Functional Astrocyte-Neuron Lactate shuttle in a human stem cell derived neuronal network

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Disclosure/ Conflict of interest

The authors declare no conflicts of interest.

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Running title

Stem cell derived model of the Astrocyte neuron lactate shuttle

Abstract

The NT2.D1 cell line is one of the most well documented embryocarcinoma cell lines, and can be differentiated into neurons and astrocytes. Great focus has also been placed on defining the electrophysiological properties of the neuronal cells, and more recently we have investigated the functional properties of their associated astrocytes. We now show for the first time that human stem cell derived astrocytes produce glycogen and that co-cultures of these cells demonstrate a functional astrocyte neuron lactate shuttle (ANLS). The ANLS hypothesis proposes that during neuronal activity, glutamate released into the synaptic cleft is taken up by astrocytes and triggers glucose uptake which is converted into lactate and released via monocarboxylate transporters for neuronal use. Using mixed cultures of NT2 derived neurons and astrocytes we have shown that these cells modulate their glucose uptake in response to glutamate. Additionally, we demonstrate that in response to increased neuronal activity and under hypoglycaemic conditions, co-cultures modulate glycogen turnover and increase lactate production. Similar results were also shown following treatment with glutamate, potassium, Isoproterenol and dbcAMP. Together these results demonstrate for the first time a functional ANLS in a human stem cell derived co-culture.

Key words

Astrocyte, Glycogen, Neuron, Stem cell, Lactate

Introduction

Neurons and astrocytes form complex relationships that actively support brain function. Recently the concept of the tripartite synapse[1] has replaced the traditional neurocentric view of the brain. This hypothesis acknowledges that astrocytes not only conduct housekeeping functions but that they also sense neuronal synaptic transmitter release and in turn can modulate neuronal activity through the release of gliotransmitters.

Astrocytes also play a vital role in neurovascular and neurometabolic coupling.[2] Their anatomical position between blood vessels and neurons make them an ideal interface for effective glucose uptake from blood.[3] They also extend numerous processes that connect via end-feet, blood vessels to the neurons and the extracellular space.[4] Some of these processes ensheath neurons whilst others are interconnected with other astrocytes via gap junctions to form astrocytic syncitia.[5]

The astrocyte-neuron lactate shuttle hypothesis (ANLS),[6] has profoundly altered our understanding of brain metabolism. The ANLS hypothesis postulates that during neuronal activity astrocytes respond to glutamatergic activation by increasing glucose utilisation, enhancing glycolysis and lactate release.[6] This lactate is then taken up by neurons via MCT2 transporters and is converted to pyruvate for ATP generation by the TCA cycle.[7]

During prolonged activity, astrocytes may also rely upon their reserves of glycogen.[8, 9] Glycogenolysis has been shown to be essential in rat hippocampal learning[10] and chick bead discrimination tests.[11]

These metabolic roles in the brain have fundamental implications within the context of stem cell derived neuronal networks. The NT2.D1 embryocarcinoma cell line is a well characterised cell line that has been shown to generate both neuronal (NT2.N) and astrocytic (NT2.A) cells.[12, 13] The neurophysiological properties of the neuronal cells derived from these cells has been well characterised. Within the NT2.N population, heterogenous sub-populations are produced such as dopaminergic, cholinergic, GABAergic and

glutamatergic neurons.[14-17] NT2.N cells have been found to generate action potentials on depolarisation.[18, 19] In addition, they also express the high voltage activated calcium channels, pharmacologically classified as L, N, P/Q and R[20] as well as calcium activated BK channels, which are involved in neuronal hyperpolarisation following action potential firing.[21] We have recently demonstrated that NT2.D1 cell derived neuron and astrocyte (NT2.N/A) networks can communicate and so have the potential to interact with each other as observed *in vivo.* [22] If this model is to be applied to the investigation of human neuronal function, it is also essential that the metabolic relationship is sufficiently representative of functional reality.

In this study, we utilised mixed cultures of NT2.N/A cells to investigate the metabolic properties of these cells and measured the response of the astrocytic network to well characterised neuromodulators. We demonstrate NT2.N/A cells express the main tenets of the ANLS model and display functional characteristics consistent with their neuron-astrocyte metabolic coupling. We also found for the first time that human stem cell derived astrocytes store glycogen and that neuromodulators such as glutamate, potassium and noradrenaline can modulate its turnover. This study establishes that stem cell derived astrocytes provide metabolic support to their neuronal counterparts, thus demonstrating a tractable human model which will facilitate the study the metabolic coupling between neurons and astrocytes and its relationship with CNS functional issues ranging from plasticity to neurodegeneration.

Methods

Cell culture

Human teratocarcinoma NT2/D1 cells used in this study were kindly donated by Professor Andrews (University of Sheffield, UK). NT2/D1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose with Glutamax, , with pyruvate (Invitrogen) containing 10% heat inactivated foetal bovine serum (Invitrogen), 100 units/ml penicillin and 100 µg/ml streptomycin. NT2/D1 cells were differentiated to produce mixed cultures of neurons and

astrocytes using the method described by Woehrling et al (2007).[23] Briefly, following treatment with 10μM retinoic acid for 4 weeks, NT2 cells were replated at a lower density (1:3) in RA free medium to separate differentiated cells from undifferentiated cells by sharp striking of the flasks. Cells were then plated into CellBind™ 12-well plates (Corning, USA). Subsequently cells were treated with mitotic inhibitors to suppress the proliferation of non-neural cell types. To supress the growth of undifferentiated cells cultures were treated with 0.1 μM cytosine arabinoside (ARAC) for 1 week, followed by 0.1 μM cytosine arabinoside (ARAC), 3 μM fluorodeoxyuridine (FDU) and 5 μM uridine (U) for 4 weeks. All cells were maintained by incubation at 37°C in a humidified atmosphere of 5% CO_2. Unless otherwise stated all experiments were carried out at 37°C in a humidified atmosphere of 5% CO_2.

