischemic stroke

Studies about proteomics concerning Eph/ephrin family in relation to different types of injuries or inflammation concentrates mainly on the fluctuations of both mRNA levels and the protein expression levels of the native form of the EphA4 receptor. However, little is known about the cleavage of these molecules, which may affect their signalling properties.

It has been argued that the cleavage of this family of proteins works as a reducer or terminator of their protein signalling via various proteases (mainly metalloproteases) (Hattori, Osterfield and Flanagan, 2000; Janes et al., 2005, 2009; Georgakopoulos et al., 2006; Tomita et al., 2006; Litterst et al., 2007; Inoue et al., 2009). Of them, only γ-secretase and caspase-3 have been shown to target EphA4 (Inoue et al., 2009). Most of the literature on Eph/ephrin cleavage focuses on the ligand (ephrin) cleavage and how it affects the relevant interacting receptor in forward/backward signalling. However, only four articles show some insight into the receptor's cleavage itself (i.e. Furne et al., 2009; Gatto, Morales, Kania, and Klein, 2014; Inoue et al., 2009 about EphA4, and Litterst et al., 2007 about EphB2).

In the case of the stroke models presented in the current thesis, there are two EphA4 fragments observed to be increased only in the ischemic hemisphere (Figure 37). A higher molecular weight fragment (~50 KDa) is produced in both of the studied models (MCAO and PIT). A smaller fragment of ~20 KDa is produced only in the PIT model, suggesting that the type of technique used to induce thrombosis affects the cleavage of EphA4. These C-terminal fragments (CTFs) are not explicitly produced by plasmin since plasminogen$^{-/-}$ animals also develop this cleavage fragment (Figure 37). These data suggest that a different

enzyme performing this cleavage might exist. As inferred by previous works, MMPs and γ-secretase are potentially-responsible enzymes for the generation of similar fragments. Therefore, EphA4 could act as their target in the models of stroke studied here, but the possibility that tPA is also producing the cleavage cannot be discarded either. Furthermore, pro-apoptotic proteases, such as the caspase family could be accountable for the production of the ~20 KDa fragment (Furne et al., 2009; Thundyil et al., 2013). In order to be able to precisely modulate the cleavage of EphA4 and other adhesion proteins and receptors, it is paramount to identify proteases responsible for this activity.

In this regard, Inoue et al. (2009) showed that EphA4 cleavage is affected by the inhibition of the γ-secretase (though an inhibitor called compound E) and it produces the accumulation of a ~50 kDa fragment corresponding to a CTF sub-product (Inoue et al., 2009). A different paper from Litterst et al. (2007) shows a similar product of cleavage, which is obtained from an unknown protease cleaving on the structure of EphB2. It is likely that these fragments are produced by a metalloprotease (MP) since MP inhibitors reduce this cleavage. Interestingly, these products are similar in size to the one I found to be produced by plasmin in the current manuscript (Figure 37). However, it is unlikely that the CTF found in our work is produced by a MP, because the generation of CTFs by MPs is produced after treating samples with the inhibitors for a minimum of 4 h and a maximum of 24 h. These times are much longer than the protease responsible for the generation of EphA4-CTF in MCAO and PIT models, which can cleave EphA4 in as short as 15 min. This suggests that the cleavage of EphA4 would be happening as a consequence of the activity of a different protease in these models.

A different study from Furne et al. (2009) identified EphA4 as a target for caspase-3, a protease related to cell apoptosis. However, the cleavage produces 19–23 kDa fragments, which could relate to the smaller EphA4 fragments observed in our PIT model but not to the ~50 KDa ones. Therefore, it could potentially mean that an apoptotic event involving EphA4 takes place after the PIT-induced ischemia, but this hypothesis would need to be validated.

What effects could this cleavage produce in the pathophysiology of stroke?

