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An investigation into the destructive and adaptive responses of neural cells to stress

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Abstract

Homeostasis within the neuro-glial unit is essential to the longevity of neurons. Conversely, loss of homeostasis, particularly of $\text{Ca}^{2+}$ levels, of redox balance and of ATP, contribute to neuronal loss and dysfunction in many neurodegenerative and neurological disorders. This thesis is centred on better understanding the vulnerability of neurons to stress, as well as adaptive responses to these stresses. Since neurodegenerative conditions associated with $\text{Ca}^{2+}$, redox and bioenergetic dyshomeostasis are often characterised by early dendritic pathology, I first studied dendritic vs. somatic responses of primary cortical neurons to these types of challenges in real-time. Using a wide range of genetically-encoded probes to measure $\text{Ca}^{2+}$, ATP, NADH, glutathione and glutamate, I show that dendrites are selectively vulnerable to oxidative stress, excitotoxicity as well as to metabolic demand induced by action potential (AP) burst activity. However, I provide evidence that neurons undergoing energetically demanding AP burst activity can adjust their metabolic output by increasing mitochondrial NADH production in a manner dependent on the mitochondrial calcium uniporter (MCU), as well as increase their capacity to buffer their intracellular redox balance. Finally, I have studied transcriptional programs in astrocytes triggered by neurons and neuronal activity to better understand adaptive signaling between different cell types in the neuro-glial unit. I developed a novel system combining neurons and astrocytes from closely-related species, followed by RNA-seq and in silico read sorting. I uncovered a program of neuron-induced astrocytic gene expression which drives and maintains astrocytic maturity and neurotransmitter uptake function. In addition I identified a novel form of synapse-to-nucleus signaling, mediated by glutamatergic activity and acutely regulating diverse astrocytic genes involved in astrocyte-neuron metabolic coupling. Of note, neuronal activity co-ordinately induced astrocytic genes involved in astrocyte-to-neuron thyroid hormone signaling, extracellular antioxidant defences, and the astrocyte-neuron lactate shuttle, suggesting that this non cell-autonomous signaling may form part of the homeostatic machinery within the neuro-glial unit.
Lay summary

The brain is made up of several cell types that are in constant communication with each other. Neurons are electrically active cells and are surrounded by ‘star-shaped’ cells called astrocytes. The electric activity of neurons requires energy that is mainly produced by the consumption of oxygen within specialized compartments in the cell called mitochondria. This, however, also causes the production of damaging molecules called reactive oxygen species (ROS). In many neurological and neurodegenerative diseases, there is an imbalance between the production of these stressors and the ability of neurons to cope with the resulting insults. This thesis is centred on better understanding the vulnerability of neurons to stress, as well as adaptive responses to these stresses. I show that certain parts of the neurons, called dendrites, are particularly vulnerable to oxidative stress and the energetic burden induced by electrical activity. However, I also show that neurons have developed a way to adjust their energy production in the face of a change in energy demand by increasing the activity of mitochondria, and that this depends on the uptake of Ca\(^{2+}\). Lastly, I show how neurons and the electrical activity of neurons can induce gene expression changes in neighbouring astrocytes. These changes support a highly debated phenomenon centred on the supply of energy in the form of lactate from astrocytes to neurons. Additionally, I show that neuronal electrical activity increases the expression of antioxidant genes in astrocytes that can potentially protect neurons from ROS.
Declaration

I have carried out the work in this thesis, with minor contributions from lab members indicated where appropriate. Data presented here has not been submitted for any other degree or professional qualification. Some of the graphs in this thesis have been taken from Hasel et al. 2015, Bell et al., 2015, Marland et al. 2015 and Qiu et al. 2013 as stated in the according figure legends.

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Chapter 1
Introduction
Chapter 1: Introduction

In the brain, neurons and astrocytes are closely associated with each other, forming a functionally and morphologically coupled unit (Araque et al., 1999). Homeostasis within this unit is crucial to guarantee neuronal health and survival. For example, astrocytes supply neurons with oxidizable substrates in the form of lactate to fuel the metabolically expensive neuronal action potential firing (Chih and Roberts, 2003). Furthermore, astrocytes shuttle precursors for the synthesis of the antioxidant glutathione to neurons to boost their defense against oxidative stress (Dringen and Hirrlinger, 2003). Consequently, homeostasis within this unit is essential to maintain neuronal health.

Conversely, loss of neuronal homeostasis, especially at the level of Ca\(^{2+}\), redox state and bioenergetics as a consequence of excitotoxicity, oxidative stress and mitochondrial dysfunction, can contribute to neuronal dysfunction and cell death, and can be found in neurodegenerative and neurological diseases (Melo et al., 2011; Zündorf and Reiser, 2011; Nakamura et al., 2012; Mehta et al., 2013). These diseases are often associated with signs of early dendritic pathology, showing spine loss and dystrophic processes that can eventually lead to changes in neuronal physiology, suggesting a selective dendritic susceptibility of dendrites in these conditions (Luebke et al., 2010).

However, neurons have developed systems to maintain homeostasis when challenged by these stressors. For example, when faced with metabolic stress induced by action potential bursting, neurons have to adjust their metabolic output to the change in metabolic demand. In addition to the non-cell autonomous support conferred by astrocytes to cope with this metabolic stress, neurons have their own machinery to maintain homeostasis. Since mitochondria are the main source of ATP in neurons during action potential bursting (Hall et al., 2012), and additionally harbour Ca\(^{2+}\) sensitive NADH producing enzymes (Denton, 2009), it has been postulated that mitochondrial Ca\(^{2+}\) can boost the mitochondrial energy output of synaptically active neurons (Kann and Kovacs, 2006).
The data presented in this thesis will focus on the consequences of excitotoxicity, oxidative stress and synaptic activity on the metabolic and redox state of cortical neurons, as assessed by using genetically-encoded imaging probes, and show a possible explanation for the selective susceptibility of dendrites in neurodegeneration. Furthermore, the role of mitochondrial Ca\textsuperscript{2+} in adjusting neuronal energy production to changes in energy demand will be studied, investigating the role of the mitochondrial Ca\textsuperscript{2+} uniporter (MCU) therein. Finally, to better understand signaling within the neuro-glial unit, a novel tool to investigate non-cell autonomous changes in gene expression will be introduced and applied, showing the dramatic transcriptional changes astrocytes undergo when in contact with neurons or when exposed to synaptic activity.

The thesis will cover an introduction to oxidative stress, antioxidant systems and the role of excitotoxicity in mitochondrial dysfunction. In addition, systems in place to adjust neuronal energy production to energy demand will be introduced, as well as neuron-astrocyte interactions at the level of metabolic coupling, glutamate signalling and the tripartite synapse.
1.1 Oxidative Stress

Oxidative stress has long been thought to be a major player in acute and chronic diseases that are characterized by the occurrence of dysfunctional neurons. Oxidative stress acts on several subcellular components of neurons, including DNA, proteins and lipids, and by causing oxidative damage, can impair neuronal health and function. This introduction will cover the definition of ROS, their main sources, what damage they can inflict and what this means in terms of neuronal dysfunction and neurodegeneration.

