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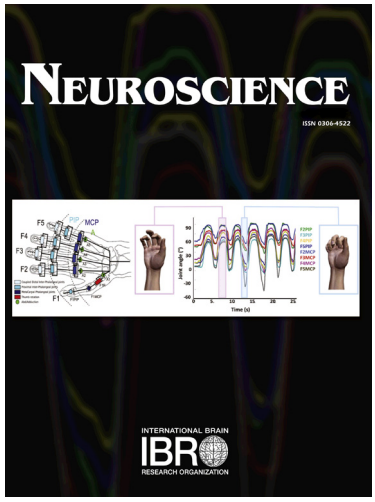

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Oleate induces K_{ATP} channel-dependent hyperpolarisation in mouse hypothalamic glucose-excited neurones without altering cellular energy charge.

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Short Title: Oleate inhibits glucose-excited hypothalamic neurones

Key words: glucose sensing, K_{ATP}, oleate, hypothalamus, mitochondria, fatty acid oxidation

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Abbreviations

ACC, acetyl-CoA carboxylase; AMPK, adenosine 5'-monophophate-activated protein kinase; AMP-PNP, 5'-adenylylimidodiphosphate; ARC, arcuate nucleus; CPT1, carnitine palmitolytransferase-1; CSF, cerebrospinal fluid; GE, glucose-excited; GI, glucose-inhibited; KATP, ATP-sensitive potassium channel; oleate; POMC, proopiomelanocortin; VMN, ventromedial nucleus; UCP, uncoupling protein

Abstract:

The unsaturated fatty acid, oleate exhibits anorexigenic properties reducing food intake and hepatic glucose output. However, its mechanism of action in the hypothalamus has not been fully determined. This study investigated the effects of oleate and glucose on GT1-7 mouse hypothalamic cells (a model of glucose-excited (GE) neurones) and mouse arcuate nucleus (ARC) neurones. Whole-cell and perforated patch-clamp recordings, immunoblotting and cell energy status measures were used to investigate oleate and glucose sensing properties of mouse hypothalamic neurones. Oleate or lowered glucose concentration caused hyperpolarisation and inhibition of firing of GT1-7 cells by the activation of ATP-sensitive K^+ channels (K_{ATP}). This effect of oleate was not dependent on fatty acid oxidation or raised AMP-activated protein kinase activity or prevented by the presence of the UCP2 inhibitor genipin. Oleate did not alter intracellular calcium, indicating that CD36/fatty acid translocase may not play a role. However, oleate activation of K_{ATP} may require ATP metabolism. The short-chain fatty acid octanoate was unable to replicate the actions of oleate on GT1-7 cells. Although oleate decreased GT1-7 cell mitochondrial membrane potential there was no change in total cellular ATP or ATP/ADP ratios. Perforated patch and whole-cell recordings from mouse hypothalamic slices demonstrated that oleate hyperpolarised a subpopulation of ARC GE neurones by K_{ATP} activation. Additionally, in a separate small population of ARC neurones, oleate application or lowered glucose concentration caused membrane depolarisation. In conclusion, oleate induces K_{ATP}-dependent hyperpolarisation and inhibition of firing of a subgroup of GE hypothalamic neurones without altering cellular energy charge.

Introduction:

The hypothalamus is critical for the continuous regulation of whole-body glucose, lipid and energy homeostasis. To perform this function, various hypothalamic nuclei (e.g. arcuate (ARC), ventromedial (VMN), lateral hypothalamic area and paraventricular) contain neuropeptide-expressing neurones that monitor circulating nutrients and hormone levels [1]. Scattered throughout these nuclei are subpopulations of neurones that sense changes in glucose levels, resulting in altered neuronal firing and modified energy homeostasis [2]. There are two main subtypes of glucose sensing hypothalamic neurones that contribute to these homeostatic mechanisms: neurones excited (glucose-excited (GE)) and inhibited (glucose-inhibited (GI)) by increased levels of glucose. GE neurones utilise ATP-sensitive potassium (K_{ATP}) channels (in a manner similar to pancreatic beta cells [3]) to modulate their electrical activity in response to changes in extracellular glucose concentration [4-6]. It is presently unclear which ion transport mechanism is responsible for transducing changes in glucose concentration to modify electrical activity in GI neurones [7]. These glucose sensing neurones, in particular GE neurones, play important roles in the feeding response to glucoprivation (as suppression of glucokinase (GK) diminishes the glucoprivic stimulation of feeding [8]), and liver glucose production [9] and have been strongly implicated in the detection of hypoglycaemia and subsequent generation of counterregulatory responses [10]. For example, loss of the K_{ATP} channel subunit, $K_{IR}6.2$, causes near complete suppression of glucagon responses to hypoglycaemia, which is driven by the loss of K_{ATP} on neural cells [11].

Similarly, energy status (i.e. lipid level) is also communicated continuously to the hypothalamus. This is performed, at least in part, by hypothalamic neurones responding to changes in the concentrations of circulating hormones (e.g. leptin and insulin), the levels of which correlate with adipose tissue depot size. It is this latter communication system that is considered to become faulty in obesity [12]. However, circulating lipids, such as long-chain fatty acids, have also been demonstrated to directly act on hypothalamic centres to modulate feeding and hepatic glucose output [13, 14]. Circulating levels can be acutely elevated, such as during fasting or hypoglycaemia, where lipolysis is elevated [15], therefore it is plausible that some neurons have a capacity to detect both reduced glucose and elevated lipid levels. Indeed the long-chain

fatty acid, oleate alters ARC neurone neuropeptide expression, electrical activity and within glucosensing neurons, can alter intracellular calcium signalling via CD36/fatty acid translocase [16, 17, 14, 18]. Furthermore, long-chain fatty acids are activators of K_{ATP} channels in pancreatic beta cells [19] and hypothalamic-delivered oleate suppresses hepatic glucose production in a K_{ATP}-dependent manner [14]. A previous study suggested that oleate alters the excitability of ARC neurones in a glucose-concentration dependent manner suggesting interaction at the level of cellular nutrient metabolism [18]. In addition, the central effects of fatty acids on glucose homeostasis have been ascribed to neuronal fatty acid metabolism [20, 21].

Alternatively, fatty acids can regulate the activity of mitochondrial uncoupling proteins (UCP) [22], including the neuronal enriched UCP isoforms, UCP4 and UCP5 [23]. In the pancreatic beta cell, UCP2 has been implicated in regulating glucose sensing behaviour [24, 9, 25]. Indeed, oleate modulates the expression of UCP2 and alters glucose-dependent insulin secretion in pancreatic beta cells and beta cell lines [26, 27]. Importantly, UCP2 has been shown to regulate the glucose-sensing behaviour of GE neurones and alter whole-body glucose homeostasis [9]. Consequently, in order to explore the mechanisms by which oleate modifies neuronal excitability we have examined the interplay between nutrient-dependent pathways and oleate responses in mouse GE-type neurones.

Methods:

Cell Culture

Immortalised mouse hypothalamic GnRH secreting GT1-7 cells (Pamela Mellon, San Diego, California, USA [13]) were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Gillingham, UK)) with 10% fetal bovine serum (PAA Laboratories, Yeovil, UK & Hyclone, Pasching, Austria) as described [28].

Immunoblotting

GT1-7 cells, seeded in 6-well dishes, were serum-starved for three hours with DMEM replaced by saline and then treated with nutrients (0 - 100 μM oleate or 2.5/0.1 mM glucose) for various times as described in the results section. We used oleate concentrations in the low micromolar (10-100 μM) as plasma concentrations of total free fatty acids have been estimated within the range of 350-500 μM under normal circumstances [29-31] and, in type 1 diabetes, can reach levels of more than 1200 μM [32]. In human CSF levels of oleate have been reported to be >10 μM in non-diabetic populations [33], and may be expected to reach much higher levels in type 1 diabetic patients. In rats, brain glucose levels have been estimated at ~2.5 and ~0.1 mM during euglycaemia and hypoglycaemia, respectively [34].

