Chronic exposure to K_{ATP} channel openers results in attenuated glucose sensing in hypothalamic GT1-7 neurons

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ARTICLE INFO

ABSTRACT

Individuals with Type 1 diabetes (T1D) are often exposed to recurrent episodes of hypoglycaemia. This reduces hormonal and behavioural responses that normally counteract low glucose in order to maintain glucose homeostasis, with altered responsiveness of glucose sensing hypothalamic neurons implicated. Although the molecular mechanisms are unknown, pharmacological studies implicate hypothalamic ATP-sensitive potassium channel (K_{ATP}) activity, with K_{ATP} openers (KCOs) amplifying, through cell hyperpolarization, the response to hypoglycaemia. Although initial findings, using acute hypothalamic KCO delivery, in rats were promising, chronic exposure to the KCO NN414 worsened the responses to subsequent hypoglycaemic challenge. To investigate this further we used GT1-7 cells to explore how NN414 affected glucose-sensing behaviour, the metabolic response of cells to hypoglycaemia and K_{ATP} activity. GT1-7 cells exposed to 3 or 24 h NN414 exhibited an attenuated hyperpolarization to subsequent hypoglycaemic challenge or NN414, which correlated with diminished K_{ATP} activity. The reduced sensitivity to hypoglycaemia was apparent 24 h after NN414 removal, even though intrinsic K_{ATP} activity recovered. The NN414-modified glucose responsiveness was not associated with adaptations in glucose uptake, metabolism or oxidation. K_{ATP} inactivation by NN414 was prevented by the concurrent presence of tolbutamide, which maintains K_{ATP} closure. Single channel recordings indicate that NN414 alters K_{ATP} intrinsic gating inducing a stable closed or inactivated state. These data indicate that exposure of hypothalamic glucose sensing cells to chronic NN414 drives a sustained conformational change to K_{ATP}, probably by binding to SUR1, that results in loss of channel sensitivity to intrinsic metabolic factors such as MgADP and small molecule agonists.

1. Introduction

Individuals with Type 1 Diabetes (T1D) often experience repeated episodes of hypoglycaemia as a consequence of exogenous insulin therapy. This recurring exposure to hypoglycaemia results in a reduction in the magnitude and altered threshold of symptomatic, hormonal and behavioural counterregulatory responses (CRR) to subsequent hypoglycaemia: a syndrome called impaired hypoglycaemia awareness (Adamson et al., 1984; Davis and Shamoon, 1991; Heller and Cryer, 1991; Inouye et al., 2002; Jacobson et al., 2006; Sanders et al., 2006). This response to recurrent hypoglycaemia can also be elicited in rodent models, leading to the identification of altered glucose sensitivity of hypothalamic neurons as an important contributory mechanism (Alquier et al., 2007; Dunn-Meynell et al., 2002; Fioramonti et al., 2013; Kang et al., 2008; McCrimmon et al., 2005, 2006; Sanders

et al., 2004; Song and Routh, 2006). Indeed it is now well established that the brain, in particular certain regions of the hypothalamus, regulates the physiological responses to hypoglycaemia in an effort to maintain whole body glucose homeostasis (Borg et al., 1994, 1995, 1997). Unfortunately, there are no effective treatments for defective CRR in type 1 diabetes other than the strict avoidance of hypoglycaemia that has only been achieved in intensive clinical trials involving small numbers of participants. Therefore, there is an urgent requirement to identify alternative therapies and/or adjunct molecules that act centrally to prevent, or restore, defective CRR, but also to define their biological and pharmacological mechanisms.

Central glucose sensing occurs in specialised neurons, many of which express the canonical proteins associated with pancreatic beta cell glucose sensing: glucokinase and ATP-sensitive potassium (K_{ATP}) channels (Ashford et al., 1990a, 1990b; Beall et al., 2012; Claret et al., 2007; Kang et al., 2004, 2006; Tarasov et al., 2004). Furthermore, the increased opening of hypothalamic K_{ATP} channels in glucose sensing neurons (resulting in neuronal hyperpolarization and inhibition of firing), in response to a fall in circulating glucose levels, is required to initiate an appropriate CRR to hypoglycaemia (Miki et al., 2001). *In vivo* rodent studies have shown that direct microinjection of the K_{ATP} channel openers (KCOs), diazoxide or NN414 into the ventromedial hypothalamus (VMH), immediately prior to hypoglycaemia amplifies adrenaline and glucagon secretion during hyperinsulinaemic-hypoglycaemic clamp studies (McCrimmon et al., 2005). Additionally, VMH microinjection of diazoxide is also able to amplify the CRR following recurrent hypoglycaemia, in a rodent model of defective CRR (McCrimmon et al., 2005). Conversely, intracerebroventricular (ICV) infusion or VMH microinjection of selective K_{ATP} inhibitors (which depolarize and increase neuronal firing), such as the sulphonylureas, glibenclamide or tolbutamide, attenuates the CRR during both systemic hypoglycaemia and cerebral glucopenia, resulting in blunted glucagon and adrenaline secretion (Evans et al., 2004).

Consequently, it has been reasoned that agents, which act selectively to increase the opening of hypothalamic K_{ATP} channels, could be useful amplifiers of glucose counter-regulatory responses. K_{ATP} channels are composed of one of the two members of the inward-rectifier K^+ channel family, $K_{IR}6$ and one of the three forms of the sulphonylurea receptor (SUR) subunit family. K_{ATP} channels that sub-serve glucose sensing in hypothalamic neurons express the SUR1 subunit (Ashford et al., 1990a; Dunn-Meynell et al., 1998; Kang et al., 2004; Lee et al., 1999; McCrimmon et al., 2005). While diazoxide is capable of activating both SUR2 and SUR1 subtypes of K_{ATP} channels, NN414 is a SUR1 selective agonist and has been shown to be a more potent activator than diazoxide of Kir6.2-SUR1 containing K_{ATP} channels (Dabrowski et al., 2003). Initial studies on rodents demonstrated encouraging results, with acute systemic delivery of NN414 resulting in an amplified CRR to hypoglycaemia, which was prevented by local delivery of glibenclamide to the VMH (Fan et al., 2008; McCrimmon et al., 2005). Unexpectedly, chronic ICV infusion of NN414 to rats during antecedent hypoglycaemia induced a larger depression of the CRR to subsequent hypoglycaemic challenge (Beall et al., 2013). Furthermore, using GT1-7 neurons, an *in vitro* model of hypothalamic glucose sensing neurons that expresses Kir6.2-SUR1 containing K_{ATP} channels (Beall et al., 2012), it was demonstrated that chronic exposure to NN414 or diazoxide profoundly impaired the up-regulation of K_{ATP} channel activity in response to the removal of intracellular ATP (Beall et al., 2013). Such an inactivation of hypothalamic K_{ATP} channels *in vivo* by chronic NN414 might explain the reduced CRR response to hypoglycaemia observed in rats. Consequently, we hypothesised that chronic hypothalamic K_{ATP} opening by KCOs may alter cellular metabolism and nucleotide levels and/or affect K_{ATP} gating directly

resulting in maintained closure channel and diminished hypoglycaemic sensing.

To investigate this phenomenon further we used GT1-7 neurons to determine the consequence of shorter-term exposure to K_{ATP} channel activators on glucose sensing and to examine whether one or more of these mechanisms underlie agonist-driven K_{ATP} channel inactivation.