1.1.1 Oxidative stress and Neurodegeneration

The free radical theory of aging suggests that free radicals, especially in the form of reactive oxygen species (ROS), are a causative agent in neuronal dysfunction associated with aging and neurodegenerative diseases, by causing damage to cell constituents such as proteins, lipids and DNA (Harman, 1956). Neurons are particularly susceptible to an oxidative burden as they show weak antioxidant defences, including low levels of glutathione and reduced catalase activity (Dringen et al., 2004; Fernandez Fernandez et al., 2012). This, in combination with the high activity, high oxygen demand and therefore high radical production of the brain, give oxidative stress a prominent role in the development of dysfunctional neurons leading to neurodegeneration (Mink et al., 1981; Cadenas and Davies, 2000; Halliwell, 2006). Signs of oxidative stress can be found in each of the major neurodegenerative diseases, including Alzheimer’s disease (AD), Parkinson’s disease (PD) and amyotrophic lateral sclerosis (ALS), and acute brain diseases such as ischemic stroke (Halliwell, 2006). Typical markers of oxidative stress found in these neurodegenerative diseases include oxidized DNA bases, such as 8-OHdG (oxidized guanosine), carbonylated proteins and lipid peroxidation products (such as 4-hydroxynonenal). It is thought that the accumulation of ROS-induced damage can further the disease progression leading to neuronal dysfunction and cell death.
1.1.2 Generation of reactive oxygen species

Oxidative stress occurs when the production of reactive oxygen species (ROS) cannot be counteracted by the cell’s antioxidant defence system (Halliwell, 2006). ROS are defined as any reactive chemical containing an oxygen (Halliwell, 2006). Free radicals are species that contain one or more unpaired electrons, and are therefore quick to react with their surrounding (Halliwell, 2006). Diatomic oxygen (O2) shows very low reactivity with non-radicals but much prefers to react with free radicals, which donate their spare electron(s) to O2 in a step-wise manner, creating ROS. This characteristic of O2 is the reason why mitochondria are a major producer of ROS in the brain (Halliwell, 2006).

The brain consumes roughly 20% of the O2 taken up by the body at resting state, of which about 80-95% are used by the mitochondrial cytochrome oxidase (complex IV) in the electron transport chain (ETC), to eventually generate adenosine triphosphate (ATP) (Mink et al., 1981; Cadenas and Davies, 2000; Halliwell, 2006). While cytochrome oxidase itself does not generate ROS, leakage of electrons from intermediate electron carriers within the ETC can cause the formation of the major ROS, superoxide, or, more correctly, superoxide radical anion \( \text{O}_2^- \), the black dot annotates the unpaired electron, Fig. 1.1) (Halliwell, 2006). O2 can readily be converted into H\(_2\)O\(_2\) by one of three superoxide dismutases (SODs) in the cell, one of which is found directly within mitochondria (SOD2 or Mn-SOD, Fig. 1.1) (Halliwell, 2006). Unlike the negatively charged O2, H\(_2\)O\(_2\) has a neutral charge and can therefore cross biological membranes, allowing it to cause damage outside the compartment in which it was formed.

![Figure 1.1: Step-wise reduction of di-atomic oxygen.](image)

Diatomic oxygen (O\(_2\)) can be converted into reactive oxygen species (ROS) by the step-wise addition of electrons (\(e^-\)). In the electron transport chain (ETC), O\(_2\) can accept one electron and be converted into superoxide radical anion \( \text{O}_2^- \). Superoxide dismutase (SOD)-2 inside mitochondria can then convert
O$_2^-$ into hydrogen peroxide (H$_2$O$_2$). H$_2$O$_2$ can leave the mitochondria due to its neutral charge and is then either neutralized by catalase or glutathione (GSH) or can be further oxidized into hydroxyl radical (OH$^*$) in a Fenton reaction with Fe$^{2+}$. Image adapted from (Imlay, 2013).

The most reactive ROS, however, is generated by further reduction of H$_2$O$_2$ (as when reacting with transition metals such as Fe$^{2+}$ in a Fenton reaction), which creates the hydroxyl radical (OH$^*$, Fig. 1.1) (Halliwell, 2006). OH$^*$ is extremely reactive and can oxidize and therefore damage DNA, proteins, carbohydrates and fatty acids (Augusto et al., 2002; Evans et al., 2004; Davies, 2005).

While the ETC is the major contributor to oxidative stress, ROS are produced by other sources as well, including NAD(P)H oxidase. The function of NAD(P)H oxidase is to transfer one electron to O$_2$ to create O$_2^-$). While initially thought to be mainly present in phagocytic cells and used in the context of an immune response, recent evidence suggests the NAD(P)H oxidase is the primary source of O$_2^-$ production following N-methyl-D-aspartate receptors (NMDAR) activation (Brennan et al., 2009).

1.2 Antioxidant systems

To cope with the constant production of ROS, cells are equipped with an array of antioxidant systems. Antioxidants are present in enzymatic and non-enzymatic form, including SODs, catalase and ascorbate. Of particular interest for the brain, however, are the thiol-based antioxidant systems involving thioredoxin, peroxiredoxin and glutathione.

1.2.1 Thioredoxins and Peroxiredoxins

Thioredoxins are involved in the antioxidant defence against several ROS, including peroxides (Yoshida et al., 2005; Halliwell, 2006). The thioredoxin system decreases cell death following an H$_2$O$_2$ insult, and its inhibition causes oxidative stress (Yoshida et al., 2005). In its reduced state, thioredoxin donates reducing equivalents to oxidized proteins in order to reduce their disulfide bonds (Fig. 1.2). In turn, the dithiol site of thioredoxin is reduced by thioredoxin reductase, which itself relies on
reducing equivalents donated from NAD(P)H (Yoshida et al., 2005). Furthermore, thioredoxin can reduce oxidized peroxiredoxins.

Figure 1.2: Interactions of the thioredoxin (Trx) and peroxiredoxin (Prx) systems.

Prxs can reduce peroxides using their Cys residues and can be reduced by Trxs. Trx can also reduce Cys residues on oxidized cellular proteins. Trx is then reduced by the Trx reductase (TrxR) in an enzymatic reaction requiring reducing equivalents from NADPH. Image taken from (Karlenius and Tonissen, 2010).

Similar to thioredoxins, peroxiredoxins can reduce and therefore detoxify H$_2$O$_2$, and additionally reduce organic hydroperoxides (R-OOH to R-OH) (Wood et al., 2003). Depending on the number of cysteinyl residues peroxiredoxins have to reduce their targets, they can be categorized into 1-Cys and 2-Cys peroxiredoxins, with the latter comprising the bigger group.