Protein isolation and immunoblotting procedures were as described previously [28]. Briefly, protein lysates were subjected to SDS-PAGE, electrotransferred to nitrocellulose membranes, and probed with primary antibodies against p-AMPKα (Thr172; 1:1000), p-ACC (Ser79; 1:1000), actin (1:5000), UCP4 (1:1000) and UCP5 (1:1000). All antibodies were obtained from Cell Signalling Technology Inc. (New England Biolabs, Hitchin, UK) except UCP4 and UCP5, which were obtained from Acris Antibodies (Herford, Germany). Proteins were detected with horseradish peroxidase-conjugated Goat anti-Rabbit IgG and immunoreactive proteins identified by chemiluminescence. Gel protein bands were quantified by densitometry, where total density was determined with respect to constant area, background subtracted and average relative band density calculated.

Hypothalamic Slice Preparation

All animal procedures conformed to the UK Animals Scientific Procedures Act (1986) and were approved by the University of Dundee institutional ethical review committee. Wild-type male C57Bl/6 mice (6-20 weeks old) were killed by cervical dislocation and the brains rapidly removed and submerged in an ice cold slicing solution as described previously [35]. Briefly, hypothalamic coronal slices containing the ARC (350 μm) were prepared using a Vibratome (St Louis, MO, USA) and stored at room temperature (22-25˚C) in an external solution containing (in mM): NaCl 125, KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 25, $CaCl_2$ 2, $MgCl_2$ 1, D-Glucose 10, D-Mannitol 15, ascorbate 1 and pyruvate 3, equilibrated with 95% O_2, 5% CO_2, pH 7.4. Immediately before use, brain slices were transferred to the recording chamber of an upright Zeiss Axioskop-2 FS plus microscope and continuously perfused with a modified external solution (containing 0.5 mM $CaCl_2$ and 2.5 mM $MgCl_2$, no ascorbate and pyruvate) at a constant flow rate of 5-10 ml min^{-1} and bath temperature of 33˚C.

Electrophysiology

GT1-7 cells were visualised by phase contrast and individual neurones of mouse hypothalamic slices by differential interference contrast optics. Whole-cell patch-clamp recordings were performed using borosilicate pipettes (4-8 MΩ) filled with an internal solution containing (mM): K-gluconate 130, KCl 10, EGTA 0.5, HEPES 10, NaCl 1, $MgCl_2$ 3, $CaCl_2$ 0.28, Na_2-ATP 3, Tris-GTP 0.3, and phosphocreatine 14; pH 7.2 for ARC neurones and with KCl 140, EGTA 10, HEPES 10, $MgCl_2$ 5, $CaCl_2$ 3.8, and Na_2-ATP 3; pH 7.2 for GT1-7 cells. Hypothalamic neurone recordings were made in external solution at 33˚C and GT1-7 cell recordings at room temperature (22-25˚C) in saline containing (in mM): NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, glucose 2.5 (pH 7.4). For perforated patch-clamp recordings 30-35 μg/ml amphotericin B and/or 2.5 mg/ml gramicidin was included in the internal solution in the absence of Na_2-ATP. As previously described, voltage and current commands were manually or externally driven using pCLAMP 9.2 software and injected into neurones via the patch-clamp amplifier (Axopatch 200B; Molecular Devices, Sunnyvale CA, USA). In the whole-cell current-clamp configuration, hyperpolarising current pulses (5-20 pA amplitude at a frequency of 0.05 Hz) were used to monitor changes in input resistance. For voltage-clamp recordings the membrane

potential was held at -70 mV and voltage steps (20 mV increments, 200 ms duration) applied over the voltage range of -160 to -20 or +80 mV. Voltage-clamp protocols were carried out immediately after whole-cell formation, prior to and during exposure to drugs and/or oleate or altered glucose concentration (following achievement of maximal responses). At least 10 minutes of stable control data were recorded before the application of drugs or oleate, which were added to the external/saline solution and applied to slices or GT1-7 cells via a superfusion system. In some slice experiments oleate was applied locally via pressure ejection using a broken-tipped pipette positioned above the recording neurone.

Nucleotide measurements

GT1-7 cells were seeded onto black-walled 96 well plates at 1 x 10^4 cells per well. Total ATP measurements were made using ATPLite assay kit (Perkin-Elmer, Seer Green, UK), as per the manufacturer's protocol, with minor modifications. Briefly, cells were lysed with shaking, using 40 µl of mammalian cell lysis buffer for 10 minutes at 700 rpm, followed by the addition of 40 µl of ATP substrate solution. For ATP/ADP ratios, cells were plated as for total ATP measurements. Ratios were determined using an ATP/ADP ratio kit (Sigma Aldrich, Gillingham, UK) as per the manufacturer's instructions.

Mitochondrial Membrane Potential

GT1-7 cells were seeded on to glass-bottom dishes (In Vitro Scientific, CA, USA) 24 hours prior to study. JC-1 was used as per the manufacturer's protocol with minor modifications. Briefly, cells were pre-incubated with 2µg/ml JC-1 for 1 hour at 37°C in a 95% O_2/5% CO_2 humidified incubator before washing twice with saline containing 10 mM glucose and studied immediately. Images were captured on a Leica SP5 laser scanning confocal microscope and fluorescence intensity analysed using the standard Leica LAS AF software and GraphPad Prism 5 for statistical analyses.

Non-ratiometric calcium imaging

GT1-7 cells were seeding into 96 well plates at 2x10^5 cells per well. Cells were loaded with Fluo4 Direct (ThermoFisher, UK) for 1 hour at 37 degrees. Fluorescence was monitored using a Pheratar FS multifunction plate reader (BMG Labtech), with oleate added by injection and cells maintained at 37 degrees during the imaging protocol.

Fluorescence is presented as relative fluorescent units (RFU), with fluorescence at the zero point being normalised to 1.

Chemicals

Amphotericin B, AMP-PNP, FA-free bovine serum albumin, diazoxide, etomoxir, gramicidin, malonyl-CoA, octanoic acid, oleic acid, and tolbutamide were purchased from Sigma-Aldrich. Compound C was obtained from Merck Chemicals Ltd (Nottingham, UK). Genipin was purchased from Wako Chemicals (Eastleigh, UK) and JC-1 was obtained from Thermo Fisher Scientific (Renfrew, UK). Oleate was prepared as a 10 mM stock solution containing 5% NEFA-free BSA.

Statistics

In slice recordings, hypothalamic neurones responding to drugs, oleate or altered glucose concentration were distinguished from non-responding neurones based on the criterion that the change in membrane potential (ΔVm) induced by the challenge was ± three times the standard deviation of the mean membrane potential prior to the challenge. Results are expressed as the mean ± S.E.M. of the defined responses, with the number of cells studied. Statistical significance was determined by Student's *t*-test or ANOVA followed by Bonferroni's *post hoc* test where appropriate. Changes in protein phosphorylation in Western blot experiments were analysed using a one-sample *t*-test for multiple comparisons with respect to control. A *P* value of less than 0.05 was considered statistically significant.

Results:

Mouse hypothalamic GT1-7 cells exhibit GE behaviour and are oleate-sensitive

Attempting to study the mechanism(s) by which oleate alters ARC neurone excitability is difficult when recording from unidentified neurones in a hypothalamic slice. Therefore, we utilised the mouse hypothalamic cell line, GT1-7, which exhibits GE-type properties. We have previously demonstrated that this neuronal cell line shows graded electrical responses over a physiological range of glucose concentrations and that these responses can be modulated by pharmacological manipulation of the classical components of glucose-sensing, namely glucokinase, adenosine 5'-monophosphate-activated protein kinase (AMPK) and K_{ATP} [36]. They therefore represent an excellent model for studying the mechanisms through which oleate may alter neurone excitability.