Cultures of NT2 astrocytes were isolated from co-cultures according to the method developed by Woehrling *et al. (*2010).[24] NT2.N/A cells were washed 3 X using PBS and then dissociated using Accutase (PAA laboratories, UK). Large NT2.N aggregates settled quickly leaving a single cell suspension containing astrocytes that were replated into a CellBIND 12 well plate (Corning, USA). After incubation for 4h any remaining NT2.N cells were washed off the more adherent NT2.A cells using media. All cells were maintained by incubation at 37°C in a humidified atmosphere of 5% CO_2.

RT PCR

Total RNA was extracted using Trizol® Reagent (Invitrogen), quantified by spectrophotometry and treated with DNase and an RNase inhibitor (Qiagen). 1 μg of total RNA was reverse transcribed using the Nanoscript reverse transcriptase kit (Primer Design, UK) and oligo dT_{15} primers (Primer Design, UK). Real-Time PCR: cDNAs were amplified in a standard 40-cycle SYBR® green real-time PCR reaction using optimised sequence specific primers for GLUT1, GLUT3, MCT2, MCT4, MCT1, GLT-1, GLAST and GLUL according to the manufacturer's instructions. The house keeping genes UBC, B2M, EIF4A2 and C14orf133 (supplied by PrimerDesign Ltd) were assayed under the same conditions as above. The expression of UBC was found to be unchanged under the conditions imposed

and was therefore used in the normalisation of qRT-CR data. Cycling conditions were as follows: 10min at 95˚C, 15s at 95˚C and 1min at 60˚C for 40 cycles, 30s at 95˚C, 30s at 55˚C and 30s at 95˚C. The following formula was used to calculate the relative amount of the transcripts in differentiated (treat) and the undifferentiated samples (control), both of which were normalized to the endogenous controls. ΔΔCT = ΔCT (treat) – ΔCT (control) for biological RNA samples or ΔΔCT = ΔCT (HBRR) – ΔCT (UHRR) for reference RNA samples. ΔCT is the difference in CT between the target gene and endogenous controls by subtracting the average CT of controls from each replicate. The fold change for each treated sample (relative to the control sample (or UHRR) = $2^{-\Delta\Delta CT}$. Fold changes in gene expression using the comparative CT method and statistical analysis were determined using the freely available Relative Expression Software Tool (REST 2009, www.qiagen.com). Fold changes >2 fold were considered significant.

Immunohistochemistry

Cells cultured on PDL/Laminin coverslips (BD Biosciences) were washed with PBS and fixed for 10 minutes with 4% paraformaldehyde. Following fixation coverslips were washed twice with PBS and permeabilized with 0.2% Triton/PBS. Subsequently cells were incubated in 2% BSA/0.2%Triton/PBS for 1 hour to avoid non-specific binding of antibody. Subsequently cells were incubated for 1 hour at room temperature with either mouse anti-GFAP (clone GA5 Millipore, 1:500) or rabbit anti- β-tubulin-III (Abcam, 1:500). After incubation coverslips were washed three times in 2% BSA/0.2% Triton/PBS and then incubated with donkey anti-mouse Rhodamine (1:200, Jackson ImmunoResearch) and goat anti-rabbit FITC (1:200; Jackson Immunoresearch).

After washing, the nuclei were visualized by Hoechst staining (Invitrogen) and mounted with ProLong® Gold Antifade Reagent (Invitrogen). The cells were examined using a Zeiss LSM510 confocal laser-scanning microscope or a Zeiss Axiovert 200M fluorescent microscope.

Glycogen staining

Cytologic localization of glycogen was determined using the periodic acid-Schiff method.[25] Briefly, the cells cultured on coverslips were washed with ice-cold PBS and fixed for 5 minutes at room temperature in methanol. After fixation coverslips were washed three times with 70% (vol/vol) ethanol. Subsequently, the cells were incubated for 30 minutes at room temperature with 1% (wt/vol) periodic acid dissolved in 70% ethanol. After incubation cells were washed three times with 70% ethanol and stained for 60 minutes at room temperature with 0.5% (wt/vol) basic fuchsin (Sigma-Aldrich) dissolved in acid ethanol (ethanol/water/concentrated HCl, 80:19:1). Finally cells were washed three times with 70% ethanol.

Determination of glycogen levels

The method used to determine levels of glycogen in biological samples was first described in.[26] Following the treatment cells were washed three times with ice-cold PBS and then scraped in 300µl of 30mM ice-cold HCl. Subsequently samples were sonicated for 15 seconds and both glycogen and protein content was determination. 10µl aliquots of cell lysate were sampled. To the first aliquot 30µl of acetate buffer (0.1M, pH 4.65) was added. To the second 30µl of 0.1mg/ml of amyloglucosidase in acetate buffer was added. Both samples were incubated for 30min at room temperature. After incubation 200µl of Tris-HCl buffer (0.1M, pH 8.1) containing $MgCl_2$ (3.3 mM), ATP (0.33mM), NADP (38µM), hexokinase (4µg/ml) and glucose 6-phosphate dehydrogenase (2µg/ml) were added to both aliquots and incubated for 30min at room temperature. Standards were prepared using a solution of glucose (1mg/ml) and 1:2 serially diluted using acetate buffer to provide standards ranging from 500µg/ml and 3.9µg/ml. Standards were incubated with acetate buffer for 30min at room temperature and then with hexokinase and glucose 6-phosphate dehydrogenase for 30min at room temperature. Fluorescence of the NADPH formed in the final reaction (excitation: 340 nm; emission: 450 nm) was obtained using SpectraMAX GeminiXS microplate luminometer (Molecular Devices, UK) and SoftMaxPro software. The first aliquot

provides the sum of glucose and glucose 6-phosphate, and the second corresponds to total NADPH derived from glycogen, glucose and glucose 6-phosphate. One mole of glycogen corresponds to one mole of glycosyl units originating from glycogen. The remaining 10 µl sample was used to determine protein content using BCA assay reagent kit from (Pierce, Thermofisher) and read at 590nm using a Thermo multiscan EX 96-well plate reader (Thermofisher).