2. Materials and methods

2.1. Cell culture

Mouse hypothalamic GT1-7 cells (Pamela Mellon, San Diego, California, USA) (Mellon et al., 1990) were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco, Invitrogen, Paisley, UK) containing 2.5 mM glucose with 10% fetal bovine serum (FBS; PAA Laboratories, Yeovil, UK), as previously described (Beall et al., 2012). For continuous exposure to NN414 (5 μM; Novo Nordisk, Copenhagen, Denmark), diazoxide (250 μM; Sigma-Aldrich) or tolbutamide (200 μM; Sigma-Aldrich), cells were grown on poly-*l*-lysine-coated glass coverslips at ~10—20% confluence. Vehicle-treated cells received 2 μl 0.1 M NaOH (NN414) or 0.1% DMSO (diazoxide, tolbutamide), with pH maintained at 7.4. In all experiments where GT1-7 cells underwent NN414 or vehicle challenge prior to electrophysiological or metabolic assessment, cells were superfused or washed thoroughly with normal saline (2.5 mM glucose) before measurements initiated.

2.2. Electrophysiology

All electrophysiology experiments were conducted using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) and analysed using PCLAMP7 software (Axon Instruments). GT1-7 cells were superfused at room temperature (22—25 °C) with normal saline (in mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 0.5 or 2.5 glucose (pH 7.4). Membrane potentials were recorded using perforated-patch or whole-cell current-clamp configurations, and membrane currents recorded using the whole-cell voltage clamp mode. In whole-cell experiments, cells were maintained in current-clamp mode to monitor resting membrane potential prior to a voltage clamp protocol to obtain current—voltage relations; whereby the cell was clamped at a holding potential of -70 mV and voltage steps ranging from -90 mV to $+30$ mV were elicited for a duration of 400 ms at intervals of 20s (net range -160 to -40 mV). Recording electrodes were pulled from thin borosilicate glass (open tip resistance of 3—5 MΩ) and filled with an intracellular solution containing (mM): 140 KCl, 5 $MgCl_2$, 3.8 $CaCl_2$, 10 EGTA, 10 HEPES, pH 7.2 (free $[Ca^{2+}]$ of 100 nM). Whole cell macroscopic currents were examined immediately (~1—2 min) after the cell membrane was ruptured (control) and after the cell had been dialysed (~12—15 min) with 0 ATP (by which time K_{ATP} channels were maximally open, termed run-up). Current- and voltage-clamp data were collected and analysed as described previously (Beall et al., 2010). For perforated-patch recordings, the electrode solution contained (in mM): 140 KCl, 5 $MgCl_2$, 3.8 $CaCl_2$, 10 HEPES, 10 EGTA (pH 7.2) and 25—40 μg/ml amphotericin B (Sigma-Aldrich). Following attainment of the perforated patch recording configuration, a minimum of 10 min of stable recording was collected in normal saline (2.5 mM glucose) before switching to a solution containing 0.5 mM glucose with or without tolbutamide (200 μM) (Sigma-Aldrich) and/or NN414 (5 μM) or diazoxide (250 μM).

For single channel recordings, patches of cell membrane were isolated and maintained in the inside-out configuration. Recording electrodes were pulled from thin walled borosilicate glass (open tip resistance of 10—12 MΩ) and filled with an intracellular solution

containing (mM): 140 KCl, 1 MgCl$_2$, 1 CaCl$_2$, 10 HEPES, pH 7.2. The intracellular aspect of the membrane was exposed to a bathing solution containing (mM): 140 KCl, 1 MgCl$_2$, 2.7 CaCl$_2$, 10 EGTA, 10 HEPES, pH 7.2 (free [Ca^{2+}] of 100 nM). When 100 μM ATP was added to the bath, the [Mg^{2+}] was raised to 1.1 mM to compensate for the Mg^{2+} chelation by ATP and maintain a free [Mg^{2+}] of 65 nM. K$_{ATP}$ channel activity was recorded for no longer than 15 min in order to avoid excessive channel run down, as previously reported (Larsson et al., 1993). 5 μM NN414, 200 μM MgADP or 100 μg/ml Trypsin was acutely applied to patches in the presence of 100 μM ATP. The average channel activity in a patch was defined as N.Po, where N = the number of functional channels in the patch and Po = the open state probability, which was determined by measuring the total time spent at each unitary current level and expressed as a proportion of the total time of the segment of recording (90—120 s) that was analysed. N.Po was calculated using the PCLAMP7 software which incorporates a 50% threshold parameter in order to detect single channel events which are >50% of a predetermined unitary current amplitude.

2.3. Glucose uptake

GT1-7 cells were exposed to NN414 (5 μM) or vehicle for 24 h prior to uptake analysis. Cells were washed in Hepes Buffered saline (HBS (in mM): 140 NaCl, 20 Hepes, 5 KCl, 2.5 MgSO$_4$, 1 CaCl$_2$, 2.5 Glucose, pH 7.4) and serum-starved (1 h) prior to incubation (12 min) with 10 μM 2-deoxy-D-[^{3}H]glucose (2DG; 24.4 kBq/ml; PerkinElmer, Cambridge UK) and 0, 0.1, 0.5 or 2.5 mM glucose at 20 °C. Non-specific uptake was determined using 10 μM cytochalasin B (Sigma-Aldrich). Following wash and cell lysis, cell-associated radioactivity was measured (Beckman LS60000IC scintillation counter; Beckman, High Wycombe, UK) and protein content quantified by Bradford reagent. Each uptake measurement was made up of 10 replicates.

2.4. Glucose oxidation

GT1-7 cells were pre-exposed to NN414 (5 μM) or vehicle for 24 h and following wash with HEPES buffered saline (HBS) were incubated for 4 h with 74 kBq/ml D-[U—^{14}C]glucose (PerkinElmer) and 0.1, 0.5 or 2.5 mM glucose in HBS at 37 °C. Subsequently, the media were transferred to 15 ml tubes and the ^{14}CO$_2$ released using 200 μl 60% perchloric acid and trapped by Whatman (GF/B) filter paper discs pre-soaked in 1 M KOH. Radioactivity was quantified by liquid-scintillation counting.

2.5. Hexokinase assay

GT1-7 cells were treated with NN414 (5 μM) or vehicle for 24 h in 2.5 mM glucose, then washed with ice-cold HBS followed by ice-cold Tris base, TES (N-tris(hydroxylmethyl)methyl-1-L-amino-ethine sulphonic acid, glacial acetic acid (TTA) buffer before undergoing three freeze/thaw cycles, spun at 12000 rpm at 4 °C for 10 min followed by lysis. The hexokinase assay reaction mixture contained (in mM): 39 TTA buffer (pH 7.6), 7.8 MgCl$_2$, 1.1 β-NADP, 0.74 ATP, 2.5 U glucose-4-phosphate dehydrogenase, 0—100 glucose plus 5—10 μg of lysate, with the ATP added last to initiate the reaction. Absorbance of β-NADPH was measured at 355 nm at 30 °C.

2.6. Cellular respiration

GT1-7 cells were seeded (30,000 cells/well) in XF 24-well culture microplates (Seahorse Bioscience, Copenhagen, Denmark) with NN414 (5 μM) or vehicle for 24 h in 2.5 mM glucose. Cells were then washed in serum-free media (2.5 mM glucose) for 1 h prior to

measurements being taken. Baseline recordings were taken to ensure a steady respiratory rate prior to injection of mitochondrial inhibitors for analysis of mitochondrial function.

2.7. Nucleotide assay

GT1-7 cell intracellular ATP/ADP ratios were determined by bioluminescence assay (ab65313, Abcam, Cambridge, UK). Cells were seeded (1000 cells/well) with NN414 (5 μM) or vehicle for 24 h in 2.5 mM glucose. Following cell lysis, luminescence intensity was determined by luminescence microplate reader (CentroPro LB 962 Luminometer, Berthold Technologies, Bad Wildbad, Germany).