In GT1-7 cells, a hypoglycaemic challenge (0.1 mM glucose) of 30 minutes resulted in increased AMPK phosphorylation (p-AMPK) (Fig. 1A). This was accompanied by increased acetyl-CoA carboxylase (ACC) phosphorylation (p-ACC), a key substrate of AMPK, indicating increased AMPK activity. As AMPK has been implicated as a key component of cellular glucose sensing in hypothalamic neurones [37], GT1-7 neurones [36] and pancreatic beta cells [38], we next examined whether oleate also alters AMPK activity. Oleate (10-100 μM; Fig. 1B) increased levels of AMPK and ACC phosphorylation with a minimum oleate concentration to elicit this response of > 50 μM (Fig. 1B). Thus, oleate mimics the effects of lowered glucose levels on p-AMPK/p-ACC levels in GT1-7 cells over a similar time course. Consequently, we next determined whether oleate also induced cell hyperpolarisation and K_{ATP} activation in GT1-7 cells as demonstrated previously for lowered glucose concentrations [39].

Hence we performed perforated patch recordings (which minimises disruption of normal cell glucose metabolism) from GT1-7 cells to monitor changes in electrical activity and firing frequency. Application of oleate (100 μM) to GT1-7 cells induced hyperpolarisation and inhibition of firing (Fig. 1C). Baseline membrane potential was -54.3 ± 3.1 mV, which hyperpolarised to -70.0 ± 1.5 mV following approximately 15 minutes of oleate treatment (n = 3, $P < 0.05$), and reversed by application of tolbutamide (200μM) suggesting activation of K_{ATP} channels. The lowest concentration at which oleate induced a detectable hyperpolarisation of GT1-7 cells was ~50 μM (data not

shown). To determine the contribution of K_{ATP}, we utilised whole-cell voltage-clamp, intermittent with current-clamp recordings to examine the effect of oleate on K_{ATP} currents following attainment of the maximal hyperpolarising response. Firstly, oleate hyperpolarised GT1-7 neurones (Fig. 1D upper traces) with a similar time course to that observed in perforated patch recordings. Secondly, this hyperpolarisation was accompanied by an increased total current (Fig. 1D lower traces), a shift in the reversal potential from –29.2 ± 6.5 mV to -58.9 ± 4.5 mV (n = 6; $P < 0.01$; Fig. 1E) and an increased slope conductance (Fig. 1F). The increase in conductance elicited by oleate was returned to control levels by concomitant application of 200 μM tolbutamide (n = 3; Fig. 1F). Additionally, GT1-7 cells under voltage-clamp exposed to 100 μM oleate and subsequently challenged by addition of the direct K_{ATP} channel opener diazoxide (250 μM) in the continued presence of oleate, demonstrated further cell hyperpolarisation (Fig. 1D), an additional negative shift of the reversal potential (to -76.0 ± 2.2 mV; n = 6; $P < 0.01$; Fig. 1E) with an enlarged current associated with a greater slope conductance (Fig. 1D, F). These actions of oleate are consistent with submaximal activation of K_{ATP}. In order to assess whether the increase in AMPK activity drives the oleate-mediated hyperpolarisation and K_{ATP} activation, we determined the effect of oleate on whole-cell recordings with the AMPK inhibitor [40], compound C (20 μM) in the pipette solution. The presence of compound C resulted in a significantly more depolarised membrane potential for GT1-7 cells, in comparison to untreated control cells, but did not prevent oleate from hyperpolarising the cells in a tolbutamide-dependent manner (Fig. 1G). Consequently, these data suggest that, in contrast to lowered glucose level [35], elevated AMPK activity is not required for oleate-mediated activation of K_{ATP} channels and cell hyperpolarisation.

GT1-7 cell response to OA is independent of fatty acid oxidation

It has previously been reported that oleate induces depolarisation and excitation of mouse ARC proopiomelanocortin (POMC) neurones via mitochondrial β-oxidation, raised ATP concentration and subsequent inactivation of K_{ATP} channel activity [41]. As GT-7 cells respond to oleate by activation of K_{ATP} and cell hyperpolarisation such a mechanism was unlikely to also explain this outcome. Alternatively, increased fatty acid oxidation could raise reactive oxygen species levels [42-44] previously implicated in pathways leading to neuronal K_{ATP} activation [45]. Thus in order to examine the role of

fatty acid metabolism via β-oxidation we prevented the delivery of oleate to mitochondria by inhibition of carnitine palmitoyltransferase-1 (CPT1). Whole-cell recordings were used to deliver directly to the interior of the cell (drug present in patch pipette solution), either malonyl-CoA (50 μM) an endogenous CPT1 inhibitor [46] or etoxomir (100 μM) an irreversible CPT1 inhibitor [47]. The presence of malonyl-CoA did not significantly alter the membrane potential of GT1-7 cells (–43.9 ± 2.3 mV; n = 10; P > 0.05 vs untreated controls), and subsequent challenge with oleate resulted in membrane potential hyperpolarisation and increased K_{ATP} conductance (Fig. 2A-C). The actions of oleate in the presence of malonyl-CoA were similar to that of control experiments (above), however, although the initial (5-10 minute) response to oleate in the presence of malonyl-CoA was indistinguishable from control, we did note that the oleate response waned with time and by 20 minutes was reduced by > 50% (Fig. 2A-C). This outcome could indicate a delayed reduction in fatty acid oxidation by malonyl-CoA, mediated perhaps by diffusion limitations. Thus, in separate experiments, we examined the effect of etomoxir (applied via pipette solution with a dialysis time of at least 20 minutes prior to oleate challenge) and showed that this drug had no effect *per se* on the resting membrane potential of GT1-7 cells and did not prevent oleate from causing cell hyperpolarisation (Fig. 2D). Consequently, it appears unlikely that β-oxidation of oleate is required to elicit the inhibition of GT1-7 cell electrical activity. We next examined whether the short-chain fatty acid octanoate (C8), which does not require CPT1-dependent transport into the mitochondria for oxidation, could mimic the hyperpolarising effect of oleate on GT1-7 cells. Surprisingly, addition of octanoate (50 μM) to GT1-7 cells caused a significant depolarisation, which was reversible on washout of the fatty acid (Fig. 2E). However, the presence of this short-chain fatty acid did not prevent oleate from hyperpolarising GT1-7 cells in a tolbutamide-dependent manner (Fig. 2F). These results indicate that GT1-7 cells respond by different effector mechanisms to short- and long-chain fatty acids. In calcium imaging studies, we found that oleate treatment did not alter intracellular calcium (Fig. 2H) suggesting that CD36 is not involved in this response.

Oleate hyperpolarisation of GT1-7 cells may be dependent on ATP metabolism

Previously it has been suggested that oleate may inhibit ATP-dependent gating of an acetylcholine-modulated potassium channel [48]. As K_{ATP} channel activity is controlled

to a large extent by the ATP/ADP ratio in cells, with ATP inhibiting and ADP activating K_{ATP} activity, respectively [49], a simple explanation for the oleate-mediated hyperpolarisation could be reduced ATP-dependent inhibition of channel activity. To examine this possibility, we first decided to replace ATP with the non-hydrolysable ATP analogue, 5'-adenylylimidodiphosphate (AMP-PNP; 3 mM) in the pipette solution. The mean GT1-7 cell resting membrane potential was significantly depolarised by the presence of AMP-PNP (Fig. 2G), compared to control experiments in which ATP was allowed to wash into the cell interior (compare with Fig. 2A). Furthermore, neither oleate (up to 200 μM) nor diazoxide (250 μM) application to cells dialysed with AMP-PNP resulted in hyperpolarisation of the membrane potential (Fig. 2G), conflicting with the idea that oleate may act to displace ATP from its inhibitory site on K_{ATP}. However, these data suggest the requirement for ATP hydrolysis for K_{ATP} activation, as previously demonstrated for diazoxide in beta cells [50].