Upon exposure to H$_2$O$_2$, one of the Cys of peroxiredoxin is oxidized, creating cystein sulfenic acid (-SOH), which is turn forms a disulfide bond with the remaining Cys. This disulfide bond is then reduced by thioredoxin (Wood et al., 2003). However, if the oxidative challenge is too strong, the Cys of peroxiredoxin can be oxidized to sulfinic (-SO$_2$H) or sulfonic (-SO$_3$H) acid. While it has been thought until recently that peroxiredoxin-SO$_2$H is irreversibly oxidized, two ATP-dependent reductases, sulfiredoxin and sestrin2, have been shown to be able to reduce the cysteines (Budanov et al., 2004).
Our lab has shown recently that sulfiredoxin and sestrin2 are regulated by synaptic activity. Primary cortical neurons undergoing 4h of intense action potential bursting show a drastic increase in sulfiredoxin and sestrin2 mRNA levels, which conferred neuronal protection against oxidative stress (Papadia et al., 2008). This boost in the thioredoxin system was further supported by the synaptic activity-induced drop of Txnip transcription, an inhibitor of the thioredoxin reductase, increasing the reduction of thioredoxin (Papadia et al., 2008).

Next to thioredoxins and peroxiredoxins, the major thiol-based antioxidant system revolves around the tripeptide GSH.

### 1.2.2 Glutathione

Glutathione (GSH) is a tripeptide antioxidant that is ubiquitously expressed throughout the human body and is the most abundant thiol compound in mammalian cells (Meister and Anderson, 1983). It consists of glutamate, cysteine and glycine and is synthesized in a two-step process. First, glutamate and cysteine are linked by the glutamate-cysteine ligase (GCL), creating γ-Glu-Cys. Then, the dipeptide is joined by glycine, a reaction catalysed by the GSH synthetase, creating the functional tripeptide GSH (Meister and Anderson, 1983; Dringen and Hirrlinger, 2003). GSH in turn will inhibit GCL in order to regulate intracellular GSH levels (Richman and Meister, 1975).

The levels of GSH synthesis in neurons are dependent on the availability of its three building blocks, glutamate, cysteine and glycine. Since glutamate and glycine act as neurotransmitters in the brain, and cysteine is neurotoxic in high concentrations, extracellular concentrations of all three amino acids are kept low (Dringen and Hirrlinger, 2003). Neuronal GSH production relies on the availability of cysteine, but neurons show low levels of the cystine/glutamate antiporter system xc- (Zhang et al., 2014) and are therefore thought to not be able to pick up cystine from the extracellular space (Kranich et al., 1996). Astrocytes, on the other hand, show high levels of xc- and are thought to take up cystine and convert it into cysteine (Sagara et al., 1993; Dringen et al., 2000). Similarly, astrocytes surrounding neurons take up extracellular glutamate via EAAT glutamate transporters, where it can either be
converted into GSH or into glutamine, the latter of which can be shuttled to neurons. Astrocytes can then release the synthesized GSH into the extracellular space, where it is converted into cysteinylglycine (Cys-Gly) by the ectoenzyme γ-glutamyl transpeptidase on astrocytes (Dringen et al., 2000). Ectopeptidases on neurons will then convert Cys-Gly into cysteine and glycine, which will subsequently be taken up. All in all, astrocytes supply neurons with all three building blocks of GSH, allowing neurons to synthesize GSH while minimising neurotoxic levels of glycine, glutamate and cysteine (Dringen et al., 2000).

Once GSH has been synthesized, it can reduce peroxides either directly or as an electron donor for glutathione peroxidases (GPX, Fig. 1.3). When reacting with ROS, the thiol-group of GSH donates its reducing equivalents to the radical, making it self reactive. However, GSH will quickly homo-dimerise into glutathione disulfide (GSSG, see below).

\[ \text{H}_2\text{O}_2 + 2 \text{GSH} \rightarrow 2 \text{H}_2\text{O} + \text{GSSG} \]

GSSG can be reduced in a process catalysed by glutathione reductase (GR), transferring reducing equivalents from NAD(P)H to GSSG, creating two GSH tripeptides (Fig. 1.3) (Dringen et al., 2000).

GPXs contain selenium groups that will get oxidized when reacting with peroxides. GSH will then reduce the selenium group in GPx and form GSSG (Halliwell, 2006). Glutathione can also act as a reducing agent for glutaredoxins (GRX) (Holmgren, 1989). GRX are similar to thioredoxins, but show a greater versatility in substrate choice. In general, GRX control the redox state of proteins, using their two thiol groups that can form dithiols upon reacting with protein disulfides (Lillig et al., 2008). GRX oxidises GSH in a redox reaction to reduce its two thiol groups (see also Grx1-roGFP2 in ‘Measuring Redox States’).
Glutathione is mainly present in its reduced state, with GSH being in the mM range and GSSG in the μM range. Therefore, estimating the redox state of a cell using [GSH]:[GSSG] ratios overestimates the effect of changes in [GSSG] (Lillig et al., 2008). [GSH] should therefore be given as [GSH]^2. With the standard glutathione redox potential being -240 mV, the cellular redox state can be calculated using the Nernst equation $E_{\text{GSH}} = -240[\text{mV}]+((R\cdot T)/2F)\cdot\ln(GSSG/GSH^2)$, with the universal gas constant R, the absolute temperature T and the Faraday constant F.

### 1.2.3 Regulation of the glutathione buffer capacity by synaptic activity

The glutathione redox potential is regulated on several levels, including the aforementioned inhibition of GCL by GSH in a negative feedback loop. We showed recently that the glutathione redox potential can be regulated by synaptic NMDAR activity (Baxter et al., 2015). Neurons show an increase in GSH demand during periods of chronic elevated synaptic activity. This is most likely because synaptic activity...
activity is metabolically challenging, increasing O$_2$ consumption and ETC activity, and therefore O$_2$\(^{-}\) generation, which in turn increases the oxidative burden on neurons and oxidises GSH to GSSG (Hongpaisan, 2004; Duchen, 2012). Additionally, NMDAR activation causes NAD(P)H oxidase activity, increasing the production of O$_2$\(^{-}\) and therefore increasing the utilization of GSH (Brennan et al., 2009). Active neurons also show a sharper drop in GSH utilization following an oxidative challenge (Baxter et al., 2015). To counteract this increase in oxidative stress, neurons undergoing chronic elevated synaptic activity increase the levels of GSH biosynthesis, as seen by an increase in glutamate-cysteine ligase (GCL) activity and its transcription (Baxter et al., 2015).

Additionally, synaptic activity increases GSH reduction, as indicated by the increase in glutathione reductase (GR) activity and its transcript levels (Baxter et al., 2015). Unlike the aforementioned supply of GSH tripeptides from astrocytes to allow neuronal GSH synthesis, this increase in GSH biosynthesis and recycling induced by synaptic NMDAR activation is cell-autonomous.

In this thesis, I have applied a genetically-encoded imaging tool (Grx1-roGFP2) to assess E$_{GSH}$ in live cells, in order to elucidate whether the boost in the GSH system described above has a functional consequence on the redox state of a neuron.

Nonetheless, the increased oxidant defence capacity conferred by synaptic activity can be increased non-cell-autonomously by astrocytes via the activation of the major antioxidant transcription factor, NF-E2-related factor 2 (Nrf2) (Baxter et al., 2015).