2.8. Statistical analysis

One-way ANOVA, with a post-hoc Bonferroni test, was used to determine statistical differences between 3 or more groups, such as in the electrophysiological recordings. All other data sets were analysed using an unpaired Student's t-test (Graphpad, Prism 5 software). Data are presented as mean ± SEM. Statistical significance was accepted at the 95% confidence value with a P value of less than 0.05. Significance was allocated to the following P values: * <0.05; **< 0.01 and ***<0.001.

3. Results

3.1. Chronic exposure of GT1-7 cells to NN414 blunts hypoglycaemia detection

As shown previously (Beall et al., 2012), perforated patch recordings from GT1-7 cells in 2.5 mM glucose demonstrated spontaneous firing activity with a mean resting membrane potential of −50.7 ± 2.7 mV (n = 4), and following challenge with 0.5 mM glucose these neurons responded by hyperpolarization to −65.5 ± 1.9 mV with cessation of firing, effects reversible on return to 2.5 mM glucose-containing solution (−52.2 ± 2.6 mV; Fig 1A). In the continued presence of 2.5 mM glucose, application of the SUR1-selective K$_{ATP}$ activator, NN414 (5 μM) also rapidly hyperpolarized these neurons, to −74.0 ± 2.0 mV, and inhibited firing (Fig 1A), an action reversible on washout of drug (not shown). When GT1-7 cells were exposed to 5 μM NN414 for 24 h, after the removal of drug (recordings initiated within 30 min) we observed no difference in the mean resting membrane potential (−47.2 ± 2.2 mV; n = 6) or excitability of GT1-7 cells in 2.5 mM glucose compared to control or vehicle treated cells (Fig. 1B). However, these antecedent NN414-exposed cells no longer responded by hyperpolarization (Fig. 1B) to challenge with 0.5 mM glucose (NN414 ΔVm = −1.7 ± 0.8 mV; Vehicle, ΔVm = −14.8 ± 2.8 mV; p < 0.001, n = 4—6; Fig. 1C) and exhibited an attenuated response to further challenge with NN414, with the membrane potential hyperpolarizing from −45.0 ± 2.6 mV to −59.2 ± 2.8 mV (NN414, ΔV$_m$ = −13.8 ± 1.8 mV; vehicle, ΔV$_m$ = −24.2 ± 2.6 mV; p < 0.005, n = 6—9). This outcome is not surprising as chronic (24 h or longer) treatment of GT1-7 cells with NN414 has been demonstrated to push K$_{ATP}$ channels into a stable inactivated state (Beall et al., 2013).

Next we examined whether a shorter-term exposure of GT1-7 cells to NN414 replicated this action on K$_{ATP}$ conductance and resulted in attenuated glucose sensing. Consequently, we tested the effects of antecedent 3-h NN414 or vehicle incubation on GT1-7 cell response to lowered glucose. In agreement with longer-term exposure, following washout of NN414, GT1-7 cell membrane potential and firing rates were comparable between groups in 2.5 mM glucose-containing solution (Fig. 1D,E). However, on challenge with 0.5 mM glucose, NN414-treated cells once again displayed a blunted hyperpolarization response to a low glucose challenge
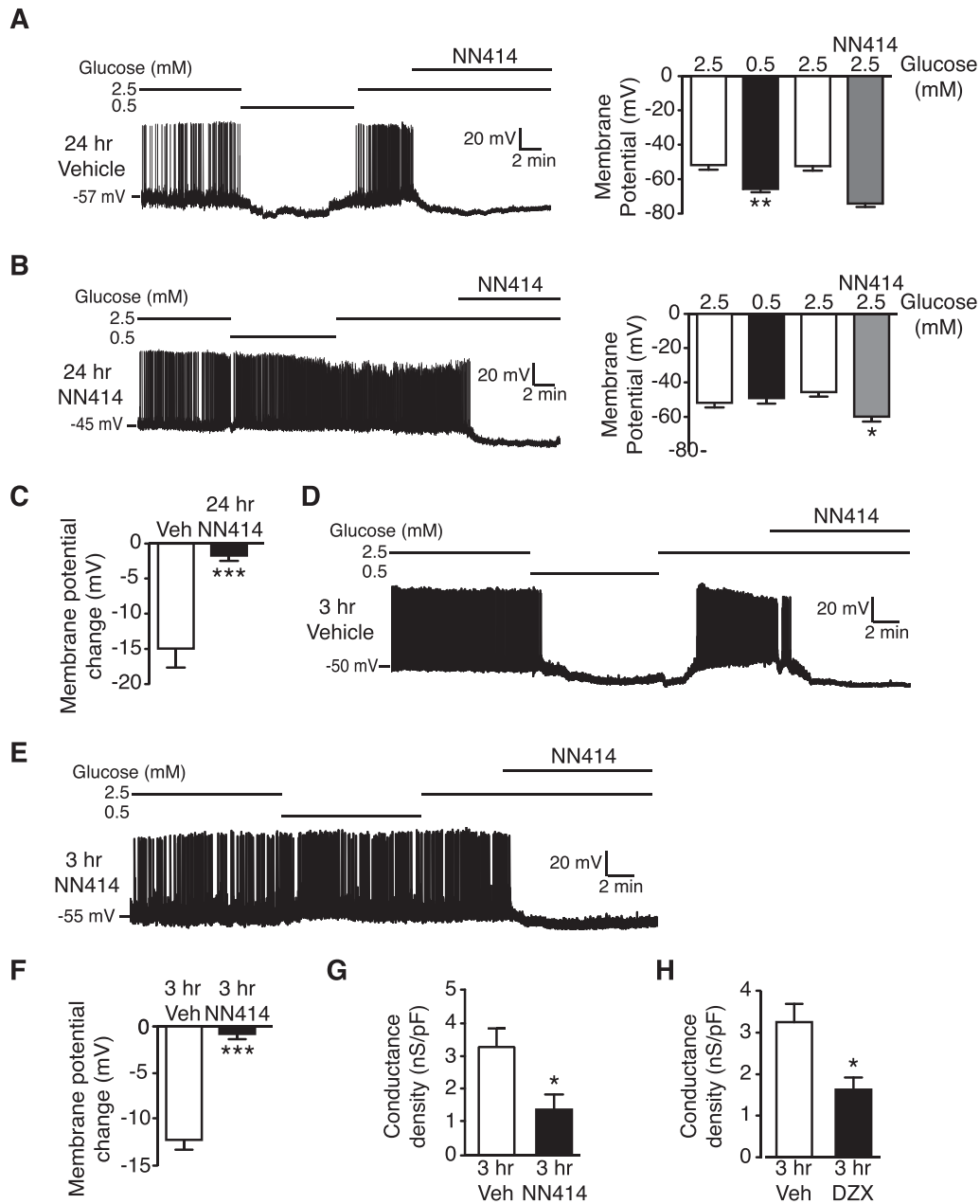
Fig. 1. Chronic NN414 treatment attenuates the hyperpolarization and conductance increase induced by low glucose and acute NN414 in GT1-7 cells. (A, B) Representative perforated-patch current clamp recordings from GT1-7 cells exposed to vehicle (A) or 5 μM NN414 (B) for 24-h. Acute application of 0.5 mM glucose to vehicle-treated cells results in membrane hyperpolarization and cessation of action potential activity, which is reversible on return to 2.5 mM glucose. In contrast, cells previously exposed to NN414 exhibit no response to 0.5 mM glucose. Subsequent acute challenge with 5 μM NN414 causes hyperpolarization in both cases, although this response is attenuated in the antecedent NN414-treated cells. Bar graphs showing mean values for membrane potentials in 2.5 mM (white bars), 0.5 mM (black bars) glucose and 2.5 mM glucose + NN414 (grey bars) for cells exposed to vehicle (A; n = 4) of NN414 (B; n = 6). (C) Bar graph showing the mean change in membrane potential of cells exposed chronically to vehicle or NN414 (24-h) on challenge with 0.5 mM glucose. (D, E) Representative perforated-patch current clamp recordings from GT1-7 cells exposed to vehicle (D) or NN414 (E) for 3-h. As observed for the 24-h exposure, antecedent treatment with NN414 for 3-h also blunted responses to acute 0.5 mM glucose and NN414 challenge. (F) Bar graph showing the mean change in membrane potential of cells exposed chronically to vehicle or NN414 (3-h) on challenge with 0.5 mM glucose. (G) Conductance density for vehicle-treated (white bar) vs 3-h NN414-treated (black bar) cells (n = 5). (H) Conductance density for vehicle-treated (white bar) vs 3-h 250 μM diazoxide-treated (black bar) cells (n = 5). Values are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