6-NBDG uptake assay

Uptake of the glucose analogue 6-NBDG (Invitrogen) in NT2.N/A and NT2.A cultures was investigated using methods described by.[27] Briefly, before measurement, culture medium was removed and the cells were washed with Krebs'–Ringer HEPES (KRH) buffer supplemented with 5mM glucose. Glucose in the medium was reduced to 0.5mM and the cells were incubated for 5min. Subsequently the buffer in each well was replaced with KRH buffer containing 0.5mM glucose and 300µM 6-NBDG. Cultures were excited at 488nm and imaged at 505–550 nm emission at 60min, 180min and 360min. Absorbance was measured using SpectraMAX GeminiXS microplate luminometer (Molecular Devices, UK).

Determination of lactate levels

Lactate was measured using the Fluorescent Lactate Assay Kit (Abcam). Briefly, the assay was carried out in a 96-well microplate. 50µl of media samples were used per well mixed with 50µl reaction mix containing 46µl lactate assay buffer, 2µl probe and 2µl enzyme mix. 50µl lactate standards prepared at 0, 0.2, 0.4, 0.6, 0.8 and 1.0 nmol/well and 50µl reaction mix. The reaction was incubated at room temperature for 30 minutes (protected from light) and absorbance at 570nm was obtained using a Thermo multiscan EX 96-well plate reader. All readings were corrected for background.

Stimulation protocol

NT2.D1 cultures were synaptically stimulated using a computer controlled constant current isolated stimulator (STG1002, Multichannel Systems, Germany) and bipolar electrodes, which were placed within close proximity to cells.

Statistics

Results were expressed as the mean of three samples ± standard error of the mean (SEM). Comparisons between treatments were performed using analysis of variance (ANOVA) followed by Dunnett's or Tukey's post-test or Student's T-test using GraphPad Prism Software. Differences were considered significant for p values <0.05.

Results

NT2.D1 derived neurons (NT2.N) and astrocytes (NT2.A) were identified visually by their characteristic morphology. NT2.A cells were identified by their flat phase dark appearance, whilst NT2.N's were typically phase bright and often observed on top of the astrocytic monolayer. These cells were further identified using immunohistochemistry for the specific markers GFAP and β-Tubulin (Fig 1). To identify glycogen in NT2.D1 derived cultures the periodic acid–Schiff method was used to determine the localization of glycogen in cultures.[26] Glycogen was found to co-localize with GFAP positive cells (Fig 1G).

Using realtime qRT-PCR, cultures were also characterised for the expression of genes involved in the ANLS following differentiation. Genes included glucose transporters (GLUT1 and GLUT3), monocarboxylate transporters (MCT1, MCT2 and MCT4), glutamate transporters (GLT-1 and GLAST) and glutamine synthase (GLUL). A comparison of undifferentiated NT2.D1 cells with differentiated co-cultures showed an upregulation of glutamate transporters (GLAST and GLT-1) and a downregulation of GLUT1, MCT1 and GLUT3, whilst other genes remained unchanged (MCT4/2 and GLUL);(Fig 2B). However, Ct values for all genes tested were relatively low (<30) suggesting a high to moderate expression of these genes (Fig 2C).

Glutamate stimulates uptake of fluorescent glucose analogue 6-NBDG in NT2.N/A cultures

Utilization of glucose in NT2.N/A and NT2.A cultures was monitored using the fluorescent glucose analogue 6-NBDG. In control cultures dye accumulated 1.68 fold ± 0.165 SEM over 360min from 0min. Following the treatment of the cultures with glutamate for 180min 6-NBDG uptake in co-cultures increased 2.09 fold ± 0.144 SEM (p<0.01) (Fig 3A). The uptake of 6-NBDG induced by glutamate was completely blocked by ouabain, a Na^+/K^+ ATPase inhibitor. Similarly treatment with Cytochalasin B, a potent inhibitor of GLUT1 and GLUT4 mediated glucose uptake, blocked the uptake of 6-NBDG. Similar results were also obtained in pure NT2.A cultures (Fig 3B).

Hypoglycaemia and neuromodulators stimulate turnover of glycogen and production of lactate

Lactate release and glycogen levels were measured in KREBS ringer HEPES buffer containing no glucose. In order to block glycogen breakdown, cells were also treated with 1,4-dideoxy-1,4-imino-d-arabinitol (DAB), a selective inhibitor of glycogen phosphorylase. Under hypoglycaemic conditions (Fig 3C), cultures released significant amounts of lactate (0.42 ± 0.05mM at 180min, p<0.001) as well as degrading glycogen to 90.62 ± 2.33% at 60min and 74.23 ± 4.48% at 180min, (p<0.01; Fig 3D) of the control. DAB treatment significantly decreased the release of lactate (180min, p<0.05) as well as the breakdown of glycogen (180min, p<0.01). Treatments with known modulators of glycogen phosphorylase, dbcAMP as well as the β_1 and β_2 adrenergic agonist Isoproterenol induced a significant decrease in glycogen levels in comparison to non-treated control (p<0.001) (Fig 4F). In comparison, lactate levels following exposure to dbcAMP did not show any significant increase whilst Isoproterenol treatment resulted in a significant decrease in lactate levels to 0.42 ± 0.03mM, (p<0.01) when compared to the control at 0.77 ± 0.05mM (Fig 4E).

Glutamate and potassium stimulate glycogen breakdown and lactate production in

NT2.N/A co-cultures

In order to test activation of the Na^+/K^+ ATPase by glutamate, cultures were exposed to glutamate both in the presence and absence of ouabain (Fig 4A and 4B). The treatment of the cultures with glutamate significantly increased the lactate levels (1mM glutamate: 0.26 ± 0.01mM, $p<0.05$). Incubation with both glutamate and ouabain reduced the release of lactate back to control levels (control: 0.21 ± 0.01mM, ouabain and 0.5mM glutamate: 0.17 ± 0.01mM) (Fig 4A). In addition, treatment with glutamate caused a significant breakdown of glycogen (1mM glutamate: 76.75 ± 2.36, $p<0.01$; 0.5mM glutamate: 81.73 ± 1.82%, $p<0.05$). This effect was completely blocked by ouabain (Fig 4B).

To determine whether the effect seen in NT2.N/A cultures was mediated by glutamate transporters and not glutamate receptors,[6] cells were exposed to glutamate in the presence of DL-*threo*-β-Benzyloxyaspartic acid (TBOA), a potent glutamate transport inhibitor. Results demonstrated that TBOA treatment blocks both the release of lactate (control: 0.29 ± 0.02mM, TBOA and 1mM glutamate: 0.23 ± 0.01mM) as well as glycogen breakdown in response to glutamate (TBOA and 1mM glutamate: 96.16 ± 2.83% of non-treated control)(Fig 4C and 4D).