Oleate reduced JC-1 mitochondrial membrane potential fluorescence without altering cellular energy charge

Previous studies have indicated an important role for uncoupling protein-2 (UCP2) as a regulator of K_{ATP} channels in glucose-sensing neurones [9], and oleate has been demonstrated to increase levels of UCP2 in beta cells [26]. Increasing cellular UCP2 levels and activity would be expected to diminish mitochondrial membrane potential and respiration, via uncoupling, and so reduce ATP levels, which could provide an explanation for oleate-mediated activation of K_{ATP} in these neurones. We examined this possibility by inhibiting UCP2 activity pharmacologically with genipin [51], which has previously been shown to reverse low glucose-mediated hyperpolarisation of pancreatic beta cells, GE-type hypothalamic neurones and GT1-7 cells [39, 38, 9]. In contrast we found that genipin was unable to reverse, or prevent, oleate from hyperpolarising GT1-7 neurones (Fig. 3A, B) suggesting that UCP2 activity is not mediating oleate action on these cells. However, on screening GT1-7 cells for additional UCP isoforms we found that they also express UCP4 and UCP5, although their protein levels were not altered by oleate treatment (Fig. 3C). Consequently, to explore further whether uncoupling by oleate was a possible mechanism we assessed GT1-7 mitochondrial membrane potential using the membrane permeant mitochondrial dye, JC-1. Oleate significantly decreased JC-1 fluorescence indicating depolarisation of the mitochondrial membrane potential

(Fig. 3D) in a time course that mimics oleate-mediated changes in membrane potential and K_{ATP} current in these cells. To determine whether the oleate-induced mitochondrial depolarisation reduced cellular energy charge, we measured both total cellular ATP and the ATP/ADP ratio. Neither measure was significantly altered by exposure to increasing concentrations of oleate (Fig. 3E, F), further indicating that the activation of K_{ATP} channels by oleate is unlikely to be caused by reduced ATP-mediated inhibition.

ARC neurones exhibit oleate and glucose sensitivity

We then sought to translate our findings in GT1-7 cells to acute brain slice preparations from mice. First we performed perforated patch recordings on unidentified ARC neurones using a physiological extracellular glucose concentration (2 mM) [52, 53], similar to the recording conditions for the GT1-7 cells. We used hypoglycaemic challenge (0.1 mM glucose) and subsequent changes in membrane potential and firing rates to identify GE- and GI-type neurones, prior to challenge with oleate. Thus, when GE ARC neurones were challenged with 0.1 mM glucose, they responded, by hyperpolarisation (from a Vm of –46.6 ± 1.2 mV to –53.8 ± 1.5 mV (n = 20); $P < 0.01$), which was reversible on return to 2 mM glucose solution (Fig. 4A). Subsequent addition of 100 μM oleate resulted in hyperpolarisation (ΔVm = –7.0 ± 1.1 mV; $P < 0.01$) and cessation of firing in 6 out of 13 GE neurones tested, with concomitant application of tolbutamide (200 μM) reversing the hyperpolarisation caused by the presence of oleate. The remaining GE neurones were unaffected by oleate (data not shown). Interestingly, in ARC neurones that responded, reversibly, to 0.1 mM glucose by depolarisation (from a Vm of –49.3 ± 0.8 mV to –38.9 ± 1.7 mV (n = 13; P < 0.01)) and were thus deemed GI-type, subsequent application of 100 μM oleate exhibited depolarisation (ΔVm = +6.4 ± 1.2 mV) in 4 out of 7 GI neurones tested (Fig. 4B), with the remaining GI neurones unaffected (data not shown). Neurones that displayed no change in membrane potential or firing rate to 0.1 mM glucose challenge were similarly electrically unresponsive to oleate (n = 5; data not shown). Consequently, subpopulations of glucose sensing GE and GI ARC neurones respond to oleate, with the change in electrical excitability mirroring the effect of hypoglycaemic levels of glucose.

As performed for GT1-7 cells, we also tested the effect of oleate on the electrical activity of mouse unidentified ARC neurones using the whole-cell current-clamp recording

configuration. Under these conditions the mean resting membrane potential for ARC neurones was –52.9 ± 0.5 mV (n = 51), similar to that reported previously for this recording mode in hypothalamic neurones [37, 54, 55]. Bath application or pressure ejection of oleate (100 μM) resulted in hyperpolarisation (Fig. 4C), with a mean ΔVm of -6.9 ± 0.6 mV (n = 32; $P < 0.001$) and inhibition of firing from 4.1 ± 0.4 Hz to 1.6 ± 0.4 Hz ($P < 0.001$). This inhibitory response was accompanied by a decrease in the mean input resistance from 2.13 ± 0.15 GΩ to 1.87 ± 0.16 GΩ ($P < 0.05$), indicative of an increased cell conductance. Application of tolbutamide (200 μM) in the continued presence of oleate, reversed the hyperpolarisation and inhibition of firing (Fig. 4C; n = 4; $P < 0.01$). In addition, naïve ARC neurones that responded with a small depolarisation (ΔVm = +2.8 ± 1.1 mV; n = 15; $P < 0.05$) and increased firing to the application of 200 μM tolbutamide (indicating the presence of active K_{ATP} channels and so likely to be GE-type) were shown to be unaffected by addition of 100 μM oleate in the continued presence of tolbutamide (Fig. 4D). These data are in general agreement with the GT1-7 findings and indicate that oleate can inhibit neuronal firing by activation of K_{ATP} channels in at least a subpopulation of hypothalamic GE neurones. In a small number of ARC neurones under these whole-cell recording conditions we found that oleate caused depolarisation (ΔVm = +5.1 ± 0.7 mV; n = 6; $P < 0.01$), which resulted in no significant change in firing frequency (4.4 ± 1.4 Hz to 6.4 ± 0.8 Hz; n = 6; $P = 0.09$), possibly because the depolarisation was sufficient in some cases to result in inactivation of voltage-gated sodium/calcium channels and inhibit action potential generation (Fig. 4E).

Discussion

Previous studies have produced disparate outcomes on whether lipid sensing hypothalamic neurones are also glucose sensing. Wang and colleagues [18] showed that there is minimal overlap between oleate sensing and glucose sensing neurones in rat ARC whereas Le Foll et al [16] demonstrated, using rat VMN neurones, that many GE- and GI-type neurones were excited and inhibited, respectively, by oleate [16, 18]. We find that glucose sensing ARC neurones are also oleate sensing in the same direction with respect to change in excitability. Thus GE neurones are inhibited by exposure to oleate and by lowered extracellular glucose concentration, via a K_{ATP}-dependent mechanism. Similarly, ARC neurones depolarised by oleate are also depolarised by lowered extracellular glucose concentration. These data indicate that for these mouse ARC neurones at least, there is a shared cellular response to increased lipid availability and glucose deprivation. Thus it is likely that such neurones play important roles in the modulation of physiological responses to the fed-fasted transition and/or in the pathophysiological outcomes associated with hypoglycaemia and/or starvation. Both insulin-induced hypoglycaemia and starvation are associated with increased lipolysis [15]. However, lipid levels are also elevated in the post-prandial period therefore perhaps the glucose level stratifies the physiological response to increased lipid availability. Therefore, it is possible that moderate changes lipid availability converge on these neurone populations to amplify an energy deficit signal or to enhance hepatic glucose production, depending on glucose availability.

To investigate the intracellular mechanism(s) responsible for oleate sensing in hypothalamic GE neurones we utilised the mouse hypothalamic GnRH-releasing GT1-7 neuronal cell line, which we have recently characterised as a GE-type glucose sensing neurone [39], with glucose-sensing properties similar to that described for ARC neurones, including POMC and AgRP neurones [37] and GnRH neurones [57]. Challenging GT1-7s with oleate elicited hyperpolarisation and reduction or cessation of firing, which was reversed or prevented by tolbutamide, indicating a K_{ATP}-dependent mechanism, similar to that observed in ARC GE neurones under the same perforated patch recording conditions. As this outcome was observed on isolated GT1-7 cells, this suggests that the oleate response is intrinsic to neurones and cell autonomous. We have

previously shown that low glucose-induced opening of K_{ATP} in mouse pancreatic beta cells, POMC neurones and GT1-7 neurones is dependent on AMPK activity [39, 38, 37]. Although oleate increases AMPK activity in GT1-7 cells (similar to that observed for low glucose stimulation), the oleate-induced hyperpolarisation of GT1-7 cells was not prevented by the presence of the AMPK inhibitor, compound C. Thus oleate-driven modulation of K_{ATP} channel activity in GT1-7 neurones is likely mediated by a mechanism distinct from that of hypoglycaemia.