### 1.2.4 The master antioxidant transcription factor NF-E2-related factor 2

NF-E2-related factor 2 (Nrf2) is the master transcriptional regulator of antioxidant responses in basal conditions and conditions of cellular stress (Ma, 2013). Nrf2 belongs to the cap ‘n’ collar (CNC) sub-family of basic leucine zipper (bZip) transcription factors (Kensler et al., 2007). Nrf2 is poly-ubiquinated and targeted for proteasomal degradation at baseline levels, which is mediated by the Keap1 protein. Keap1 uses its Kelch domain to heterodimerize with Nrf2 via Nrf2’s Neh2 domain, recruiting an E3-ligase complex. Keap1 is also attached to the cytoskeleton by binding to actin-filaments and thereby keeps Nrf2 in the cytoplasm. When cells
experience oxidative stress, cysteine residues in Keap1 are oxidized, causing Keap1 to undergo conformational changes and liberating Nrf2. Once Nrf2 has dimerised with another transcription factor, such as small MAFs (Itoh et al., 1997) or AP-1 (Venugopal and Jaiswal, 1998), it induces transcription of antioxidant genes in the nucleus (Kensler et al., 2007). In the nucleus, Nrf2 can bind to promoter regions containing the antioxidant response element (ARE), which induces the transcription of a wide array of antioxidant genes, including SOD1, homooxygenase-1 (hmox1), catalase and glutamate-cysteine ligase catalytic subunit (Gclc) (Dreger et al., 2009; Hayes and Dinkova-Kostova, 2014).

Nrf2 expression in neurons is extremely low, while astrocytes show high expression levels. Consequently, activation of Nrf2 with pharmacological activators such as tert-butyl hydroquinone (tBHQ) only induces antioxidant gene expression in astrocyte-neuron co-cultures and not pure neuronal cultures (Bell et al., 2015). Additionally, only astrocytes suffer greater cell death following an oxidative insult in Nrf2 KO mice. Hence, unlike the induction of the glutathione system in neurons following NMDAR stimulation, the Nrf2-dependent protection of neurons following an oxidative insult is a non-cell autonomous process. While over-expressing Nrf2 in neurons can confer neuroprotection against oxidative stress (Soriano et al., 2008), it has detrimental effects on early neuronal development (Bell et al., 2015). This is due to the fact that neurons change their redox state early in development to allow redox sensitive JNK- and Wnt-mediated neurite outgrowth, and they do so by epigenetically silencing Nrf2 (Bell et al., 2015).

1.2.5 Measuring Redox States

The redox state of a cell plays an important role in many physiological and pathophysiological events, such as cell development, signalling and death (Dröge, 2002; Balaban et al., 2005). It is therefore important to have tools to investigate the redox state of cells in these different stages and conditions.
The routinely used redox indicators are usually based on chemical reporters that change their fluorescence or absorbance upon oxidation or reduction (Gomes et al., 2005). However, these classical reporters come with several drawbacks. They often require cell lysis, which does not allow for spatial or fast temporal analysis of changes in the redox state, often involve error-prone chemical assays, and lack the sensitivity to measure small redox fluctuations. Additionally, classic redox probes can interfere with the complex interlinked redox systems in the cell, interact with several different radicals and even cause oxidative stress (Rota et al., 1999). Examples of fluorescent redox reporters are the widely used redox probe hydroethidine (HE) and its mitochondria targeted analogue mitoSOX (Kalyanaraman, 2011). Both probes form fluorescently measurable products upon oxidation by O$_2$-$\cdot$. While these probes allow live cell imaging in intact cells, the increase in fluorescence upon oxidation is a one-way reaction and does not allow to investigate the recovery of the redox state following an oxidative insult, as well as undergoing non-specific oxidations (Kalyanaraman, 2011).

These shortcomings prompted the more recent development of a set of genetically encoded redox reporters, including cpYFP, HyPer and roGFP (Hanson et al., 2004; Belousov et al., 2006; Schwarzländer et al., 2014; Shen et al., 2014). The major advantage of these probes is that they can be designed to only measure a particular molecule, as is the case with HyPer, which uses a bacteria-derived protein that only senses H$_2$O$_2$ (Belousov et al., 2006). Additionally, genetically encoded probes can also be targeted to subcellular compartments by adding a targeting sequence to the plasmid expressing the reporter. cpYFP, for example, has been targeted to mitochondria and is often used to measure ‘mitoflashes’, stochastic bursts of mitochondrial O$_2$-$\cdot$ production (Shen et al., 2014). However, cpYFP also shows the limitations of imaging probes that are not designed to specifically measure a certain oxidant or antioxidant, as it was recently identified as actually measuring changes in pH and not O$_2$-$\cdot$ at all (Schwarzländer et al., 2014).

Similar problems arise from roGFP, a GFP containing an artificial dithiol-disulfide pair, which makes its fluorescence redox sensitive (Hanson et al., 2004). While roGFP does show an increase in fluorescence upon oxidation, it is not clear which cellular redox system is actually responsible for roGFP oxidation, or, since roGFP is
reversibly oxidised, its reduction. One major advantage of roGFP, however, is the fact that it is a ratiometric probe. This allows comparing cells or sub-cellular compartments even if they express different levels of the sensor (alternatively, non-ratiometric probes can be calibrated with reagents that give the minimal and/or maximal fluorescence). However, roGFP shows very slow responses to changes in the cellular redox state, which is thought to be due to its non-selectivity concerning redox partners (Gutscher et al., 2008).

Recently, roGFP has been linked to the glutathione (GSH) system by fusing roGFP2 to glutaredoxin1 (Grx1), creating Grx1-roGFP2 (Gutscher et al., 2008). Since Grx1 is using GSH and GSSG as a redox partner, Grx1-roGFP2 can be used to measure the glutathione redox potential, $E_{GSH}$ (Fig. 1.4). This probe shows high temporal resolution and is able to pick up nanomolar changes in GSSG against a backdrop of millimolar GSH. Additionally, since Grx1-roGFP2 interacts with both, GSH and GSSG, Grx1-roGFP2 is reversibly oxidized and allows studying reduction-oxidation dynamics in live cells (Morgan et al., 2011).

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**Figure 1.4: Grx1-roGFP2 reports the GSH:GSSG ratio.**

Grx1-roGFP2 undergoes redox reactions with the cell’s GSH system to report the GSH:GSSG redox potential in terms of fluorescence (image from (Morgan et al., 2011)). See main text for description.

The redox-reaction of Grx1-roGFP2 involves GSSG attacking the nucleophilic cysteine on Grx1, creating S-glutathionylated Grx1 and a free GSH molecule. One of the cysteine residues on roGFP2 then attacks the S-glutathionylated Grx1 intermediate, and in turn, the two cysteine residues on roGFP2 form a disulphide bond, causing a change in the Grx1-roGFP2 fluorescence ratio and the formation of another free GSH molecule (Morgan et al., 2011). This process is entirely reversible by the reaction of Grx1-roGFP2 with GSH instead of GSSG.