Potassium has also been shown to directly activate the Na^+/K^+ ATPase.[28] The levels of lactate following treatment with ouabain alone actually decreased, suggesting a block of basal Na^+/K^+ ATPase activity (Fig 4E). Treatment with potassium at both 15 and 60mM triggered a significant increase in lactate (60mM potassium: 0.77 ± 0.05mM, $p<0.001$, 15mM potassium: 0.86 ± 0.02mM, $p<0.01$). This effect was completely inhibited in the presence of ouabain (Fig 4E). In addition, glycogen levels were significantly reduced following treatment with potassium (60mM potassium: 83.25 ± 0.62%, $p<0.001$, 15mM potassium: 86.28 ± 0.54%, $p<0.01$). This effect was again completely blocked with ouabain (Fig 4F).

NT2.N/A network activity induces glycogen turnover and lactate production

In order to test whether neuronal activity triggers the lactate shuttle in NT2.N/A cultures, the cells were stimulated electrically for 30 minutes using a computer controlled constant current isolated stimulator and bipolar electrodes in the presence and absence of TBOA (Fig 5). Following electrical stimulation the levels of lactate in the media were significantly increased (0.67 ± 0.04mM $p<0.01$) in comparison to control (0.36 ± 0.03mM) (Fig 5C). In addition, glycogen levels inside the cells were significantly decreased (77.98 ± 3.62% of the non-stimulated control $p<0.01$) (Fig 5E). These effects were completely blocked following treatment with TBOA (Fig 5D and 5E). Experiments were carried out at 37^{o}C.

Discussion

The ANLS was first proposed by Pellerin and Magistretti[6] and has since been extensively studied. Experiments supporting the ANLS have been carried out using brain slices, cultured primary neurons and astrocytes as well as isolated nerves and sympathetic ganglia from rat, mouse and chick.[29] In addition, a number of studies have used ^{13}C-NMR spectroscopy to comprehensibly investigate cerebral metabolism both in vivo and in vitro. [30, 31] However, to date, no studies have determined the existence of the ANLS in human stem cell derived cultures.

We have previously demonstrated that neuronal networks derived from NT2.D1 cells signal to astrocytes, and that astrocytic networks communicate via gap junction mediated and gliotransmitter signalling.[22] We now demonstrate for the first time that human stem cell derived astrocytes synthesize glycogen as well as markers associated with its metabolism, suggesting that these cells are well differentiated and resemble mature astrocytes.[32] Co-cultures also respond to neuromodulators and neuronal activity by enhancing glucose uptake as well as inducing glycogenesis and glycogenolysis. Whilst postmitotic co-cultures derived from NT2.D1 cells are widely accepted as a model of the human CNS, some studies have suggested that NT2.NA resemble human fetal primary cells.[33] As such caution in the interpretation of NT2 derived cultures to adult cells should be taken.

Our results support the hypothesis that astrocytes respond to glutamate and potassium, by inducing glucose uptake and aerobic glycolysis, resulting in lactate production via activation of Na^+/K^+ ATPase (an effect that was blocked by cytochalasin B, TBOA and ouabain). We also demonstrate that lactate production and glycogen breakdown occurs in astrocytes following neuronal activation and hypoglycaemia and is blocked using DAB.[34-37]

These results, together with glycogen staining, suggest that NT2.A cells possess the machinery required for glycogen synthesis and glycogen breakdown. The decrease in glycogen levels and subsequent increase in lactate suggest that NT2.A respond to glutamate and potassium, not only by increasing glucose uptake, but also by degrading glycogen as demonstrated in vivo and in primary cultures.[34-36, 38]

Treatments of co-cultures with dbcAMP and the β_1 and β_2-adrenoreceptor agonist isoproterenol induced glycogenolysis within astrocytes. dbcAMP activates glycogen phosphorylase through activation of protein kinase A leading to glycogen breakdown[39] whilst isoproterenol in turn acts by elevating levels of cyclic AMP.[40] Interestingly, although isoproterenol increased glycogen turnover in these cultures, there was a decrease in the level of lactate in the media. However, it has previously been shown that norepinephrine enhances the expression of MCT2 and subsequent lactate uptake in cortical neurons.[41]

The effect of neuronal activity on glycolysis and glycogenolysis was also investigated using electrical stimulation. After 30 minutes, glycogen levels dropped significantly, whilst lactate levels increased. Stimulation-induced glycogen breakdown and lactate release was inhibited by TBOA suggesting that neuronal glutamate release and subsequent astrocytic uptake directly induced glycolysis and glycogeneolysis in these cultures.

Whilst the majority of lactate produced in these co-cultures is predicted to be derived from astrocytes, it cannot be unambiguously proved that neurons do not produce lactate in these cultures. As such, future experiments will aim to determine metabolic flux from both cell

types in monocultures. In addition, more sensitive methods such as ^{13}C-NMR flux analysis could be used in order to comprehensively analyse energy metabolism in these cultures.

Glycolysis and glycogenolysis are important processes in normal functioning of the brain but also in a number of disease processes such as ischemia, hypoglycaemia and Alzheimer's. The focus of this report demonstrates the metabolic coupling of neurons and astrocytes for the first time in a human derived stem cell model. This has important implications in the study of memory formation, plasticity, and neurodegeneration in vitro. In addition, this model may facilitate the future study of the active contribution of astrocytes to the activity of neuronal networks in vitro and indeed, in other basic aspects of human brain function.

Disclosure/Conflict of Interest

The authors declare no competing financial interests

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Titles and legends to figures

Fig 1. Immunofluorescent image of NT2.N/A. Images showing β-Tubulin positive A) NT2.N (green), B) GFAP (Red) positive NT2.A, C) Nuclei stained with Hoechst and D) overlay of GFAP positive astrocytes and β-Tubulin positive neurons. E) Immunostaining of Astrocytes (Green) and F) staining for Glycogen (Red) using the periodic acid Schiff assay. G) Representative image of co-localisation of glycogen and GFAP (yellow), Hoechst (Blue). No glycogen staining was observed in NT2.N. Scale bar 40 and 33.7μM.