(NN414, $\Delta Vm = -0.8 \pm 0.5$ mV; vehicle; $\Delta Vm = -12.4 \pm 1.8$ mV; $p < 0.001$, n = 5; Fig. 1F) along with an attenuated response to acutely applied NN414 (NN414, $\Delta Vm = -13.8 \pm 2.9$ mV; vehicle, $\Delta Vm = -27.0 \pm 3.8$ mV; $p < 0.01$, n = 5). Consistent with a reduced K_{ATP}-responsiveness to either metabolic challenge or direct agonist application, whole-cell voltage clamp experiments on GT1-7 cells exposed to antecedent 3-h NN414 revealed a significantly reduced maximal K_{ATP} current compared to vehicle-treated cells (Fig. 1G). In

addition, short-term exposure of cells to diazoxide (n = 5) produced a similar inhibition of maximal K_{ATP} conductance (Fig. 1H).

3.2. Chronic exposure to NN414 blunts hypoglycaemia detection in GT1-7 cells even following a 24hr wash out period

We have previously shown that the chronic NN414 mediated suppression of K_{ATP} conductance in GT1-7 cells was completely

reversible after 24 h following washout of the drug (Beall et al., 2013). This recovery of K_{ATP} conductance was also observed 24 h after washout from a 3-h exposure to NN414 and was indistinguishable from vehicle-treated cells (Fig. 2A–C). In addition, GT1-7 cells with antecedent vehicle exposure and 24-h washout exhibited the typical response to lowered glucose and acute NN414 challenge (Fig. 2D, E). However, there remained a significant reduction in responsiveness to hypoglycaemia challenge in the antecedent NN414 exposed cells (Vehicle, ΔVm = −14.3 ± 1.4 mV; NN414, ΔVm = −3.8 ± 1.7 mV, n = 4–6; Fig. 2F), and a diminished response to acute NN414 challenge (NN414, ΔVm = −16.9 ± 1.5 mV; vehicle, ΔVm = −23.4 ± 1.6 mV; $p < 0.005$, n = 9–10; Fig. 2D, E). Consequently, although the K_{ATP} channel availability (as defined in whole-cell conditions following washout of intracellular ATP) is recoverable 24 h after washout of NN414, the channel appears to be much less available for opening by metabolic stimulus and so GT1-7 cells continue to display impaired glucose sensing.

3.3. Chronic NN414 exposure does not alter the GT1-7 metabolic response to hypoglycaemia

To dissect the mechanism behind the loss of glucose sensing following chronic NN414 exposure, we first investigated whether the metabolic responsiveness of GT1-7 cells to hypoglycaemia was altered. We chose 24-h continuous NN414 as this duration of agonist exposure ensured the most robust attenuation of glucose sensing. The hypothesis tested was that chronic NN414 exposure caused increased glucose uptake and/or more efficient metabolism, generating increased levels of ATP and so maintaining K_{ATP} closure during subsequent hypoglycaemic challenges. However, we could not detect any alteration in glucose uptake (Fig 3A) or glucose oxidation and incorporation (Fig. 3B,C) between chronic NN414-treated cells compared to vehicle controls, under physiological euglycaemic (2.5 mM) or hypoglycaemic (0.1 and 0.5 mM) conditions. Additionally, we did not observe any significant difference in hexokinase activity between NN414- and vehicle-treated cells (Fig 3D). To examine whether NN414 exposure resulted in altered mitochondrial oxidative metabolism and cellular ATP production we examined the real-time oxygen consumption rate (OCR) of NN414- and vehicle-treated GT1-7 cells. The basal OCR was unaltered by NN414 in GT1-7 cells (Fig 3E). In a further examination (Fig. 3F–K) of mitochondrial efficiency we utilised a modified Mitochondrial Stress Test (Seahorse Bioscience). Thus ATP synthase was inhibited by the addition of oligomycin to measure the proportion of mitochondrial respiration dedicated to ATP production, followed by assessment of mitochondrial leak (addition of rotenone
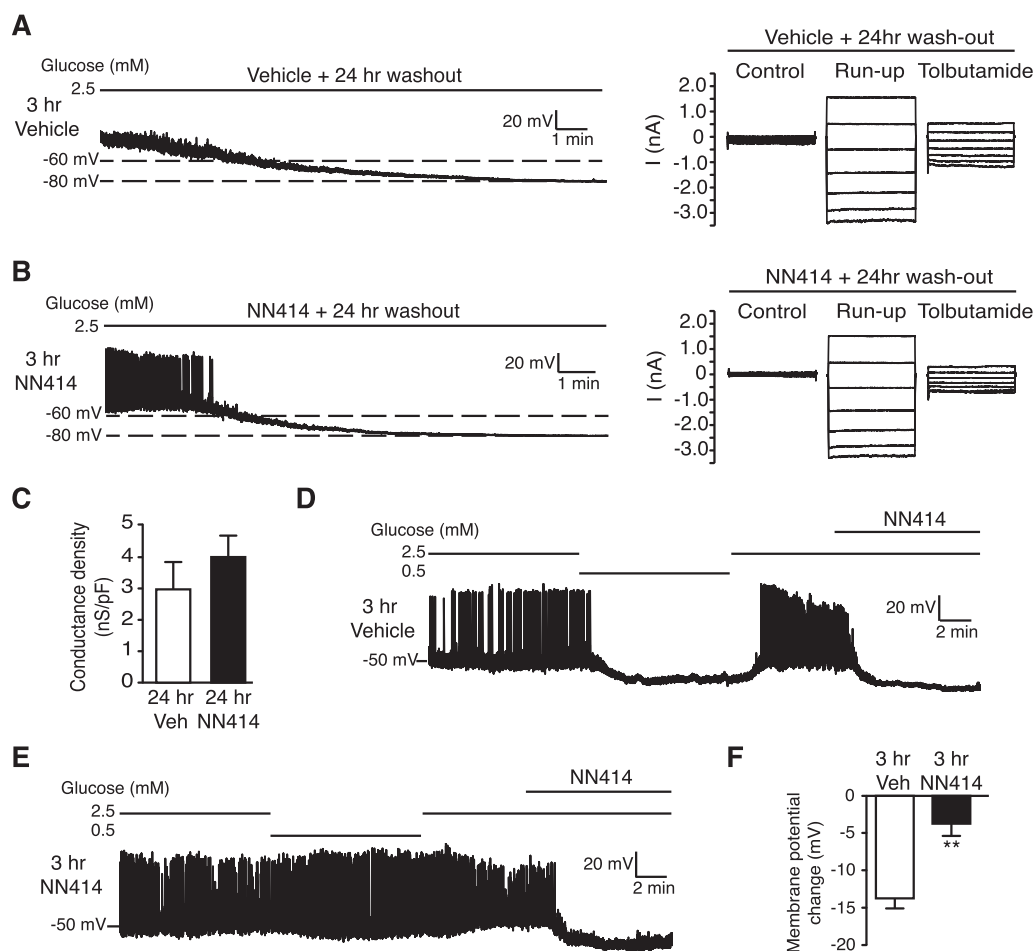