Previous studies have demonstrated that EphA4 is upregulated after traumatic brain injury in rodents and primates (Boyd, Bartlett and Lackmann, 2014). Accordingly, increased levels of the receptor have been found in brains of patients after traumatic brain injury (Frugier et al., 2012). In this line of research, other works suggest that interfering with EphA4 activation might have therapeutic effects after brain trauma. The most convincing case is a study in which EphA4 depletion (using EphA4$^{-/-}$ mice) enhances motor function after stroke (Lemmens et al., 2013). In other models of injury such as spinal cord injury, the delivery of EphA4 antagonists reduces glial scars and enhances functional recovery of injured animals as well as axon regeneration (Goldshmit et al., 2011). The data from the models used in the current thesis do not reveal any change in the overall levels of EphA4 in its native form in the hippocampus (Figure 37). Instead, I found that the levels of some of the EphA4 cleavage variants do increase. It has been argued that the cleavage of Eph/ephrin molecules leads to the termination of these molecules' signalling (Lackmann, 2010). Therefore, it would be expected that this is also the case in the models of stroke studied here and that the reduction of EphA4 cleavage signalling could potentially generate a beneficial effect after brain trauma.

CONCLUSIONS

The main focus of our laboratory are animal models in which stress and anxiety may play a crucial role; however, in this chapter, models of epilepsy, stroke and Alzheimer's disease were also explored.

There is no significant difference in the cleavage of EphA4 in the Alzheimer's rTg4510 model of tauopathy in protein samples extracted from whole hippocampi and analysed by Western blot technique.

With regard to epilepsy models, the results show an increase in the intensity of the main cleavage band of EphA4 after 2 h of the induction of PTZ seizures in mice when analysing hippocampi samples by Western blot technique. Further

analysis would need to be carried out to address the consequences of the fracture of EphA4 and the molecular pathway originating of this cleavage.

In a likewise manner, two models of stroke included in the current thesis (MCAO and PIT models), present an increase in EphA4 cleaved fragments in the ischemic hemisphere. This increased shedding of EphA4 is not produced by plasmin. However, the engagement of tPA into this process cannot be discarded. Although none of the cleaved variants of EphA4 has been related to any effects in the pathophysiology of stroke models, experiments on EphA4$^{-/-}$ rodents and patients would open the possibility for further investigation of the cleavage mechanisms of EphA4 in stroke.

FUTURE WORKS

The unavailability plasmin-deficient and tPA-deficient mice made impossible to assess whether EphA4 cleavage observed in models of epilepsy or stroke are plasmin- or tPA-dependent. Therefore, a crucial next step would be to determine the requirement of these proteases for the cleavage of EphA4 and to explore if any other proteases may be involved in the process.

The use of tPA$^{-/-}$ and plasminogen$^{-/-}$ mice upon KA and PTZ treatments would assess the role of these enzymes in the context of chemically-evoked seizures and would allow to confirm whether the cleavage of EphA4 observed in the model depends on any of these proteases. Additionally, since the overexpression of ephrin-A3 (a known EphA4 ligand) in astrocytes downregulates glutamate transporter levels and this affects glutamate excitotoxicity and PTZ seizures, it would be interesting to study the requirement of EphA4 in this process.

Similarly, the use of tPA$^{-/-}$ mice would confirm whether the observed EphA4 cleavage in the PIT and MCAO models is tPA-dependent. Furthermore, pro-apoptotic proteases, such as the caspase family produce low-molecular-weight fragments of EphA4 that resemble the ones found in the models of stroke studied here. It would be of interest to investigate the influence of these intracellular

proteases in the process of EphA4 cleavage after ischemia.

Alternatively, the fragments produced in all the models described here could be analysed by mass spectrometry to assess the structural domain affected by the different cleavages. These results would shed some light on what protease could be producing the fragments based on their sequence specificity.

CONCLUSIONS OF THE STUDY

Anxiety disorders are, as a group, the most prevalent class of mental disorders worldwide and the sixth leading cause of disability in high-income and low-income countries (Craske et al., 2016). The neurobiological mechanisms that regulate anxiety and its disorders in the brain are still not well understood. Previous members of our laboratory discovered a new proteolytic reaction involving tPA/plasmin proteases and EphA4 receptor, and that other members of the Eph family of receptors may be able to modify anxiety-like behaviours (Attwood et al., 2011, 2016). Based on these facts, in the current work, the proposed hypothesis is that molecular mechanisms involving these mentioned extracellular proteases and the EphA4 membrane receptor following stress can lead to alternative regulations of anxiety-like behaviours in mice.