In this thesis, I will validate the use of this probe in primary neuronal cultures, show its dynamic range in response to $H_2O_2$, investigate whether Nrf2 over-expression can
boost the $E_{\text{GSH}}$ and probe for a possible subcellular difference in the neuronal redox state and response to oxidative stress.

### 1.3 Excitotoxicity, metabolic stress and mitochondrial dysfunction

It is clear now that excitotoxicity plays a crucial role in acute neurotoxic events, such as ischemic stroke, as well as in chronic neurodegenerative disorders, such as HD and AD (Rothman and Olney, 1986; Choi, 1988; Lipton and Rosenberg, 1994). Excitotoxicity can cause neurons to suffer a bioenergetic collapse, as $\text{Ca}^{2+}$ from chronically stimulated NMDARs can depolarize mitochondria, causing ATP levels to drop and thereby rendering neurons dysfunctional (Abramov and Duchen, 2008). It is believed that excitotoxicity-induced mitochondrial dysfunction and metabolic stress play crucial roles in the aetiology of neurodegenerative diseases. Here, the interplay between excitotoxicity, mitochondrial $\text{Ca}^{2+}$ uptake and mitochondrial dysfunction will be introduced.

#### 1.3.1 Excitotoxicity

It has been known for more than 50 years that the prolonged stimulation of neurons with glutamate can cause cell death (Lucas and Newhouse, 1957) and that glutamate plays a role in neuronal excitation (Curtis et al., 1960). The excitatory and toxic properties of glutamate were later amalgamated into the term excitotoxicity (Olney, 1969). The cell death of neurons following glutamate stimulation has been ascribed to excessive $\text{Ca}^{2+}$ influx through glutamatergic N-methyl-D-aspartate receptors (NMDAR) (Choi, 1992). It was later shown that the $\text{Ca}^{2+}$-dependent induction of neuronal cell death is dependent on the source of $\text{Ca}^{2+}$ entry, as $\text{Ca}^{2+}$ influx following glutamate bath application causes significantly more cell death than $\text{Ca}^{2+}$ influx following global membrane depolarization (while having the same peak $\text{Ca}^{2+}$ response) and $\text{Ca}^{2+}$ influx through NMDARs is far more neurotoxic than $\text{Ca}^{2+}$ influx through non-NMDARs (Tymianski et al., 1993).

When investigating whether inhibition of NMDARs could rescue glutamate-induced cell death, it became clear that NMDAR activity is necessary for neuronal
development, as NMDAR inhibition killed developing neurons, and that NMDAR blockade increased neuronal death following an ischemic insult (Ikonomidou et al., 1999; 2000). These observations suggested that hypo-as well as hyperactivity of NMDARs could induce neuronal cell death, and that the amount of Ca\(^{2+}\) overload following NMDAR stimulation is the determining factor for the severity of neuronal dysfunction and death.

However, the latter theory has been challenged by findings that the stimulation of extra-synaptic NMDARs couples preferentially to cell death, while the stimulation of synaptic NMDARs actually has the opposite effect (Hardingham et al., 2002; Hardingham and Bading, 2010). The activation of extra-synaptic NMDARs has down-stream consequences that include the inhibition of the neuroprotective ERK1/2 and CREB pathways, activation of the pro-apoptotic calpain-STEP-p38 pathway and pronounced mitochondrial membrane (Ψm) depolarization (Hardingham and Bading, 2010).

While all these downstream consequences come together to induce neuronal cell death, the depolarization of Ψm has far reaching consequences for neurons, as mitochondria are central not only for ATP production but also harbour the machinery to initiate cell death (Duchen, 2012).

### 1.3.2 Mitochondrial Ca\(^{2+}\) uptake

Under physiological conditions, mitochondria act as a giant Ca\(^{2+}\) sink that takes up and stores Ca\(^{2+}\) at baseline and when Ca\(^{2+}\) is entering neurons following synaptic stimulations (Kann and Kovacs, 2006).

Ca\(^{2+}\) enters the mitochondrial matrix via the mitochondrial Ca\(^{2+}\) uniporter (MCU). MCU is the 40 kDa pore-forming subunit of a multi-protein complex residing in the inner mitochondrial membrane (IMM), and its genetic identity has been discovered recently by two independent research groups (Baughman et al., 2011; De Stefani et al., 2011). Before its genetic identification, MCU has been studied using the inhibitor Ruthenium Red (RR). RR, however, binds non-specifically to a number of glycoproteins and cation channels and cannot penetrate intact cell membranes (Robert et al., 2001; Hajnóczky et al., 2006).
The existence of MCU has been known for almost 50 years, so it had been well characterised as a low-affinity, high capacity Ca\(^{2+}\) channel with unidirectional cation conductance that is inhibited by RR and resides in the IMM (Raffaello et al., 2012). Its genetic identification was strongly helped by the recent creation of a mitochondrial protein database named MitoCarta, a mass spectrometry based analysis of mitochondrial fractions from 14 different mouse tissues, uncovering 1098 proteins resident in mitochondria (Pagliarini et al., 2008). Using an unbiased in silico approach and an RNAi screen based on the MitoCarta database, candidate gene ccdc109a had been identified to encode the MCU (De Stefani et al., 2011). As expected, its overexpression causes increased mitochondrial Ca\(^{2+}\) uptake, while its RNAi mediated knock-down abolishes Ca\(^{2+}\) uptake into the mitochondrial matrix (De Stefani et al., 2011).

MCU has two transmembrane domains linked by a 9 amino acid linker with a DIME domain protruding into the mitochondrial intermembrane space, while the N- and C-termini face the mitochondrial matrix, which has been confirmed by electron microscopy (Baughman et al., 2011; Martell et al., 2012; Marchi and Pinton, 2014). MCU has been suggested to form a tetramer in the IMM, with eight helices facing inward and a cluster of pore-forming charged residues, creating a negative electrostatic potential facilitating the passage of cations (Raffaello et al., 2013).

Nonetheless, MCU is only the pore-forming subunit of an elaborate multi-subunit complex. MICU1 (mitochondrial Ca\(^{2+}\) uptake 1) was first thought to be the pore-forming subunit, as MICU1 knock-down abolishes mitochondrial Ca\(^{2+}\) uptake (Perocchi et al., 2010), but was later shown to coordinate mitochondrial Ca\(^{2+}\) uptake in combination with its binding partner MICU2 (Csordás et al., 2013; Plovanich et al., 2013; Patron et al., 2014). Both MICU1 and MICU2 have EF-hand domains that allow monitoring Ca\(^{2+}\) levels. While MICU1 will facilitate mitochondrial Ca\(^{2+}\) uptake at high concentrations, MICU2 will inhibit mitochondrial Ca\(^{2+}\) uptake at low Ca\(^{2+}\) levels, allowing for a tight control of MCU activity and therefore mitochondrial Ca\(^{2+}\) uptake (Patron et al., 2014). Interestingly, a cohort of subjects with myopathy and learning difficulties has been identified as carrier of a mutation in MICU1 (Logan et al., 2014; Xu, 2015).
MCUR1 (mitochondria Ca\(^{2+}\) regulator 1) was initially also identified as a subunit of the MCU complex, as MCUR knock-down causes a decrease of stimulation-induced mitochondrial Ca\(^{2+}\) uptake (Mallilankaraman et al., 2012), but was later shown to actually be a mitochondrial cytochrome c assembly factor (Paupe et al., 2015).