Interestingly, the oleate-induced neuronal hyperpolarisation reported here is in contrast to a previous study, which demonstrated that oleate depolarised mouse ARC POMC neurones, which are GE-type neurones [54], by inhibition of K_{ATP} channels, an outcome ascribed to increased mitochondrial beta-oxidation of oleate and raised cellular ATP levels [41]. An alternative mechanism by which mitochondrial oxidation of oleate could increase K_{ATP} activity and cause cell hyperpolarisation is via the enhanced production of reactive oxygen species [42, 43, 19]. Importantly, the effects of oleate on energy and glucose homeostasis have been shown to be dependent on hypothalamic CPT1 activity [21, 58], indicative of fatty acid oxidation regulating hypothalamic neurones. CPT1 is endogenously inhibited by malonyl-CoA, the product of the reaction carried out by acetyl CoA carboxylase. However, we found that oleate-induced GT1-7 neurone hyperpolarisation was not altered by direct intracellular application of malonyl-CoA or by etomoxir, a pharmacological inhibitor of CPT1, suggesting that oleate-induced K_{ATP} channel activation and subsequent hyperpolarisation does not require mitochondrial fatty acid metabolism. Furthermore, the short-chain fatty acid, octanoic acid induced a significant depolarisation of GT1-7 cells, but did not block the hyperpolarising action of oleate. Moreover, oleate did not alter intracellular calcium in the GT1-7 cells, suggesting that the oleate-induced changes are unlikely to be mediated by CD36 driven store calcium release [16] or conventional PKCs . Consequently, there is no evidence to indicate that an alteration in fatty acid beta-oxidation or fatty acid transport via CD36 are involved in the oleate-mediated hyperpolarisation of hypothalamic neurones. Perhaps this is unsurprising as mitochondrial FA metabolism predominantly generates ATP, which would be expected to maintain K_{ATP} channel closure, as described previously [41].

It has recently been suggested that coupling of the extracellular glucose concentration to K_{ATP} in pancreatic β-cells and GT1-7 neurones may also be dependent on the expression and activity of UCP2 [36, 38]. UCP2 acts to dissipate the proton gradient across the mitochondrial membrane and is expressed throughout the VMH [9] and in GT1-7 cells [36]. Furthermore, UCP2 is activated by long-chain fatty acids [59], including oleate [24, 60] or indirectly via fatty acid-induced production of reactive oxygen species such as superoxide [61] or lipid peroxidation intermediates [62, 63]. Thus oleate activation of UCP2 is expected to decrease the mitochondrial membrane potential and mitochondrial respiration, resulting in reduction of cellular ATP levels. Acute pharmacological inhibition of UCP2 with genipin did not prevent nor reverse the oleate-induced hyperpolarisation of GT1-7 neurones, indicating that UCP2 does not play a role in oleate-induced changes in membrane potential. Nonetheless, indirect measurement of the mitochondrial membrane potential indicated that oleate induced a mild uncoupling of the mitochondrial membrane potential in a time course that correlated with the activation of K_{ATP}. This led us to screen GT1-7 neurones for other UCP isoforms, with UCP4 and UCP5, the brain-enriched isoforms [64, 65], which display proton transport capacity within lipid membranes [23]. Both UCP4 and UCP5 were detected by immunoblotting, although their expression was not significantly modified over the time course of the oleate treatment. Importantly however, the reduced JC-1 fluorescence caused by oleate did not translate to a reduction in either total ATP levels or a change in the ATP/ADP ratio. Therefore it is possible that oleate quenched the JC-1 fluorescence in a non-specific manner [66, 67] and that none of the UCP isoforms are involved in oleate-induced K_{ATP} channel opening.

This leaves open the question of what intracellular mechanism(s) mediates oleate-induced K_{ATP} channel activation and hyperpolarisation of GT1-7 and ARC GE neurones. Since ATP levels are not altered and oleate hyperpolarises GE neurones and GT1-7 cells (where ATP levels are clamped to maintain K_{ATP} channels closed), it is unlikely that activation is mediated by a deficit in ATP availability. It is possible that oleate may reduce ATP availability in the microdomain around the K_{ATP} channel that is not detectable with whole-cell ATP measurements, or that oleate (or a metabolite) alters K_{ATP} ATP sensitivity or displaces ATP from the nucleotide binding domain of the channel. An alternative possibility is that oleate is converted to a long-chain fatty acyl CoA (LC-

CoA) in neurones. This process requires ATP hydrolysis and LC-CoA molecules have been demonstrated to activate K_{ATP} by binding to a specific site on the channel [68, 19].

There are a number of limitations to our studies. Firstly, we have not been able to address the mechanism of oleate-induced depolarisation in GI ARC neurones, in large part due to the lack of a suitable cell culture model. Therefore, the current that underlies hypoglycaemic and oleate induced neuronal depolarisation remains elusive. A recent mouse model developed by Stanley and colleagues [69] allowing identification of GI neurones for slice electrophysiology may be useful for identifying this current.

The concentration of oleate used here is larger than that previously used in some studies of hypothalamic glucose sensing neurones [16, 18], although a recent study in humans measured an oleate level of > 10 μM in the CSF of non-obese humans [33]. Furthermore, our data demonstrating phosphorylation (inhibition) of ACC in response to 100 μM oleate corresponds with that previously shown to inhibit ACC and fatty acid synthesis in astrocytes [70]. Therefore the concentration used here probably lies in the upper end of the physiological level or in the pathophysiological range seen in type 1 diabetes [32] and/or obesity. However, there may be a number of reasons for the discrepancies in oleate sensitivity. Previous studies have utilised acutely dissociated VMH neurones, the preparation of which may induce a stress response sufficient to alter the sensitivity of these neurones to alternate fuel sources. Secondly, we have used whole-cell and perforated patch-clamp studies to measure whole cell macroscopic currents, membrane potential and firing frequency whereas other studies have utilised Ca^{2+} imaging. Indeed, oleate can induce changes in intracellular calcium by mobilising $[Ca^{2+}]_i$ stores [71], which may change independently of cellular membrane potential, although we did not observed in the current study.

The ability of oleate to reproduce the effects of low glucose on glucose sensing neurones in the hypothalamus might be expected to drive food intake and increase hepatic glucose production since direct pharmacological opening of hypothalamic K_{ATP} channels drives glucagon and adrenaline release in the periphery during hypoglycaemia [72]. However, central oleate (under normoglycaemic conditions) has been shown to inhibit feeding and suppress glucose production, [73, 14]. This is further complicated by the observation

that the effect of oleate on feeding and glucose production requires the activity of K_{ATP} [14], indicating that the action of oleate on feeding and glucose production may utilise a similar intracellular mechanism described here. To examine this relation in more detail, it would be interesting in future experiments to examine changes in ARC neuropeptide ratios (NPY/POMC and AgRP/POMC) and counterregulatory hormone responses to insulin-induced hypoglycaemia under conditions of high (oleate infusion) or low (acipimox-treated) plasma fatty acid levels. It is plausible that separate subpopulations of ARC neurones, which are involved in discrete physiological functions [74, 75] respond differentially to oleate (by depolarisation, hyperpolarisation or no response). Further analysis of electrophysiological responses of identified ARC neurones to oleate and other common dietary fatty acids, in conjunction with mouse metabolic phenotypic analysis is required to resolve this issue. Moreover, our studies did not examine the influence of hormones on low glucose and oleate responses. It is plausible that leptin, insulin and ghrelin, for example, modulate oleate and glucose responses, depending on the physiological context of energy surfeit or deficit.

In summary, we have demonstrated that the long-chain unsaturated fatty acid, oleate alters the activity of glucose sensing hypothalamic neurones in a manner similar to that of a hypoglycaemic stimulus. Furthermore, by utilising a hypothalamic glucose sensing cell line, we show that oleate-induced K_{ATP} channel activation occurs independently of changes in energy charge and may involve an oleate metabolite that directly alters K_{ATP} channel activity. The assimilation of fatty acid and glucose sensing neuronal populations, along with the influence of hormones such as insulin and leptin are likely to be important drivers of an integrated physiological response to changing nutrient availability. Clearly, additional research is required to delineate the network integration of glucose and fatty acid sensing neurones within the hypothalamus and their relation to energy homeostasis.