Fig 2. Characterisation of ANLS components in NT2.N/A following differentiation. A) Schematic diagram of the ANLS. 1=GLUT1; 2=GLAST/GLT-1; 3=Na+/K+ ATPase; 4=MCT1/4; 5=MCT2. B) mRNA expression of GLUT1, GLUT3, MCT2, MCT4, MCT1, GLT-1, GLAST and GLUL as well the house keeping gene UBC expressed as the average fold change ± SEM (n=3) and C) Ct values ± SEM (n=3).

Fig 3. Effects of glutamate, hypoglycaemia and neuromodulators on 6-NBDG uptake, lactate production and glycogen breakdown in NT2.D1 derived cultures. Uptake was measured in the presence of Glutamate with and without ouabain or Cytochalasin B in, A) NT2.N/A, B) pure NT2.A culture, C) Lactate production under hypoglycaemic conditions (ND = non detected), D) Glycogen turnover under hypoglycaemic conditions, E) Lactate production in cells treated with DbCAMP and Isoproterenol for 180min, F) Glycogen turnover in cells treated with DbCAMP and Isoproterenol for 180min. Results are expressed as the mean ±

SEM (n=3). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

Fig 4. Effects of glutamate and potassium on neuron and astrocytes co-cultures. A) Lactate production in response to glutamate in the presence/absence of ouabain. B) Glycogen turnover in response to glutamate in the presence/absence of ouabain. C) Lactate production in response to glutamate in the presence/absence of TBOA. D) Glycogen turnover in response to glutamate in the presence/absence of TBOA. E) Lactate production in response to potassium in the presence/absence of ouabain. F) Glycogen turnover in response to potassium and in the presence/absence of ouabain. Results are expressed as the mean ± SEM (n=3). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

Fig 5. Effects of induced NT2.N/A network activity on glycogen turnover and lactate production. (A,B) Schematic diagram of the experiment and protocols for electrical stimulation. Production of lactate (C,D) and breakdown of glycogen (E) were measured in response to high frequency electrical activity in the presence and absence of TBOA. Results are expressed as the mean ± SEM (n=3). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

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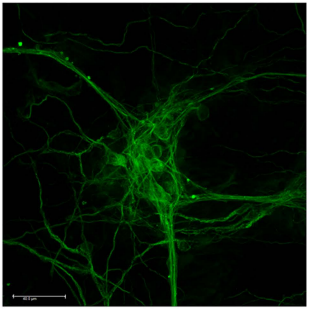

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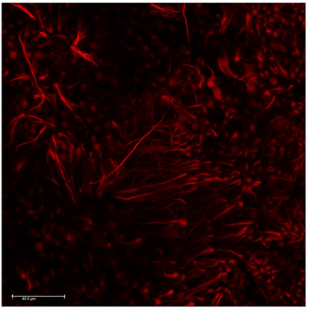

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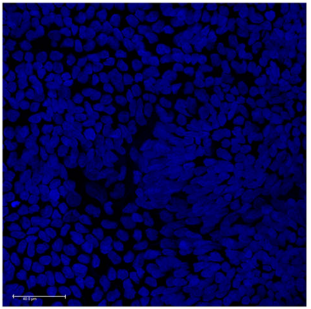

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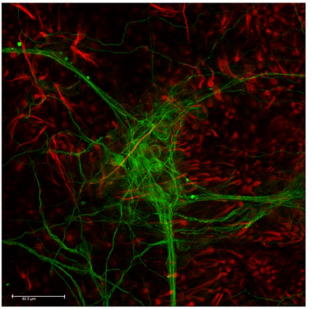

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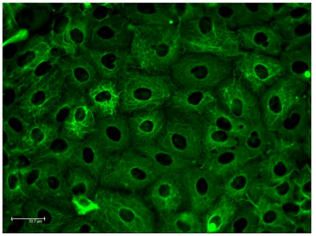

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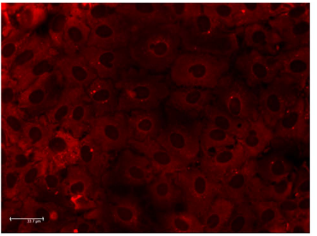