Another subunit of the MCU complex is MCUB (ccdc109b), which shares 50% sequence homology with MCU and acts as a dominant negative pore-forming subunit, strongly reducing mitochondrial Ca\(^{2+}\) uptake through the MCU (Raffaello et al., 2013).

The latest addition to the MCU complex is EMRE, or essential MCU regulator, a single membrane spanning, 10 kDa subunit which serves as a linker between MCU, MICU1 and MICU2, and which abrogates mitochondrial Ca\(^{2+}\) uptake when knocked down (Sancak et al., 2013).

Next to the MCU, other proteins have been suggested to mediate mitochondrial Ca\(^{2+}\) influx. These include LETM1, UCP2, UCP3 and RYR1 (see (Rizzuto et al., 2012) for review). LETM1 has recently been shown to be a H\(^+\)/Ca\(^{2+}\) antiporter (Jiang et al., 2009) that can mediate mitochondrial Ca\(^{2+}\) uptake at low cytoplasmic Ca\(^{2+}\) levels and has also been suggested to regulate mitochondrial bioenergetics (Doonan et al., 2014). We have shown recently that the transcripts of these mitochondrial proteins are differentially expressed in different cell types and hippocampal regions, and that their expression is regulated by synaptic activity (Márkus et al., 2016).

### 1.3.3 Mitochondrial membrane depolarization in excitotoxicity

Ca\(^{2+}\) entering the cytoplasm is driven through the MCU into mitochondria due to the strong negative charge of the mitochondrial matrix compared to the cytoplasm, which is created by action of the electron transport chain (ETC). The ETC, located in the inner mitochondrial membrane (IMM), creates an electrochemical proton (H\(^+\)) gradient across the IMM by pumping H\(^+\) into the intermembrane space, creating the mitochondrial membrane potential (Ψm) (Raffaello et al., 2012). The Ψm is around 150-180 mV, negative compared to the cytoplasm, and is necessary for the F1F0 ATPase to synthesise adenosine triphosphate (ATP), by using the energy created when H\(^+\) follow their electrochemical gradient across the IMM into the
mitochondrial matrix (Kann et al., 2003b; Kann and Kovacs, 2006; Raffaello et al., 2012). When Ca\(^{2+}\) enters the neuronal cytoplasm it follows its steep electrochemical gradient through MCU into the mitochondrial matrix, depolarizing \(\Psi_m\) as a result of the flux of positively charged ions into the negatively charged (relative to the cytoplasm) mitochondrial matrix (Fig. 1.5) (Kann and Kovacs, 2006; Glancy and Balaban, 2012).

During an excitotoxic insult, which in primary neuronal cultures can be mimicked by applying high levels of glutamate or the NMDAR agonist NMDA, neurons experience a cytoplasmic Ca\(^{2+}\) overload that cannot be readily cleared by ATP-dependent Ca\(^{2+}\) pumps on the cell membrane. The cytoplasmic Ca\(^{2+}\) overload will quickly create a mitochondrial Ca\(^{2+}\) overload, as Ca\(^{2+}\) is entering the mitochondrial matrix through MCU, causing a \(\Psi_m\) depolarisation (Abramov and Duchen, 2008; Qiu et al., 2013). This NMDAR stimulation-dependent depolarisation of \(\Psi_m\) is thought to lead to the energetic collapse of the neuron, since the F1F0 ATPase requires the \(\Psi_m\) to synthesise ATP (Khodorov et al., 1996; Stout et al., 1998; Vergun et al., 1999). Neurons unable to generate ATP from oxidative phosphorylation cannot easily switch to glycolysis (Herrero-Mendez et al., 2009) and will eventually die from bioenergetic collapse (Duchen, 2012).

![Figure 1.5: Mitochondrial Ca\(^{2+}\) dysregulation in excitotoxicity.](image-url)
Ca\(^{2+}\) entering through NMDARs accumulates in mitochondria through the mitochondrial Ca\(^{2+}\) uniporter (MCU), depolarizing the mitochondrial membrane potential \(\Psi_m\) and depleting NADH levels by affecting the TCA cycle activity and activating PARP. NMDAR stimulation also causes the production of the reactive oxygen species (ROS) \(O_2^-\) by the NADPH oxidase (Nox), which can be converted into \(OH^+\), damaging cellular components and further activating PARP, which in turn releases the pro-apoptotic ‘apoptosis-inducing factor’, AIF. The Ca\(^{2+}\)- and PARP-induced drop in NADH will eventually cause further \(\Psi_m\) depolarisation, and cells undergo energetic failure, as the \(\Psi_m\) is required for ATP production. Image from (Duchen, 2012).

Moreover, our lab has shown recently that when MCU is knocked-down during NMDAR stimulations, mitochondrial Ca\(^{2+}\) uptake and \(\Psi_m\) depolarisation are reduced, resulting in a decrease in neuronal cell death (Qiu et al., 2013). This confirms the notion that the mitochondrial Ca\(^{2+}\) overload is the main driving force depolarising \(\Psi_m\). This had already been shown earlier, as abrogating mitochondrial Ca\(^{2+}\) uptake by inhibiting the MCU using RR completely inhibits \(\Psi_m\) depolarisation (Abramov and Duchen, 2008). Ca\(^{2+}\) entry into mitochondria is thought to only be the initial step in \(\Psi_m\) depolarisation, and poly(ADP-ribose) polymerase-1 (PARP1) activation has been suggested to play a major part in \(\Psi_m\) depolarisation in excitotoxicity (Abramov and Duchen, 2008).

### 1.3.4 Poly(ADP-ribose) polymerase

Poly(ADP-ribose) polymerases (PARPs) are a group of proteins involved in the synthesis and addition of polymers of ADP-ribose to enzymes (D'Amours et al., 1999). PARPs contain a DNA binding domain, and it is now well established that PARPs play a major role in the repair process of DNA nicks and breaks (D'Amours et al., 1999; Dawson and Dawson, 2004). A subset of PARPs reside on DNA strands and, upon DNA damage, will undergo auto-poly(ADP-ribose)tation, which is thought to cause the recruitment of the DNA repair machinery. The substrates of PARPs include proteins involved in the modulation of chromatin structures, DNA synthesis and repair, and transcription factors, but the physiological consequence of poly(ADP-ribose)tation is not known (although it has been suggested it decreases the activity of the targets) (D'Amours et al., 1999; Dawson and Dawson, 2004).