Author Contributions:

M.L.J.A conceived the study. S.D., C.B., J.V.W., M.P.M.S, R.J.M. and M.L.J.A. contributed to experimental design, analysis of data and writing of the manuscript. S.D. and C.B. performed the patch-clamp studies and S.D. and M.P.M.S performed the Western blotting

studies and C.B. performed the imaging studies. J.V.W performed the nucleotide measurements and calcium imaging studies. All authors read and approved the final manuscript.

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Figure Legends

Figure 1. Oleate activates AMPK and K_{ATP} in glucose sensing GT1-7 neurones

(A) Representative immunoblots showing the effect of lowering glucose concentration from 2.0 to 0.1 mM on p-AMPK and p-ACC levels with time. Bar graphs show relative mean levels of p-AMPK and p-ACC as a function of time after challenge with 0.1 mM glucose (n = 6). (B) Representative immunoblots showing the effect of increasing concentration of oleate (10, 50 and 100 μM) on the levels of p-AMPK and p-ACC. Bar graphs show relative mean levels of p-AMPK and p-ACC as a function of oleate concentration (*n* = 5-8). (C) Oleate hyperpolarises GT1-7 cells within 5-15 minutes of

application, an action reversed on application of tolbutamide. Bar graph shows mean values for membrane potential of cells exposed to oleate (n = 3). (D) Whole-cell recording under current-clamp and switched to voltage-clamp for short periods as denoted by (●) showing that the oleate (100 μM) hyperpolarisation is sustained but submaximal with addition of diazoxide (250 μM) causing further hyperpolarisation (upper traces). The families of currents shown are from a holding potential of -70 mV with tests pulses between -160 and -20 mV and obtained at times marked (●) during the experiment shown in upper traces. (E) Current-voltage relationships formed with current amplitude values from the cell shown in (D). (F) Bar graph denotes mean slope conductance values (derived from best fit to current-voltage curves over -160 to -20 mV range) in control and after addition of 100 μM oleate, oleate + tolbutamide and oleate + Dzx (n = 6). (G) Effect of oleate (100 μM) on the membrane potential of a GT1-7 cell in which compound C (20 μM) was present in the electrode solution and allowed to dialyse into the cell for 20 minutes prior to oleate application. Note that oleate hyperpolarised the cell, which was reversed by the addition of tolbutamide (200 μM). Bar graph shows mean values for membrane potential of cells dialysed with compound C ~ 2 minutes after formation of whole-cell configuration and after 20 minutes dialysis, followed by the addition of oleate, tolbutamide and extensive wash (n = 4-7). Values are means ± SEM. *p<0.05, ** p<0.01, *** p<0.001

Figure 2. K_{ATP} activation by oleate is independent of AMPK, fatty acid oxidation and not mimicked by octanoic acid

(A) Effect of oleate (100 μM) on membrane potential of a GT1-7 cell in which malonyl-CoA (50 μM) was present in the electrode solution and allowed to dialyse into the cell for 20 minutes prior to oleate application. (B) Families of currents shown are from a holding potential of -70 mV with tests pulses between -160 and -20 mV and obtained at the marked times (●) during the experiment shown in (A). (C) Bar graph denotes mean slope conductance values (derived from best fit to current-voltage curves over -160 to -20 mV range) in control and in the presence of 100 μM oleate (n = 4), 10 and 20 minutes after oleate addition. (D) Effect of oleate (100 μM) on membrane potential of a GT1-7 cell in which etomoxir (100 μM) was present in the electrode solution and allowed to dialyse into the cell for 20 minutes prior to oleate application. Bar graph shows mean values for membrane potential of untreated (concurrently performed) cells (Cont),

etomoxir (Etom) and Etom + oleate (n = 4). (E) Octanoic acid (50 μM) reversibly depolarises GT1-7 cell membrane potential. Bar graph shows mean values for membrane potential of cells exposed to control (Cont), octanoic acid (OctA) and following extensive washout of OctA (n = 8). (F) Oleate (100 μM) hyperpolarises GT1-7 cells in the presence of OctA (50 μM), an action reversed by tolbutamide (200 μM). Bar graph shows mean values for membrane potential of cells exposed to control (Cont), octanoic acid (OctA), OctA + oleate and OctA + oleate + Tolb (n = 3-4). (G) The presence of AMP-PNP (3 mM) in the electrode solution (and thus dialysed into cell) resulted in cell depolarisation and prevented hyperpolarisation by oleate (100 μM) and DZX (250 μM). Bar graph shows mean values for membrane potential of cells exposed to control (Cont: ~2 minutes after formation of whole-cell configuration and after 20 minutes dialysis, followed by the addition of oleate and oleate + DZX (n = 4). (H). Calcium imaging traces following addition of BSA (control) of oleate (100 μM; n = 5). Values are means ± SEM. *p<0.05, *** p<0.001

Figure 3. Oleate hyperpolarises GT1-7 neurones independent of UCP2 activity but depolarises the mitochondrial membrane potential

(A) Representative perforated patch recording from a GT1-7 neurone showing that 100 μM oleate-induced hyperpolarisation is unaffected by the presence of 100 μM genipin, and depolarised by subsequent application of 200 μM tolbutamide. Bar graph shows mean values for membrane potential of cells before and after application of oleate, followed by oleate + genipin and oleate + tolbutamide (n = 4). (B) Bar graph showing data from similar experiment to (A), but with 100 μM genipin added prior to challenge with oleate + genipin, followed by oleate + tolbutamide (n = 3). (C) Representative immunoblots showing the levels of UCP5, UCP4, p-ACC, p-AMPK and actin for control, vehicle (BSA) and oleate-treated cells. (D) Confocal images of GT1-7 cells pre-incubated with JC-1 under control conditions and treated with oleate (100 μM) for 20 minutes. Conventional light images of cells are shown on the right panels. Graph showing the change in JC-1 fluorescence intensity with time (relative to starting point of recording) for control (filled squares) and oleate-treated cells (n = 8). (E) GT1-7 total cellular ATP levels and (F) ATP:ADP ratios are unchanged by the addition of oleate (10, 50 and 100μM). Values are means ± SEM. *p<0.05, *** p<0.001

Figure 4. Subpopulations of mouse ARC neurones respond electrically to oleate.

(A) Representative perforated patch recording from a GE neurone, as demonstrated by the reversible hyperpolarisation elicited on switch from 2 mM to 0.1 mM glucose. Subsequent application of oleate (100 μM) in the presence of 2 mM glucose hyperpolarises this GE neurone. Addition of tolbutamide (200 μM) in the presence of oleate depolarises the neurone. Note the loss of action potentials in the presence of oleate + TOLB. Bar graph shows mean values (n = 6) for membrane potential of neurones exposed to 2.0 mM and 0.1 mM glucose and 2 mM glucose + oleate. (B) Representative perforated patch recording from a GI neurone, as demonstrated by the reversible depolarisation elicited on switch from 2 mM to 0.1 mM glucose. Subsequent application of oleate (100 μM) in the presence of 2 mM glucose depolarises this GI neurone. Bar graph shows mean values (n = 4) for membrane potential of neurones exposed to 2.0 mM and 0.1 mM glucose and 2.0 mM glucose + oleate. (C) Representative whole-cell current-clamp recording from an unidentified ARC neurone, with application of 100 μM oleate producing hyperpolarisation of the membrane potential and inhibition of firing, actions reversed by concomitant addition of tolbutamide (200 μM). Bar graph shows mean values (n = 4) for membrane potential of neurones exposed to: Control (Cont), 100 μM oleate and tolbutamide (TOLB). (D) Tolbutamide (200 μM) induced depolarisation and increased firing in an unidentified ARC neurone (indicating the presence of active K_{ATP} channels) under whole-cell current-clamp. Subsequent addition of oleate (100 μM) had no effect on membrane potential or firing rate. Bar graph shows mean values (n = 15) for membrane potential of neurones exposed to: Cont, TOLB and oleate + TOLB. (E) Representative whole-cell current-clamp recording from an unidentified ARC neurone demonstrating the addition of oleate (100 μM) depolarised some neurones with an overall decrease in firing and truncation of action potential amplitude. Bar graph shows mean values (n = 13) for membrane potential of neurones in Cont and exposed to oleate. Values are means ± SEM. *p<0.05, ** p<0.01, *** p<0.001