In this context, tissue-plasminogen activator (tPA)/plasmin proteolytic system has been linked to the expression of anxiety-related behaviours in rodents, as well as mechanisms that underlie different forms of synaptic plasticity (Melchor and Strickland, 2005). However, the specific substrates and downstream signalling pathways behind these effects are still an open field of study. Here, I confirmed that a type of erythropoietin-producing human hepatocellular (Eph) receptor, namely EphA4, is a target for this cascade and mediates stress-induced morphological plasticity (Chapter 4), molecular interactions (Chapter 5) and anxiety-like behaviours in the mouse amygdala (Chapter 6).

Chapter 3 shows that both, tPA and plasmin, are able to cleave EphA4 *ex vivo* (homogenised brain tissue from the amygdalae and hippocampi of adult mice) and *in vitro*. However, plasmin is more efficient than tPA in doing so. tPA and plasmin produce proteolysis at five cleavage sites within the extracellular motifs of EphA4, and, interestingly, three of these five cleavage points are shared by both enzymes. The most effectively cleaved point is located at one of the fibronectin type III domains, which is known for its interactions with other proteins.

tPA and plasmin highly co-localise with EphA4 in the central amygdala (Chapter 3), which is an essential area for the expression of anxiety and memory formation related to events of negative valence (Tovote et al., 2015). *In vivo* experiments

on tPA/plasmin cascade using the restraint stress mouse model of anxiety and the Western blot technique show a strong EphA4 cleavage after 1 h of stress in central amygdala homogenates. Additionally, experiments on $tPA^{-/-}$ and $plasminogen^{-/-}$ mice suggest that this effect is tPA- and plasmin-dependent. This cleavage is also consistent with tPA/plasmin activity during the stress response (Chapter 6).

Inhibitory interneurons of the central amygdala can regulate anxiety by adjusting the activity of their downstream output target cells. Specifically, interneurons of the lateral division of the central amygdala (CeL) expressing protein kinase C - delta ($PKC\delta^{+}$) are critical for this function (Ciocchi et al., 2010; Habuensak et al., 2010). Nonetheless, the molecular underpinnings of this inhibitory control at the synaptic level are not well understood. Here, we show that $PKC\delta^{+}$ (mainly *tPA*+) cells are able to interact with downstream GABAergic synapses. Particularly, the cleavage in EphA4 produced by (tPA)/plasmin proteolytic cascade triggers the dissociation of EphA4 from gephyrin (a key scaffold protein anchoring GABA-receptors to the GABAergic synapse), which would potentially lead to the regulation of the molecular composition of GABAergic synapses (Chapters 3 and 5).

GABAergic circuits of the central amygdala are necessary for some types of synaptic plasticity associated with aversive events and not just a passive node connecting the basolateral amygdala with downstream structures (Wolff et al., 2014; Botta et al., 2015; Tasan et al., 2011; Gilpin, Herman and Roberto, 2015). It is known from the relevant literature that tPA, plasmin, EphA4 and their effectors regulate the morphology of dendritic spines, a feature that reflects spine plasticity and gives shape to postsynaptic terminals (Summarised in the introduction of Chapter 4). In our work, EphA4 cleavage events (represented by cleavage resistance or truncation that mimics cleavage) in murine neuronal primary cultures shows no differences in dendritic arborisation or spine density. However, full-length EphA4 variants (wild-type and cleavage-resistant mutant [R516Q]) induce more mature (wider) spine heads, whereas EphA4 truncated variant forms spines with elongated shapes, which commonly correlate with young, newly-formed synaptic structures (Chapter 4).

Stress-induced anxiety-like behaviour (measured by the elevated plus maze) is attenuated by lentiviral overexpression of the cleavage-resistant and native forms of EphA4 in the central amygdala and enhanced by the overexpression of the predominant form of cleaved EphA4 produced by tPA/plasmin in the same area (Chapter 6).

Additional experiments in the present work show that variations and interactions of EphA4 could affect the outcome of neurological conditions other than anxiety disorders. These include two models of stroke (photochemically induced thrombosis [PIT] and the middle cerebral artery occlusion [MCAO]) and a model of epilepsy (pentylenetetrazol [PTZ] model) (Chapter 7).

Taken together, the findings presented in this work identify the acute, stress-related cleavage of EphA4 by the tPA/plasmin cascade in the mouse central amygdala as a significant event in the development of anxiety-like behaviours.

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