If an insult is severe, PARP1 can initiate cell death pathways, including the translocation of apoptosis-inducing factor (AIF) from mitochondria into the nucleus (Fig. 1.5) (Yu et al., 2002). Pharmacological inhibition or genetic deletion of PARP1
can reduce cytotoxicity in models of ischemia, oxidative stress and excitotoxicity (Eliasson et al., 1997; Endres et al., 1997; Yu et al., 2002; Zhang et al., 2002b). During an excitotoxic insult, mitochondria increase ROS production as a consequence of the Ca\(^{2+}\) overload (Reynolds and Hastings, 1995), which in turn will activate PARP1 as a response to the damage caused by ROS (Fig. 1.5) (Duan et al., 2007). This activation will cause a steep drop in the levels of NAD\(^+\), as it is required to synthesize poly(ADP-ribose). This has severe effects on the metabolic state of the cell, as NAD\(^+\) molecules are required for glycolysis and oxidative phosphorylation (albeit in the reduced form NADH) (Abramov and Duchen, 2008). Additionally, it has been shown that PARP1 activation can inhibit glycolysis by poly(ADP-ribosyl)ating hexokinase, aggravating the bioenergetic stress (Andrabi et al., 2014). Thus, PARP-induced depletion of NAD\(^+\) will induce a neuronal energy collapse and consequently cause \(\Psi_m\) depolarisation and cell death. The PARP1 induced decrease in NADH by excitotoxic Ca\(^{2+}\) is entirely dependent on the uptake of Ca\(^{2+}\) into mitochondria, emphasizing the pivotal role of mitochondrial Ca\(^{2+}\) uptake in the excitotoxicity-induced bioenergetic collapse and cell death (Abramov and Duchen, 2008; Qiu et al., 2013).

1.4 Susceptibility of Dendrites in Neurodegeneration

Neurodegenerative diseases such as AD and PD share the common factors of oxidative stress, excitotoxicity and energy failure. The interplay between these three factors will induce substantial damage to neurons, leading to neuronal dysfunction and eventually cell death. Neurons are very polarized cells, with thin axonal and dendritic processes extending from the cell body. Each of these regions is highly specialized, and can therefore be expected to show distinct biochemical characteristics. Consequently, dendritic, axonal and somatic compartments are thought to react differentially to a pathological insult.

Indeed, in mouse models of neurodegeneration, neurons show changes in dendritic and axonal morphology prior to the occurrence of cell death (Luebke et al., 2010). These changes include loss in spine density, stunted dendritic growth, change in
dendritic growth trajectory and dystrophic swellings (Gomez-Isla et al., 2008; Luebke et al., 2010). In AD, axons and dendrites located near amyloid plaque deposits often show a dystrophic morphology with dendritic spine loss and axonal swelling (Gomez-Isla et al., 2008). Moreover, these changes in dendritic morphology in mouse models of AD can happen in advance of signs of neuronal dysfunction (Rocher et al., 2008), but can eventually lead to severe defects such as neuronal hyperexcitability (Šišková et al., 2014). The inclusion of α-synuclein in PD causes oxidative stress in mitochondria that is more prominent in dendrites when compared to the soma, which indicates a potential vulnerability of dendrites in PD (Dryanovski et al., 2013). Additionally, when comparing young to old nonhuman primates, spine densities in older subjects are strongly reduced, suggesting that even aging can cause changes in dendritic integrity (Dickstein et al., 2007).

Furthermore, during an excitotoxic insult, which is a prominent phenomenon in neurodegeneration and which can be induced in culture by NMDA bath application or oxygen-glucose deprivation, neurons show alterations in neurites in the form of dendritic beading and spine loss (Park et al., 1996; Greenwood and Connolly, 2007). The formation of beads is exclusive to neurites, which is thought to be due to the high density of glutamatergic receptors at synapses compared to the soma (Greenwood and Connolly, 2007). While the mechanism of dendritic beading during excitotoxic insults is not fully understood, it is thought that the drop in ATP following the depolarisation of the mitochondrial membrane potential is causing the accumulation of Na$^+$ inside the dendrite due to a drop in the activity of ATP-dependent Na$^+$ pumps. This in turn causes the accumulation of water and the subsequent swelling of the dendrite (Greenwood and Connolly, 2007; Mizielinska et al., 2009), accompanied by dissociation of microtubules leading to a loss of dendrite integrity (Hoskison et al., 2007). Hence, neurites seem to be particularly vulnerable to excitotoxic insults, resulting in changes in morphology and function that can have detrimental effects on overall brain function.

However, this dendrotoxicity is mostly observational and cellular mechanisms underlying dendrotoxicity and synaptotoxicity have not been fully elucidated yet. In this thesis, I will investigate whether dendrites show a selective vulnerability to excitotoxic, oxidative and bioenergetic challenges by using live-cell imaging of the
neuronal metabolic and redox state, in order to find a possible explanation for the dendritic phenotypes found in neurodegeneration.

1.5 Synaptic activity and metabolic stress

The electrical activity of neurons is an energetically and metabolically expensive process (Harris et al., 2012), which is reflected in the fact that the brain uses 20% of the O\textsubscript{2} consumed at resting state, while constituting only 2% of the total body mass (Mink et al., 1981). In the brain, glucose taken up from the blood will be completely oxidized via glycolysis and oxidative phosphorylation, with the latter producing 93% of the total ATP generated (Sokoloff, 1960). This ATP is mainly spent at the post-synapse where it is used to reverse ion fluxes after an action potential (Harris et al., 2012).

While only 7% of the total ATP produced in the brain is coming from glycolysis (Sokoloff, 1960), it is thought that astrocytes take up glucose from the blood stream, undergo glycolysis and convert pyruvate into lactate and shuttle it to neurons (Pellerin and Magistretti, 1994).

Since neurons heavily rely on ATP for synaptic transmission, and given that the rate of action potential firing can change rapidly, it is pivotal for neurons to have a system in place that adjusts energy demand to energy output. There are several mechanisms suggested in neurons working to avoid catastrophic ATP loss, and since oxidative phosphorylation is the key producer of ATP, it is not surprising that several of these mechanisms are thought to be located on or within mitochondria.

1.5.1 Adapting energy output to energy demand at the mitochondrial level: A central role of Ca\textsuperscript{2+}

Neuronal activity can change drastically over time and therefore neurons have to adapt to changes in metabolic demand by regulating their energy output. At a first regulatory level, a change in substrate availability, by the law of mass action, will automatically result in a change in the rate of product generation (Chance and Williams, 1955). Additionally, many metabolic enzymes are end-product inhibited,
thereby regulating their own activity. However, in excitable cells, adapting energy production to demand needs to be more tightly regulated. Since neurons primarily use oxidative phosphorylation as a source of ATP during neuronal activity (Hall et al., 2012), it is mainly at this level that neuronal energy levels are regulated. The metabolic adaptation response that has been studied most thoroughly (albeit not in neurons) is performed by three dehydrogenases residing within the mitochondrial matrix, and is made up of pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (α-KGDH) and isocitrate dehydrogenase (IDH) (see (Denton, 2009) for review). All three dehydrogenases supply the ETC with reducing equivalents in the form of NADH and thereby facilitating ATP synthesis through oxidative phosphorylation. Studies from as early as the 70s showed that purified protein extracts of PDH (Denton et al., 1972), α-KGDH (McCormack and Denton, 1979) and IDH (Denton et al., 1978) show an increase in activity upon the addition of Ca$^{2+}$ (Fig. 1.6). Moreover, using isolated mitochondria, the Ca$^{2+}$-dependent increase in dehydrogenase activity of PDH and α-KGDH was later shown to be regulated by mitochondrial Ca$^{2+}$ (as shown by RR inhibition of mitochondrial Ca$^{2+}$ uptake) (Denton et al., 1980).