Fig. 2. The NN414-associated suppression of K_{ATP} current is readily reversible but the response to low glucose and acute NN414 remains blunted, following drug washout. (A,B) Representative current-clamp recordings of membrane potential and voltage-clamp recordings of currents from GT1-7 cells after 3-h treatment in control (vehicle-treated; A) or NN414 (5 μM; B) and following washout. Voltage-clamp currents are shown immediately after rupture, following run-up (ATP washed from cell) and after addition of 200 μM tolbutamide. (C) Conductance density for vehicle-treated (white bar) and NN414-treated (black bar) cells (n = 6). (D, E) Representative perforated-patch current clamp recordings from GT1-7 cells exposed to vehicle (D) or 5 μM NN414 (E) for 3-h followed by washout. The hyperpolarizing responses to low (0.5 mM) glucose and acute NN414 are blunted in the antecedent NN414-treated vs vehicle-treated cells. (F) Bar graph showing the mean change in membrane potential on challenge with 0.5 mM glucose of cells with antecedent exposure to vehicle or NN414 (3-h) followed by washout. (n = 6). Values are mean ± SEM. **p < 0.01.
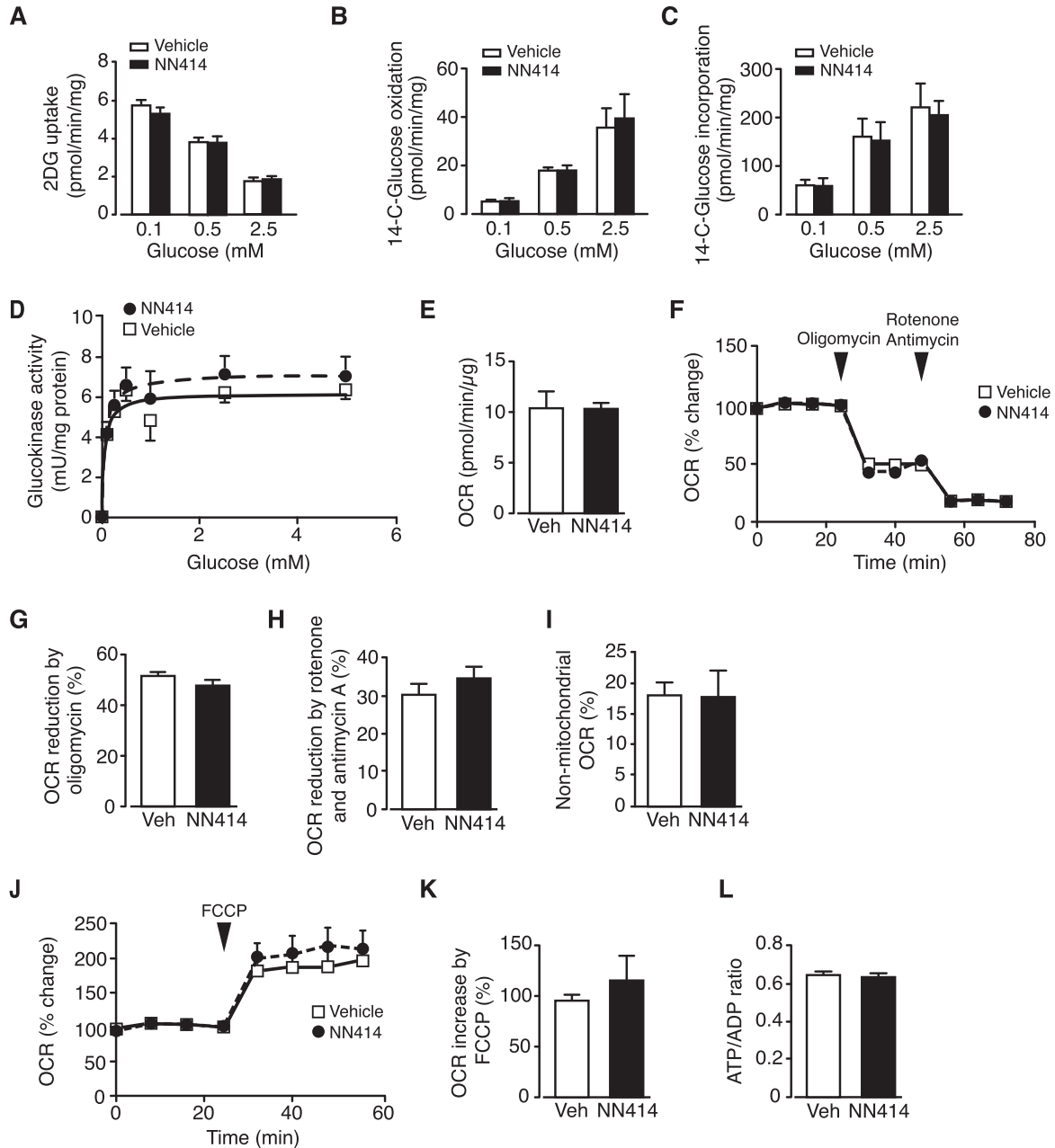
Fig. 3. Chronic NN414 exposure does not alter glucose uptake and oxidation, mitochondrial function or nucleotide ratios in GT1-7 cells. (A–C) 2-deoxyglucose (2DG) uptake (A; n = 4), [^{14}C]-glucose oxidation (B; n = 3) and total [^{14}C]-glucose incorporation (C; n = 3) in 24 h vehicle- and NN414-treated GT1-7 cells, measured in 0.1, 0.5 and 2.5 mM glucose. (D) Activity of hexokinase as a function of glucose concentration in GT1-7 cells following 24-h exposure to vehicle (□) or NN414 (●). The curved lines (vehicle, solid; NN414, broken) represent lines of best fit to the data points (n = 4 determinations per point). (E) Oxygen consumption rate (OCR) of 24-h vehicle- and NN414-treated GT1-7 cells. (F) Mitochondrial stress temporal profile for 24-h vehicle- (□) and NN414-treated (●) GT1-7 cells showing the effects of the percentage change in normalized OCR of the sequential addition of 1 μM oligomycin and 2 μM rotenone and antimycin A (n = 7). (G–I) 24-h NN414 exposure had no effect on ATP-linked OCR (G), or on the OCR required to maintain the mitochondrial leak (H) or the non-mitochondrial OCR (I) compared to vehicle-treated cells (n = 7). (J) Temporal profile showing the normalized baseline OCR for vehicle- (□) and NN414-treated (●) cells and the maximum OCR (reserve capacity) attained following addition of 0.2 μM carboyl cyanide-trifluoromethoxyphenylhydrazone (FCCP). (K) 24 h NN414 exposure had no effect on the percentage OCR increase following FCCP addition compared to vehicle-treated cells (n = 4). (L) the ATP-ADP ratio of GT1-7 cells was unchanged following 24 h NN414 exposure, compared to vehicle controls (n = 9). Values are mean ± SEM.

and antimycin A) and non-mitochondrial OCR, with no difference detected in any of these parameters (Fig. 3F–I). Furthermore the cellular reserve ("spare respiratory") capacity of GT1-7 cells, as determined by the addition of the proton ionophore, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), to induce maximal respiration, was also unaffected by previous exposure to NN414 (Fig. 3J,K). Finally we measured the ATP/ADP ratio and, in agreement with the results above, NN414 exposure produced no

change in this ratio (Fig. 3L). In summary, we detected no adaptations in glucose metabolism or mitochondrial function, which would help explain the attenuated glucose sensing following chronic NN414. These results, in conjunction with the finding that direct activators of K_{ATP} exhibit reduced efficacy following NN414 washout (even after recovery of K_{ATP} conductance in whole-cell recordings), suggested that alterations to the K_{ATP} channel itself may be responsible.