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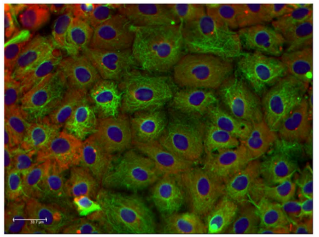
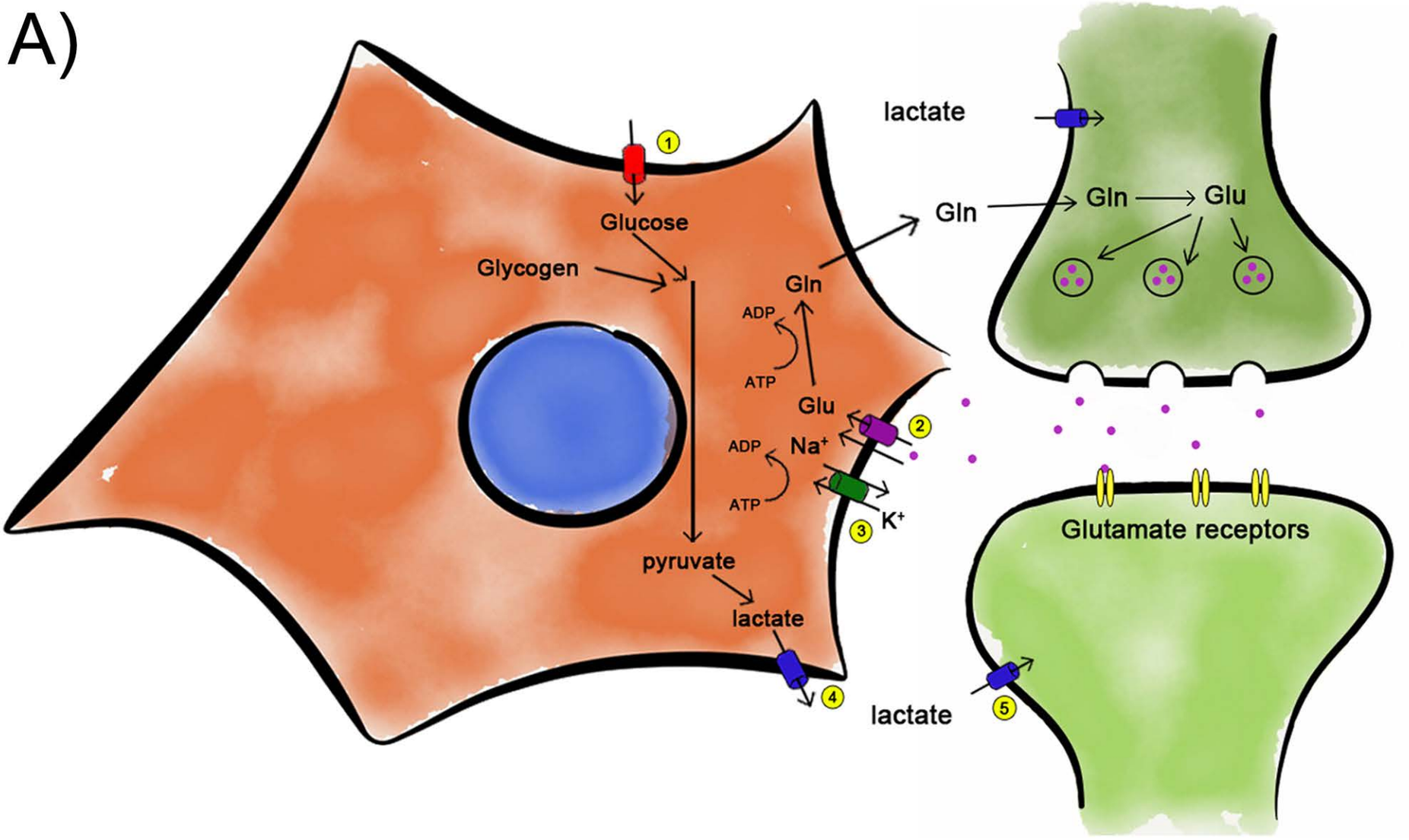


A)