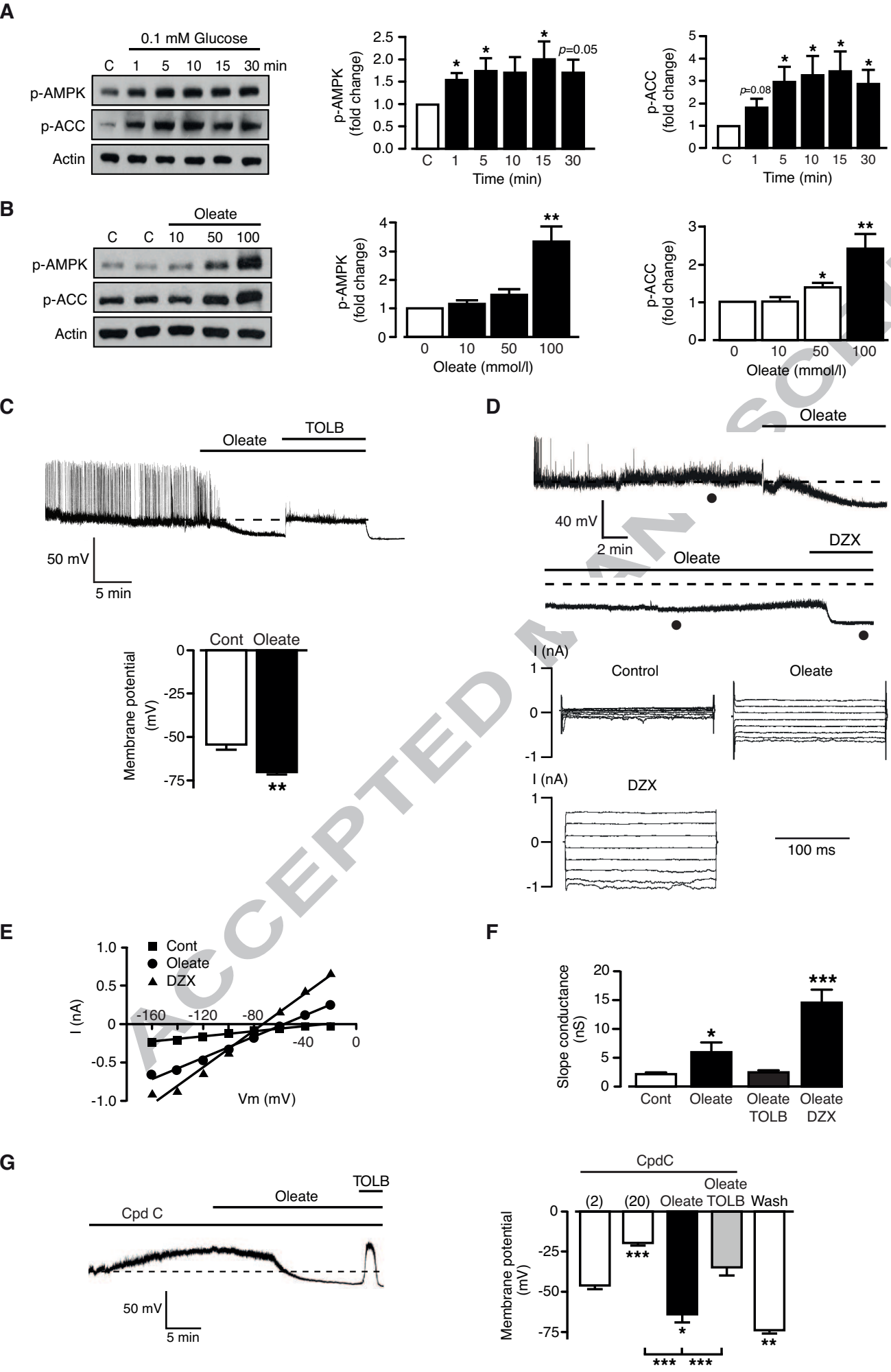
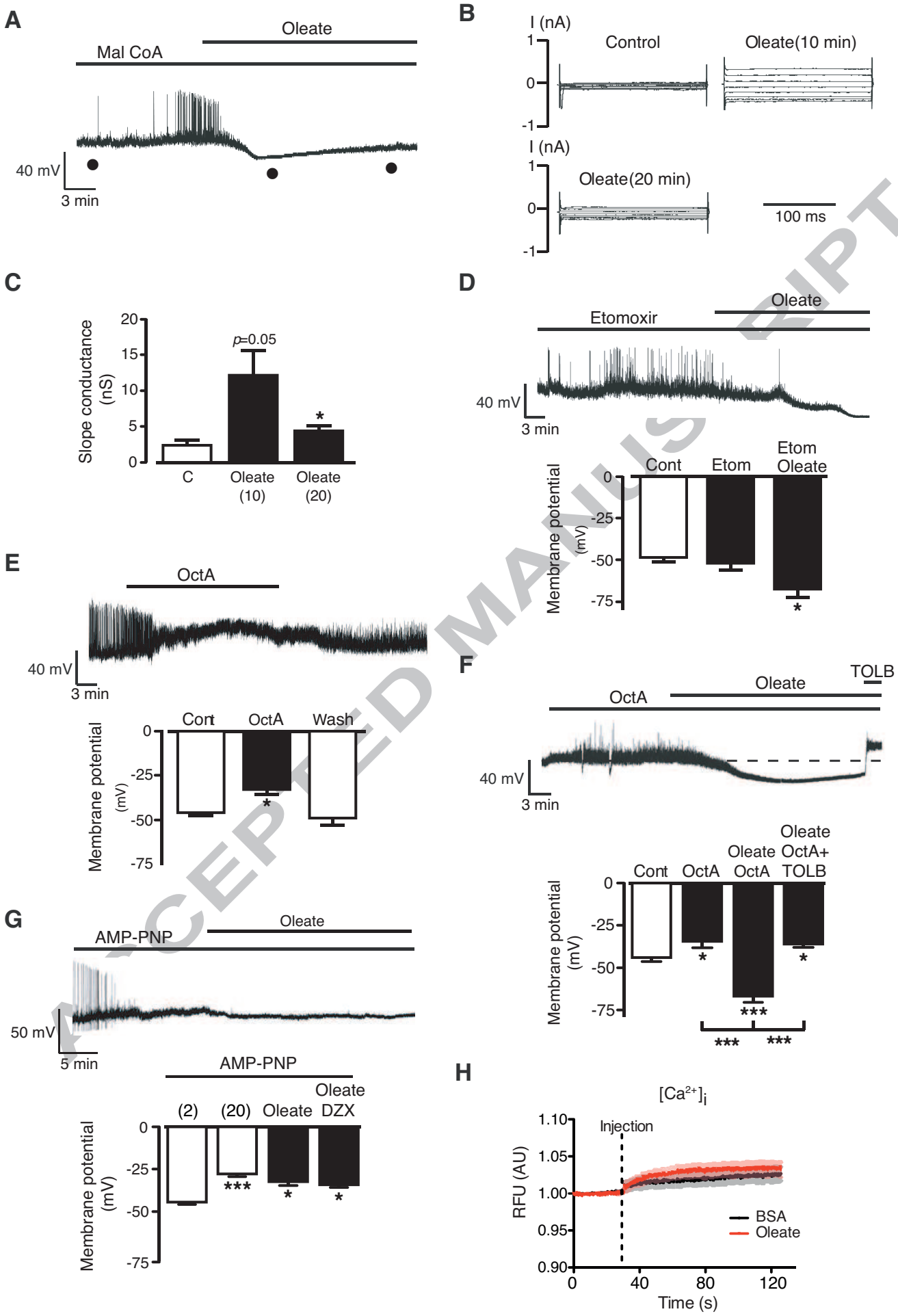