3.4. NN414 binding to and opening of the K_{ATP} channel complex is required for NN414 induced K_{ATP} inactivation

Next we investigated the possibility that the induction of prolonged cell hyperpolarization resulted in a stable "inactivated" conformation of the K_{ATP} channel. To test this we increased the extracellular concentration of K^+ to 13.5 mM in order to chemically-clamp the cell at resting membrane potential but still allow K_{ATP} channel opening by addition of NN414 without the attendant hyperpolarization, as shown in perforated-patch recordings from GT1-7 cells following acute exposure to NN414 (Fig 4A). Under chemical-clamp, we tested the effect of a 24-h exposure of GT1-7 cells to NN414 or vehicle on the subsequent K_{ATP} conductance measured by whole-cell voltage clamp. The maximal conductance values determined from these cells following washout of ATP showed that chronic exposure to NN414 significantly attenuated the whole-cell K_{ATP} current and maximum conductance (Fig. 4B−D) in a manner identical to that observed for cells undergoing hyperpolarization. A similar outcome was also obtained with a shortened (3-h) exposure to NN414 in the presence of 13.5 mM K^+ solution (Fig 4E). Consequently, we went on to examine whether the K_{ATP} inactivating effect of chronic NN414 exposure was dependent on the ability of the K_{ATP} channel to open. To effect maintained channel closure, GT1-7 cells were exposed to the K_{ATP} channel inhibitor, tolbutamide (200 μM) and subsequently challenged with acute NN414, thus demonstrating prevention of the hyperpolarizing response to the K_{ATP} activator (Fig 5A). For chronic NN414 treatment, tolbutamide was present 1 h prior to NN414 addition and continuous for the 24-h NN414 incubation period, with appropriate vehicle controls. Subsequent whole-cell current- and voltage-clamp recordings demonstrated that, in the presence of tolbutamide + NN414, the maximal cell conductance following removal of the drugs was unchanged compared to their vehicle controls (Fig. 5B−D), suggesting that NN414 is required to bind to the channel complex and open K_{ATP} for inactivation to occur.

3.5. Chronic NN414 exposure induces a refractory state for neuronal K_{ATP} channels

Although the availability of K_{ATP} to activate maximally is severely attenuated following continuous NN414 exposure, mRNA for the channel subunits $K_{IR}6.2$ (*Kcnj11*) and SUR1 (*Abcc8*) or the protein levels of the fully assembled channel complex at the plasma membrane were unchanged under these conditions (Beall et al., 2013). Therefore, we performed single channel recordings on isolated inside-out membrane patches in order to determine if adaptations were occurring in the intrinsic activity of the K_{ATP} channel following prolonged NN414 exposure that contribute to the changes observed in glucose-sensing and subsequent K_{ATP} agonist
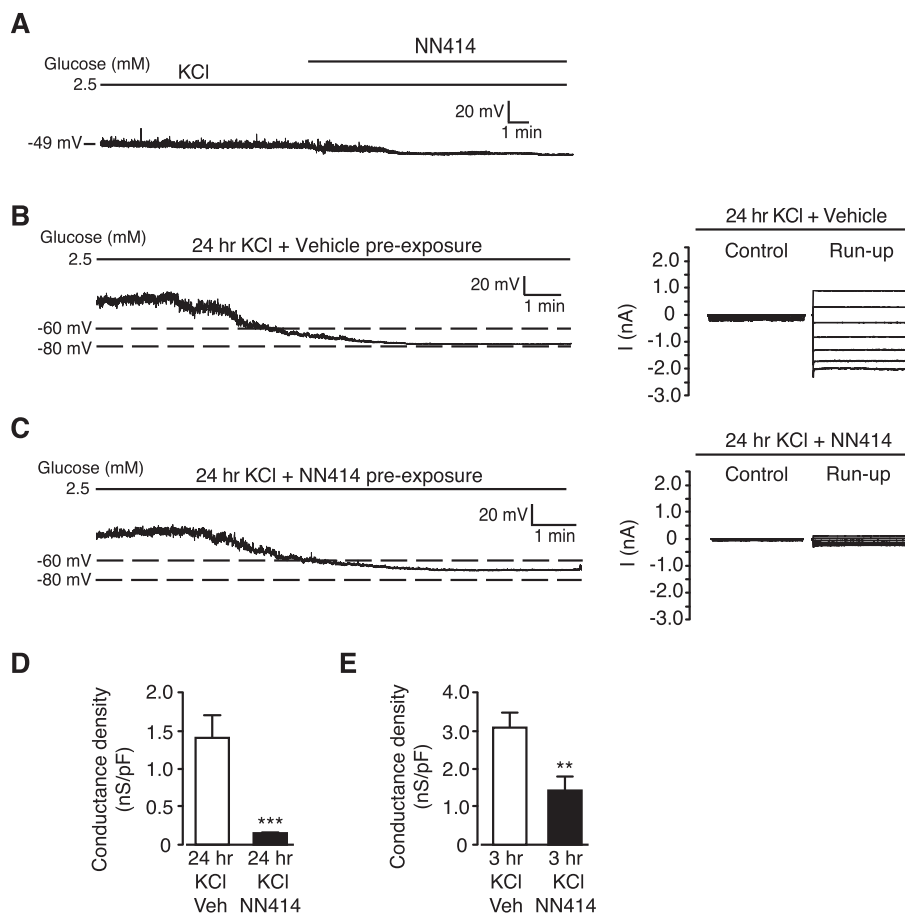

Fig. 4. Chemical-clamp of cell membrane potential does not prevent chronic NN414 from reducing K_{ATP} conductance. (A) Representative perforated-patch current clamp recording of a GT1-7 cell demonstrating that acute NN414 application does not hyperpolarize the membrane potential in the presence of high extracellular (13.5 mM) KCl. (B, C) Representative whole-cell current-clamp recordings of membrane potential and voltage-clamp recordings of currents from 24-h KCl + vehicle- (B) and 24-h KCl + NN414-treated GT1-7 cells (C). (D) Pooled conductance density at run-up from 24-h KCl + vehicle-vs 24-h KCl + NN414-treated cells (n = 11−12). (E) Conductance density at run-up for 3-h KCl + vehicle-treated vs 3-h KCl + NN414-treated (black bar) cells (n = 11). Values are mean ± SEM. **p < 0.01, ***p < 0.001.
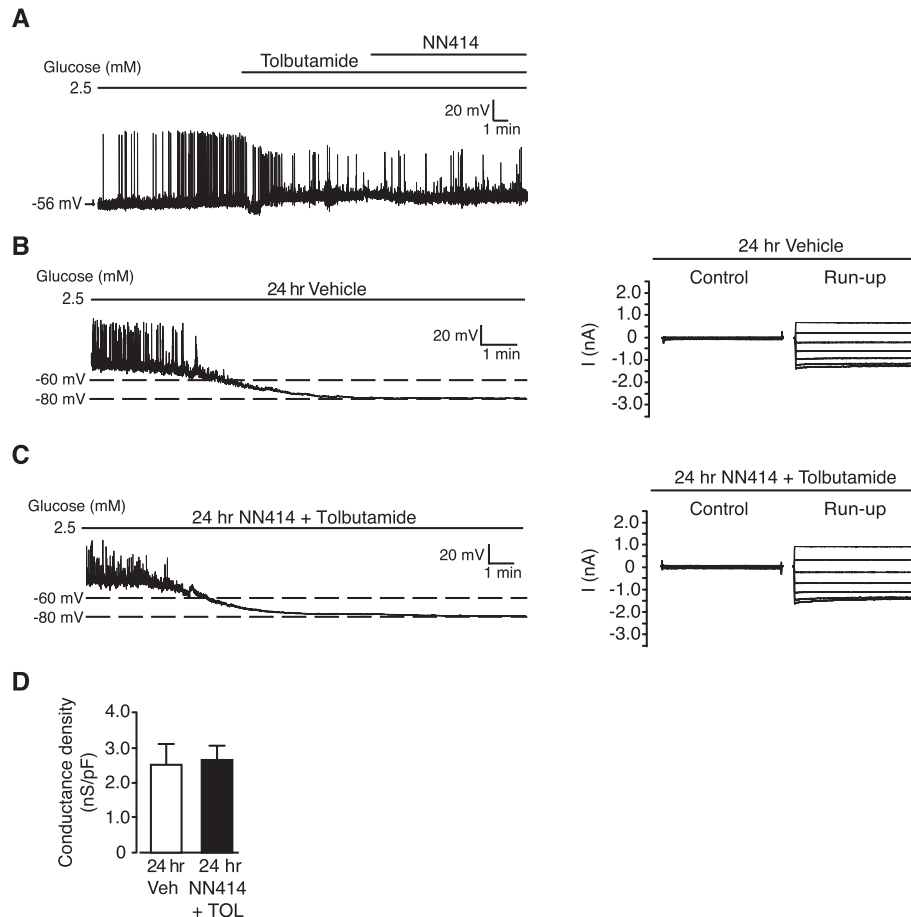
Fig. 5. Tolbutamide prevents chronic NN414-associated attenuation of K$_{ATP}$ channel conductance. (A) Representative perforated-patch current clamp recording of a GT1-7 cell demonstrating that 5 μM NN414 does not hyperpolarize the cell membrane when applied in the presence of 200 μM tolbutamide. Note that addition of tolbutamide causes a small depolarization as reported previously (Beall et al., 2012). (B, C) Representative whole-cell current-clamp recordings of membrane potential and voltage-clamp recordings of currents from 24-h vehicle- (B) and 24-h tolbutamide + NN414-treated GT1-7 cells (C). (D) Pooled conductance density at run-up from 24-h vehicle (white bars; n = 7) vs. 24-hour tolbutamide + NN414-treated cells (black bars; n = 5). Values are mean ± SEM.