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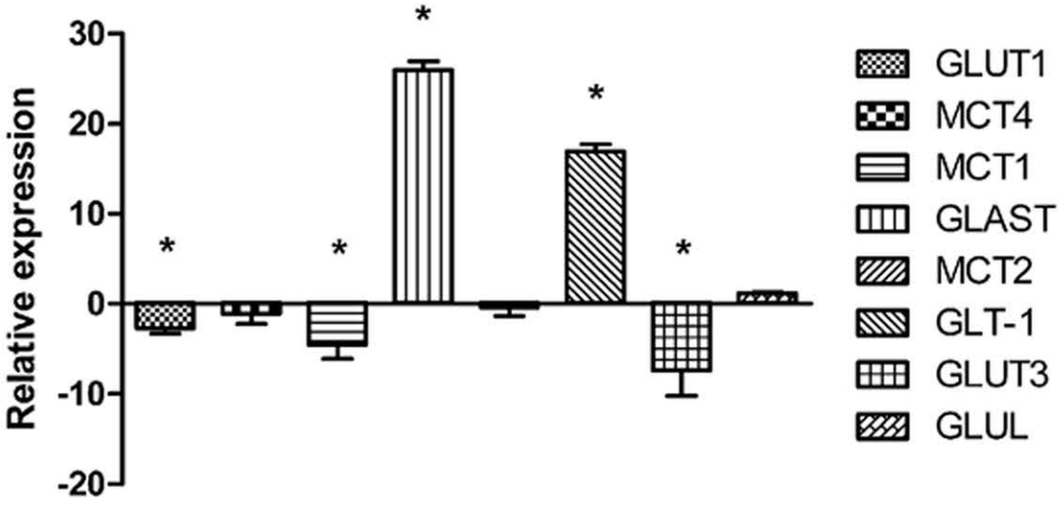
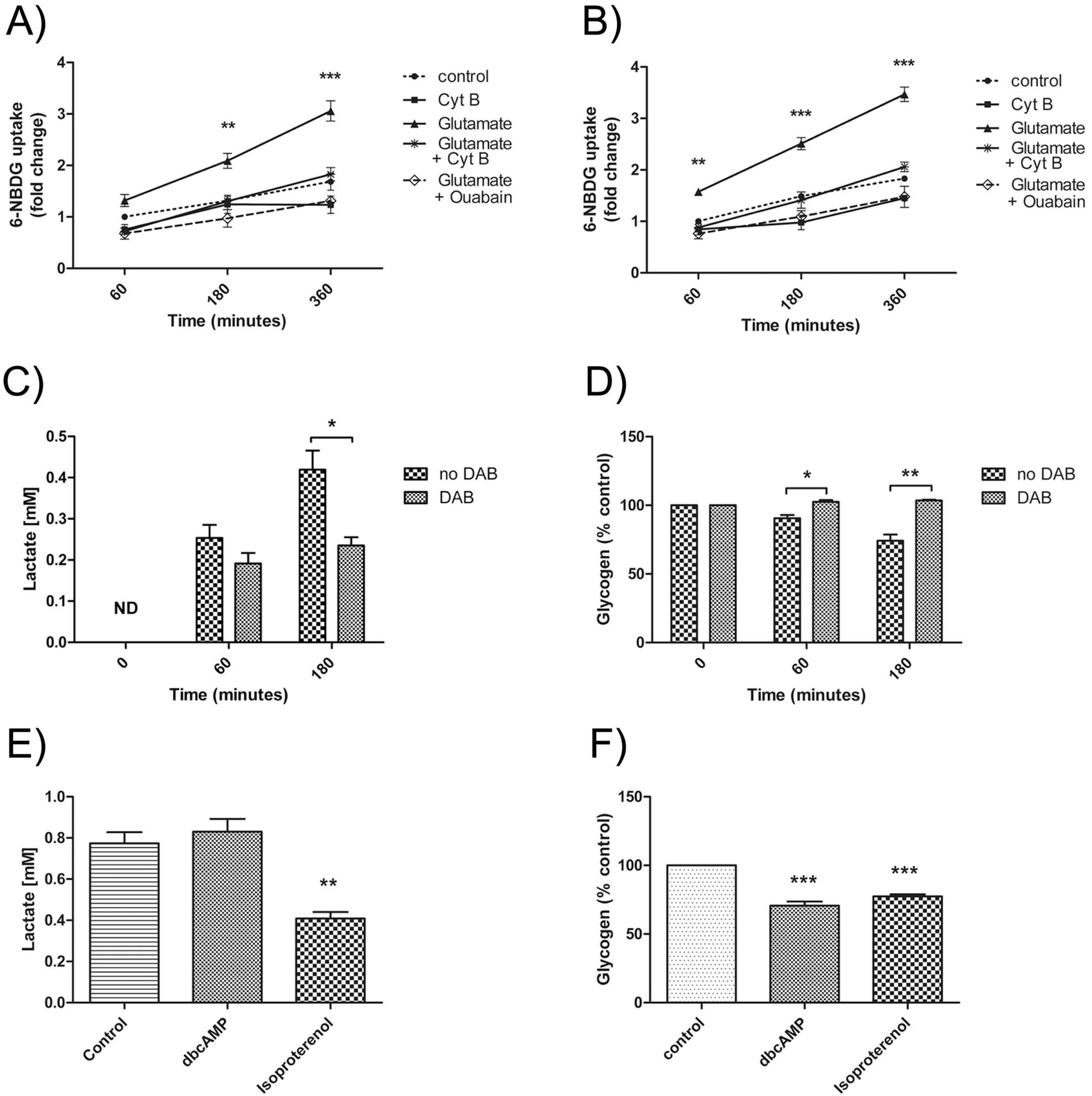
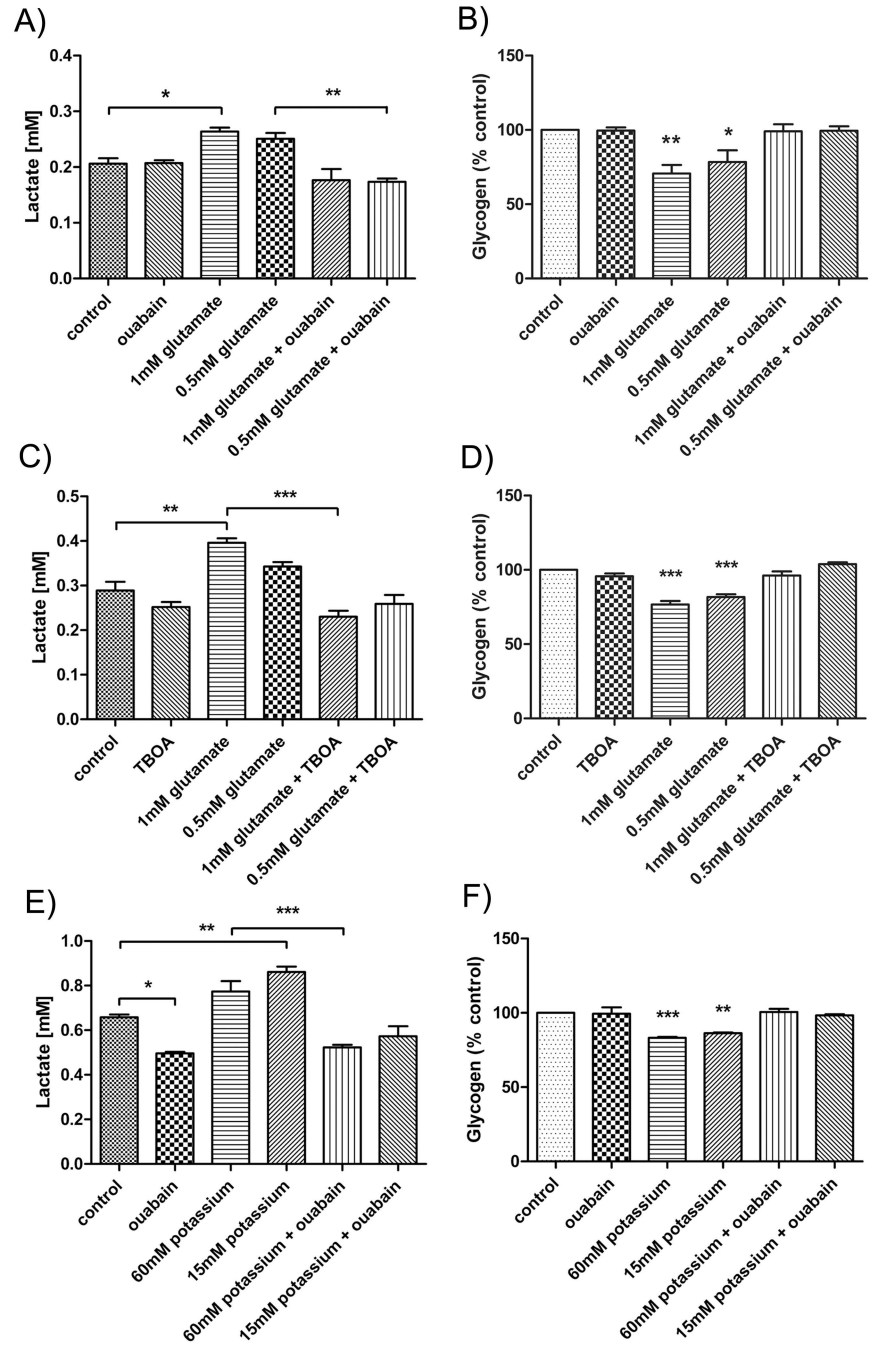
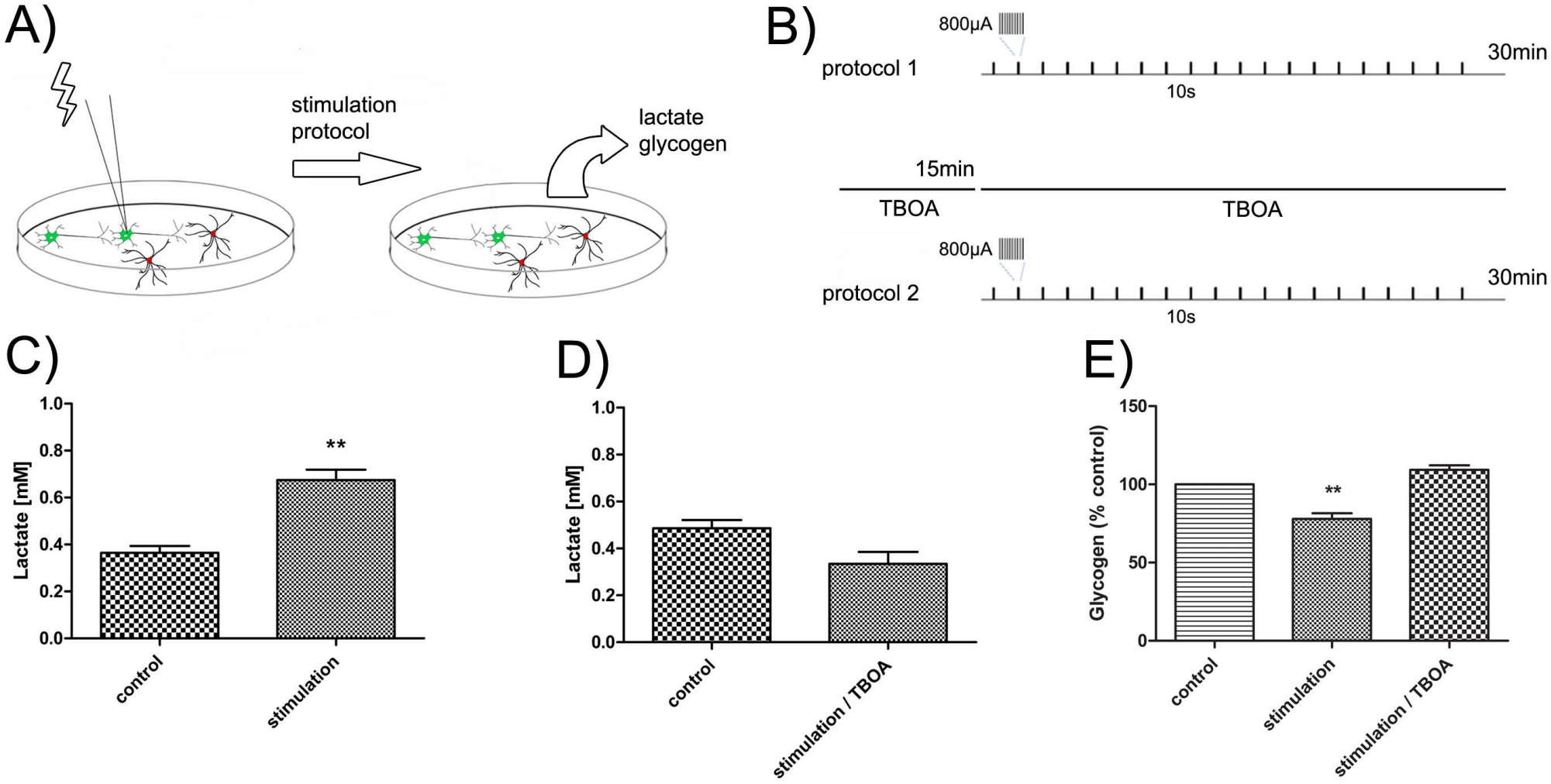