Figure 2



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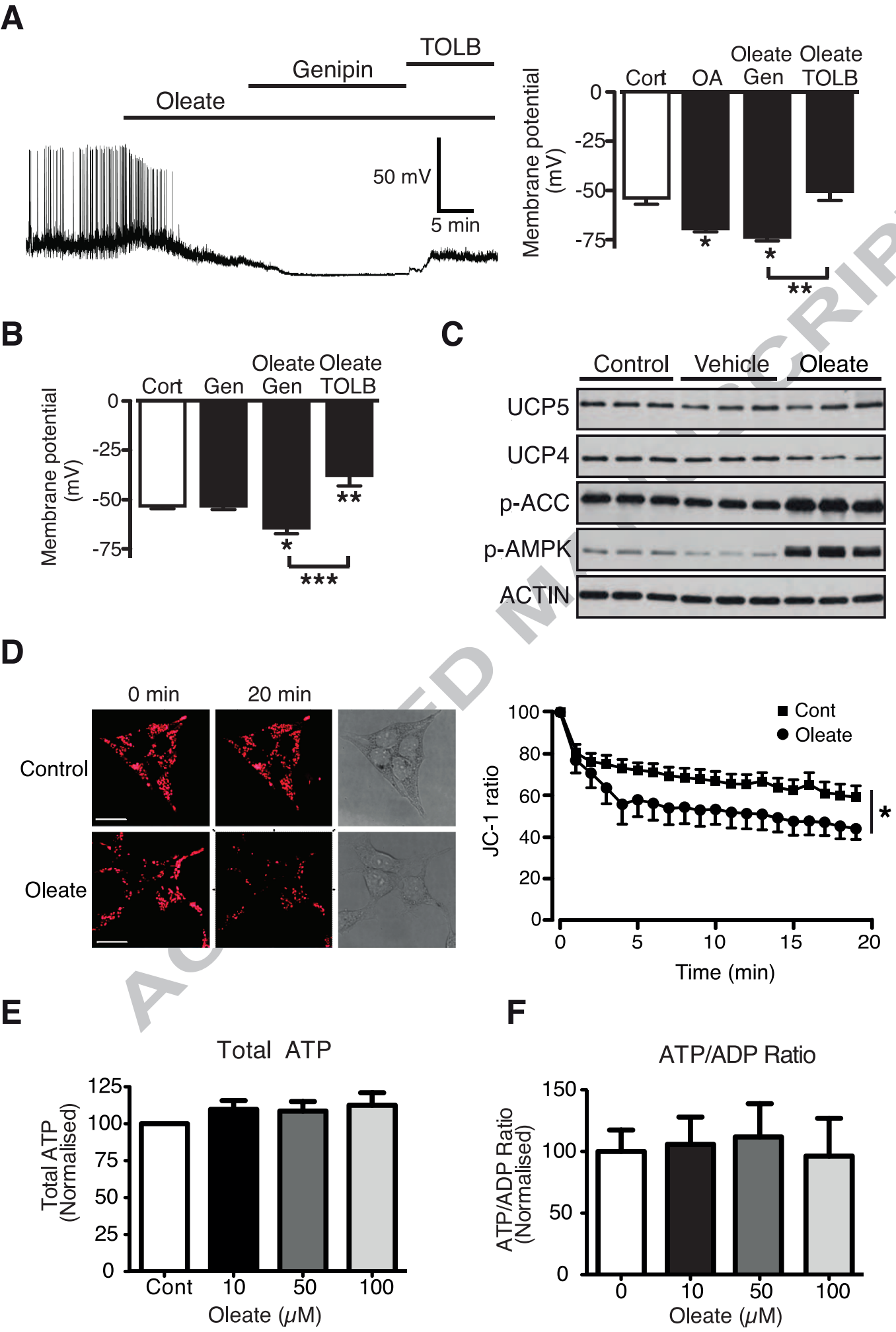
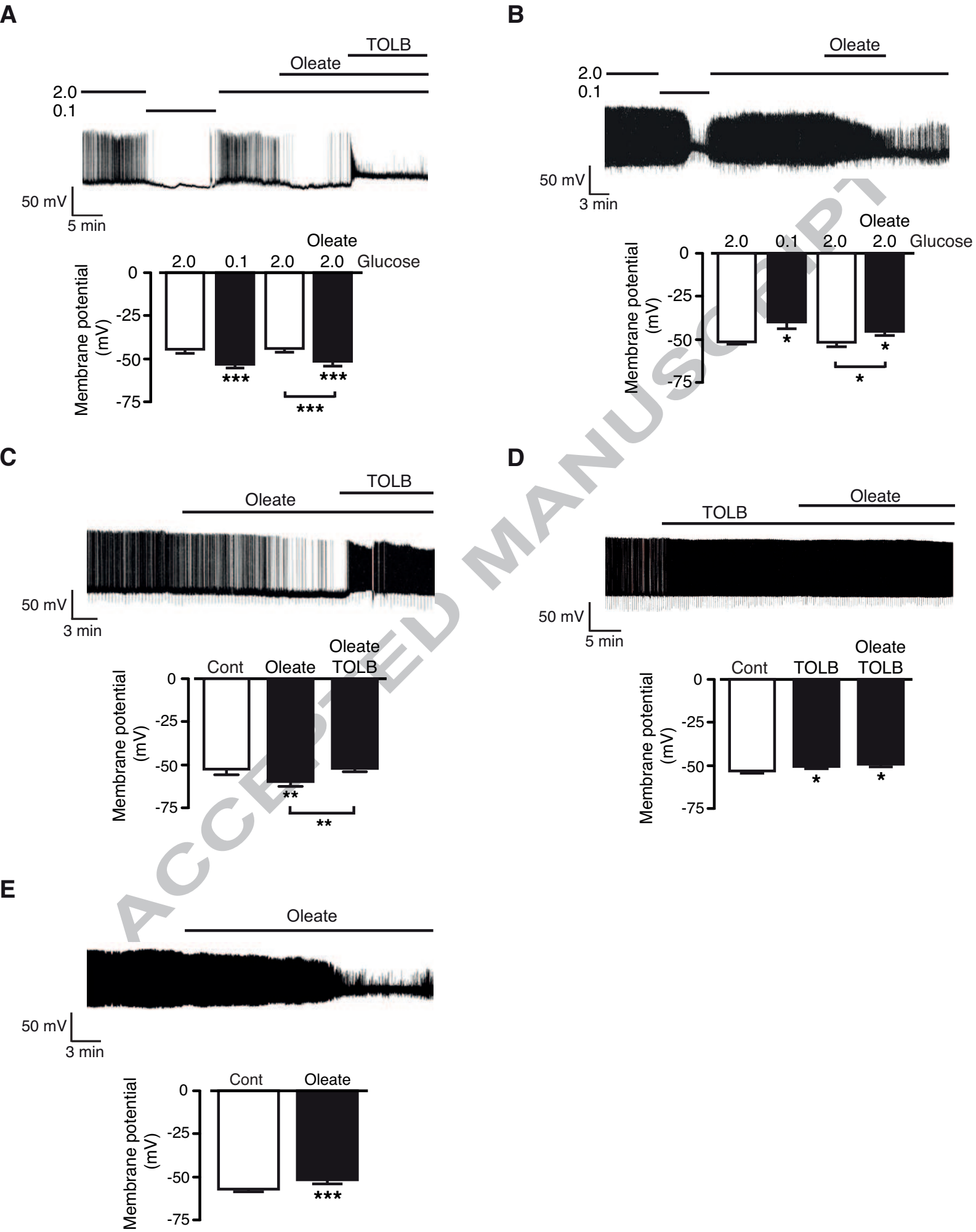


Figure 4



Highlights

- Oleate and low glucose hyperpolarise and inhibit GT1-7 and mouse GE neurones by activation of K_{ATP}
- Oleate inhibition of GT1-7 neurone activity is not mediated by AMPK or fatty acid oxidation
- Activation of K_{ATP} by oleate requires ATP hydrolysis but does not reduce the levels ATP or the ATP:ADP ratio
- GT1-7 hyperpolarisation by oleate is not dependent on UCP2
- Oleate and low glucose depolarise a subpopulation of hypothalamic GI neurones