sensitivity. Following patch excision into the inside-out configuration in symmetrical 140 mM K$^+$ solutions and assurance of stable recording conditions, the membrane potential was clamped at −50 mV. Patches isolated from GT1-7 cells exposed for 24 h to vehicle-containing solution exhibited typical K$_{ATP}$ single channel activity, which was reversibly inhibited by the application of 100 μM MgATP to the cytoplasmic aspect of the membrane (Fig 6A). Furthermore, application of 200 μM MgADP to the membrane elicited K$_{ATP}$ channel activation (Dunne and Petersen, 1986; Kakei et al., 1986), and 100 μM NN414, in the presence of 100 μM MgATP (Dabrowski et al., 2003), induced a large increase in channel opening (Fig. 6B, C), as expected. In contrast, inside-out patches excised from GT1-7 cells following 24-h exposure to NN414 were characterised by the relative absence of channel openings, indicating a substantial reduction in the intrinsic activity of the channel (Fig. 6B,C top trace), with complete insensitivity to activation by application of 200 μM MgADP or 100 μM NN414 + 100 μM MgATP (Fig. 6B,C). Finally, to ensure antecedent NN414-exposed cell-derived patches contained channels capable of being activated, patches were subsequently exposed to trypsin (100 μg/ml), which has previously been demonstrated to re-activate "run-down" K$_{ATP}$ channels (Lee et al., 1994a; Proks and Ashcroft, 1993). Exposure of patches to trypsin (2 min) yielded a comparable level of K$_{ATP}$ activation in both treatment groups (NN414: N.Po = 0.265 ± 0.161; Vehicle: N.Po = 0.318 ± 0.20; Fig. 6B,C).

4. Discussion

There is a clear need to develop novel approaches to prevent hypoglycaemia in individuals with long-term T1D, as current strategies are relatively ineffective and hypoglycaemia remains the major limitation to intensive insulin therapy. Studies on rats by microinjection of the non-selective K$_{ATP}$ channel opener, diazoxide into the VMH (McCrimmon et al., 2005) or by systemic delivery of the SUR1-selective opener, NN414 (Fan, et al., 2008) demonstrated promising results with the amplification of the CRR to hypoglycaemia. Subsequently, ingestion of diazoxide by T1D subjects under hyperinsulinemic hypoglycaemic clamp demonstrated an improved CRR, which was diminished in individuals with a K$_{ATP}$ channel polymorphism that is associated with diabetes (George et al., 2015).

Conversely, we have previously demonstrated that continuous central NN414 exposure, rather than defending against the development of defective CRR in response to repetitive hypoglycaemic challenge, attenuates the CRR to hypoglycaemia *in vivo* and reduces K$_{ATP}$ channel conductance density *in vitro* (Beall et al., 2013). Here we show that continuous NN414 application for 24 h also blunted low-glucose sensing in the GT1-7 cell line. Such an effect *in vivo* could therefore account for the suppression of the CRR to hypoglycaemia following chronic NN414 exposure. Although termination of NN414 administration *in vivo* partially restored the CRR
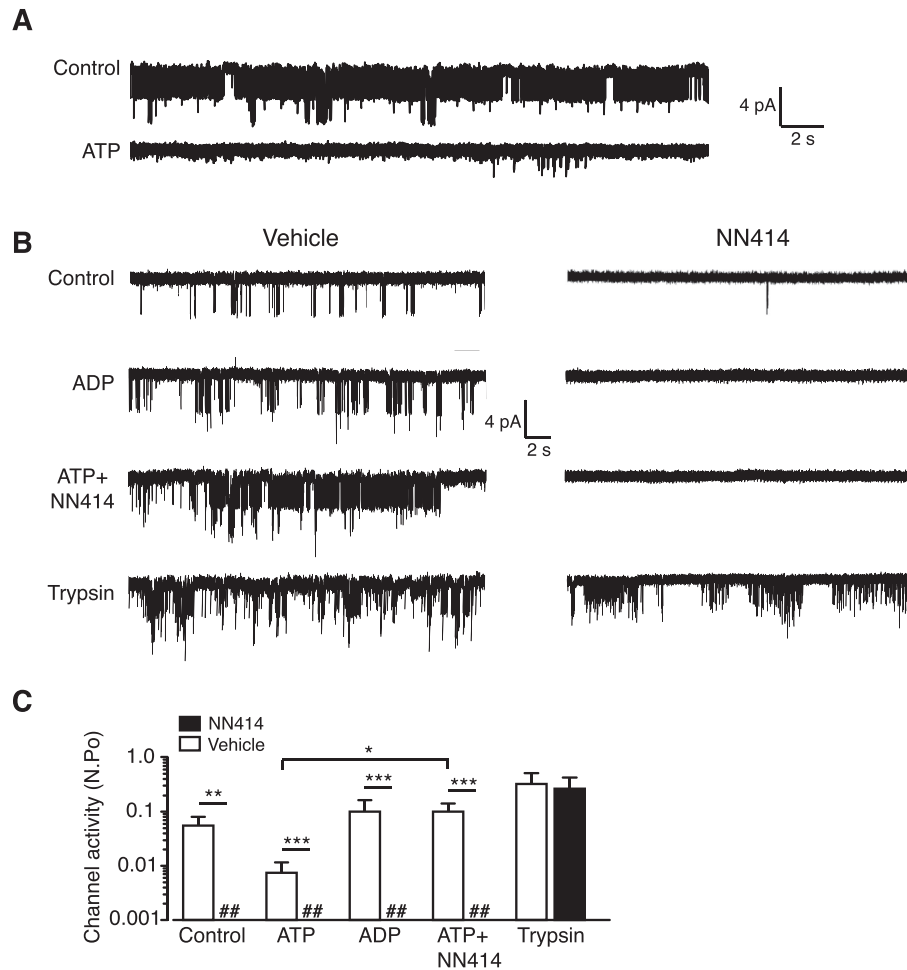
Fig. 6. Chronic NN414 treatment drives K_{ATP} channels into an inactive state unresponsive to channel agonists. (A) Representative single channel recording from an isolated inside-out membrane patch from control GT1-7 cells, recorded in symmetrical 140 mM KCl at a membrane potential of −50 mV. Channel openings (inward current) are represented as downward deflections. The addition of 100 μM MgATP almost completely inhibits channel activity. (B) Representative inside-out single channel recordings from 24-h vehicle-treated and 24-h NN414-treated GT1-7 cells. This demonstrates that the intrinsic K_{ATP} channel activity is higher in vehicle-vs. NN414-treated cells (top trace) and that the activation induced by addition of 200 μM MgADP (second trace) or 100 μM MgATP + 100 μM NN414 (third trace) to the cytoplasmic aspect of the membrane observed in 24-h vehicle-treated cells is absent in the 24-h NN414-treated cells. In contrast, 100 μg/ml trypsin application to the cytosolic domain of the inside-out patches elicited comparable responses between vehicle- and NN414-treated cells (bottom trace). (C) Bar graph of mean K_{ATP} channel activity (N.Po) from inside-out patches obtained from 24-h vehicle- (white bars) and 24-h NN414- (black bars) treated cells (n = 4–11). ## denotes an N.Po value < 0.001. Values are mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