C)

Gene	Ct value ± SEM
GLUT1	19.96 ± 0.54
MCT4	19.97 ± 0.52
MCT1	16.25 ± 0.58
GLAST	16.15 ± 0.28
MCT2	22.35 ± 0.54
GLT-1	17.12 ± 0.42
GLUT3	16.25 ± 0.63
GLUL	14.51 ± 0.27
UBC	14.69 ± 0.26


A)
6-NBDG uptake (fold change)
Time (minutes)
control
Cyt B
Glutamate
Glutamate + Cyt B
Glutamate + Ouabain
B)
6-NBDG uptake (fold change)
Time (minutes)
control
Cyt B
Glutamate
Glutamate + Cyt B
Glutamate + Ouabain
C)
Lactate [mM]
ND
Time (minutes)
no DAB
DAB
D)
Glycogen (% control)
Time (minutes)
no DAB
DAB
E)
Lactate [mM]
Control
dbcAMP
Isoproterenol
F)
Glycogen (% control)
control
dbcAMP
Isoproterenol


A)
Lactate [mM]
0.4
0.3
0.2
0.1
0.0
*
**
control
ouabain
1mM glutamate
0.5mM glutamate
1mM glutamate + ouabain
0.5mM glutamate + ouabain
B)
Glycogen (% control)
150
100
50
0
**
*
control
ouabain
1mM glutamate
0.5mM glutamate
1mM glutamate + ouabain
0.5mM glutamate + ouabain
C)
Lactate [mM]
0.5
0.4
0.3
0.2
0.1
0.0
**
***
control
TBOA
1mM glutamate
0.5mM glutamate
1mM glutamate + TBOA
0.5mM glutamate + TBOA
D)
Glycogen (% control)
150
100
50
0
***
***
control
TBOA
1mM glutamate
0.5mM glutamate
1mM glutamate + TBOA
0.5mM glutamate + TBOA
E)
Lactate [mM]
1.0
0.8
0.6
0.4
0.2
0.0
*
**
***
control
ouabain
60mM potassium
15mM potassium
60mM potassium + ouabain
15mM potassium + ouabain
F)
Glycogen (% control)
150
100
50
0
***
**
control
ouabain
60mM potassium
15mM potassium
60mM potassium + ouabain
15mM potassium + ouabain


A)
stimulation
protocol
lactate
glycogen
B)
800μA
protocol 1
30min
10s
15min
TBOA
TBOA
800μA
protocol 2
30min
10s
C)
Lactate [mM]
1.0
0.8
0.6
0.4
0.2
0.0
**
control
stimulation
D)
Lactate [mM]
1.0
0.8
0.6
0.4
0.2
0.0
control
stimulation / TBOA
E)
Glycogen (% control)
150
100
50
0
**
control
stimulation
stimulation / TBOA