response to hypoglycaemia and removal of NN414 from GT1-7 cells recovered whole-cell K_{ATP} conductance (Beall et al., 2013), we find that the responsiveness of GT1-7 cells to hypoglycaemic challenge remains impaired. One plausible explanation we explored was that chronic exposure to the K_{ATP} openers resulted in a "metabolic adaptation" leading to maintained levels of intracellular ATP under hypoglycaemic conditions, as has been reported for diazoxide in islets (Elmi et al., 2000). Thus we examined whether chronic NN414 exposure induced adaptations in cellular glucose metabolism or mitochondrial function in GT1-7 cells. We found no significant changes in glucose uptake, hexokinase activity or glucose oxidation in normo- or hypoglycaemic conditions nor did we observe any alteration in the oxygen consumption rate, mitochondrial electron transfer function or cytosolic ATP:ADP ratio in GT1-7 cells following chronic NN414 treatment in comparison with vehicle controls. As such a change in neuronal bioenergetics following K_{ATP} activation is unlikely to explain the persisting defect in glucose sensing.

Consequently, we next focused on the possibility that long-term exposure to these channel openers resulted in a functional change in the K_{ATP} channel itself, either via prolonged hyperpolarization (voltage-dependent) per se or by binding of the K_{ATP} openers to the channel protein complex. Using increased extracellular K^+ concentration to chemically-clamp the membrane potential to ~ -50 mV and prevent hyperpolarization by NN414, we demonstrated that chronic exposure to this channel opener still resulted in severely attenuated K_{ATP} cellular conductance. In contrast, the presence of a selective K_{ATP} inhibitor, the sulphonylurea tolbutamide, which maintained the channel in the closed conformation prior to and during exposure to NN414, prevented the NN414-mediated reduction in whole-cell K_{ATP} conductance. This outcome indicates that either the presence of tolbutamide prevents NN414 from binding to the K_{ATP} channel or that prolonged opening by the agonist, allosterically prevented by tolbutamide binding, results in the channel entering a stable inactivated state. Although the exact binding site for the K_{ATP} openers has not yet been fully established, the selective agonist activity of NN414 is dependent on intact Walker A motifs of the nucleotide binding domains of SUR1 (Dabrowski et al., 2003). Furthermore, [3H]-glibenclamide binding to SUR1 can be displaced by NN414 (Nielsen et al., 2006) and tolbutamide (Niki et al., 1989) which has been demonstrated to bind to the COOH terminal group of SUR1 transmembrane domains (Ashfield et al., 1999) and may also interact with diazoxide at these

and additional sites on SUR1 (Babenko et al., 2000).

Nevertheless, regardless of the exact physical interaction site, prevention of K_{ATP} opening protects the channel from entering a long-lived closed or inactivated state. In an attempt to examine this further, we assessed single channel activity in excised patches following chronic NN414 or vehicle exposure of GT1-7 cells. This revealed a severe loss of channel activity in patches excised from NN414-treated cells compared to vehicle controls, indicative of inhibition of the intrinsic gating activity of the channel in the absence of regulatory nucleotides. Furthermore, K_{ATP} opening could not be elicited by the natural agonist MgADP applied to the intracellular aspect of the patch, in comparison to vehicle-treated channels, which responded with increased activity as expected (Dabrowski et al., 2003; Gribble et al., 1997a) or indeed by NN414 in the presence of MgATP (Dabrowski et al., 2003). The lack of responsiveness to MgADP is also consistent with the proposition that chronic NN414 treatment affects the gating capability of the channel through actions on SUR1 (Gribble et al., 1997b). In an attempt to demonstrate that functional channels were still present in excised patches from NN414-treated GT1-7 cells, the protease trypsin was applied to the intracellular aspect of the membrane. Previous studies have shown that trypsin re-activates K_{ATP} channels, which have been allowed to undergo the slow inactivation process termed "run-down" observed in inside-out patches from pancreatic β-cells (Lee et al., 1994a, 1994b). Indeed, we show that trypsin treatment re-activates channels in patches from chronic NN414-treated cells to the same extent as patches from vehicle-treated cells. This outcome is consistent with our finding that chronic NN414 does not affect K_{ATP} channel subunit trafficking to the plasma membrane (Beall et al., 2013). Interestingly, trypsin treatment of the intracellular aspect of isolated patches severely reduces the sensitivity of K_{ATP} to inhibition by sulphonylureas, along with complete loss of [^{3}H]-glibenclamide binding (Lee et al., 1994a). A simple explanation for this outcome is that trypsin cleaves SUR1 (or at least the portion of SUR1 containing the binding site for sulphonylureas) from the K_{ATP} complex, thus also potentially removing any NN414 still bound to this site. Alternatively, trypsin treatment cleaves that part of the channel complex responsible for the sustained conformational change underlying the inactivated state, as has been described for other types of K^+ channels (Hoshi et al., 1990; Kirsch and Brown, 1989; Solaro and Lingle, 1992). An interesting question that arises from these findings is whether KCOs induce this effect exclusively on hypothalamic neurons or it is a generalised action on K_{ATP} channels and may be observable in other tissues such as pancreatic beta cells? Additionally, to enable detailed examination of the biophysical nature of the KCO effect similar experiments should be performed on recombinant Kir6.2/SUR1 (or other subunit combinations) channels. Such studies are currently underway.

In conclusion, although short-term application of K_{ATP} channel openers, such as the SUR1-selective opener NN414, appear to have excellent therapeutic potential for normalising defective CRR, the results from *in vivo* rodent work and our *in vitro* cellular data strongly suggests there may be a potential drawback with the use of such an agent given chronically as a preventative therapy. Perhaps the development of structurally modified SUR1-selective K_{ATP} openers with lower efficacy or openers that act at a different site on the channel complex will circumvent this problem. Alternatively, lower dose NN414 or diazoxide and/or intermittent dosage regimes could be tested using this neuronal model and rodents in an attempt to obviate this difficulty prior to further trials in humans.

Author contributions

EH, LH, JF and CB performed experiments and analysed data. EH,

LH, JF, CB, RM and MA contributed to the conception and design of experiments, interpretation of data and drafting and revising the manuscript. RM and MA supervised the study and EH and MA wrote the manuscript. All authors approved the final version. MA and RM are the guarantors of this work.

Acknowledgements

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