1.5.2 α-Ketoglutarate dehydrogenase and Isocitrate dehydrogenase

α-KGDH and IDH are both present within the TCA cycle and subject to a complex activity regulation, as both are rate-limiting enzymes of the TCA cycle (Denton et al., 1980). α-KGDH catalyses the conversion of α-ketoglutarate to succinyl-CoA and is end-product inhibited by NADH and succinyl-CoA (Smith et al., 1974). Ca$^{2+}$ activates α-KGDH by binding directly to the enzyme, although the binding sites have not yet been identified (Rutter and Denton, 1988; Denton, 2009). When ATP levels drop, the affinity of α-KGDH for its substrate α-ketoglutarate and for Ca$^{2+}$ is increased (Rutter and Denton, 1988).

Upstream of α-KGDH, IDH produces NADH in the conversion of D-isocitrate to α-ketoglutarate. ATP and NADH inhibit IDH, and similar to α-KGDH, a drop in ATP/ADP will increase its affinity for Ca$^{2+}$ (Rutter and Denton, 1988). Ca$^{2+}$ directly
binds to IDH, increasing its activity, but as with α-KGDH, the Ca\(^{2+}\) binding site has not yet been established (Nichols et al., 1995).

Thus, α-KGDH and IDH are NADH producing dehydrogenases within the mitochondrial matrix, which, upon an increase in Ca\(^{2+}\) levels will boost NADH production and TCA cycle progression, and they will do so particularly effectively when ATP levels are low.

![Mitochondrial Ca\(^{2+}\) regulates cellular energetics.](image)

**Figure 1.6: Mitochondrial Ca\(^{2+}\) regulates cellular energetics.**

Ca\(^{2+}\) entering mitochondria through the mitochondrial Ca\(^{2+}\) uniporter MCU activates matrix dehydrogenases, leading to an increase in tricarboxylic acid (TCA) cycle activity, generation of NADH and hence ATP production by the electron transport chain (ETC). α-ketoglutarate dehydrogenase (α-KGDH) and isocitrate dehydrogenase (IDH) reside within the TCA cycle and bind Ca\(^{2+}\) directly, while pyruvate dehydrogenase (PDH) connects the extra-mitochondrial production of pyruvate with the TCA cycle and is regulated by the Ca\(^{2+}\) sensitive pyruvate dehydrogenase phosphatase (PDP). Adjusted from (Rizzuto et al., 2012).

### 1.5.3 Pyruvate dehydrogenase

PDH catalyses the conversion of pyruvate to acetyl-CoA, a step that connects the extra-mitochondrial production of pyruvate (either through glycolysis or conversion
of lactate) to the intra-mitochondrial TCA cycle. In mammalian cells, PDH is present in a complex (PDHC) consisting of 3 subunits, with the E2 subunit (dihydrolipoamide acetyltransferase) making up the 60-subunit core. Attached to the E2 core are the subunits E3 (dihydrolipoamide dehydrogenase) and E1 (pyruvate decarboxylase), creating the 8 MDa PDHC (Patel et al., 2014). E1 and E2 constitute the component of the PDHC that recognizes and binds the substrate pyruvate (Perham, 1991). E1 itself consists of two E1α and two E1β subunits and the reaction catalysed by E1, the decarboxylation of pyruvate, is the first and irreversible step in the production of acetyl-CoA (Perham, 1991; Denton, 2009). Similar to IDH and α-KGDH, PDHC is inhibited by its end products NADH, acetyl-CoA and ATP (Patel and Roche, 1990). However, the PDHC is also subject to phosphorylation-dependent activity control, and because of its central role within the PDHC, E1 is the subunit that is targeted by phosphorylation (Rardin et al., 2009). E1 activity is controlled by phosphorylation of Ser\(^{232}\), Ser\(^{293}\) and Ser\(^{300}\) of the E1α subunit (PDHE1α), with Ser\(^{293}\) being the most powerful inhibitory phosphorylation site (Linn et al., 1969; Rardin et al., 2009). The inactivating phosphorylation of PDH is catalysed by 4 PDH kinase (PDK) isoenzymes in humans (3 in rodents) and show different affinities for the 3 serine phosphorylation sites on PDHE1α (Korotchkina and Patel, 2001). Activating de-phosphorylation of PDHE1α, on the other hand, is catalysed by two PDH phosphatases (PDP1 and PDP2) (Teague et al., 1982). While PDHE1α phosphorylation by PDKs is not regulated by Ca\(^{2+}\) (Sugden and Holness, 2003), PDP1 can bind Ca\(^{2+}\), which increases its affinity for the E2 subunit of PDH (Pettit et al., 1972). This in turn increases the affinity of PDP1 for the phosphorylation sites on PDHE1α, de-phosphorylating PDHE1α and by that increasing PDH activity (Pettit et al., 1972). Therefore, increasing mitochondrial Ca\(^{2+}\) will cause PDP1 to de-phosphorylate PDHE1α, which in turn increases PDH activity and therefore NADH production and TCA cycle activity. It is noteworthy, however, that PDK and PDP activity are also regulated by the PDH products acetyl-CoA, NADH as well as ATP (Glancy and Balaban, 2012).

However, despite being extensively studied in other tissues, it is not known what the role of PDH phosphorylation is in neuronal bioenergetics and whether PDH
phosphorylation can be regulated by synaptic activity. In this thesis, I will investigate whether synaptic activity can regulate PDH activity, and investigate the role of mitochondrial Ca\(^{2+}\) and the MCU in PDH phosphorylation during network bursting.

1.5.4 Regulating mitochondrial energy output in neurons

The Ca\(^{2+}\) regulation of PDH, \(\alpha\)-KGDH and IDH link the mitochondrial uptake of Ca\(^{2+}\) ions to an increase in energy output, which is particularly interesting in the context of synaptic activity, since an increase in neuronal activity is metabolically challenging and at the same time induces mitochondrial Ca\(^{2+}\) transients. However, the role of matrix dehydrogenases in neurons has not been described so far. Nonetheless, the mitochondrial energy production of neurons has been studied using NAD(P)H autofluorescence.

1.5.5 Neuronal metabolic adaptation measured by NAD(P)H autofluorescence

NADH and NADPH have autofluorescent properties that are widely utilized to measure energy production in neurons (Shuttleworth, 2010). Cells are excited at 340-360 nm and the emission measured >400 nm. Since NADH levels are 10 fold higher than NADPH levels in the mouse brain (Klaidman et al., 1995), NAD(P)H autofluorescence is mainly due to NADH.

In neurons, Ca\(^{2+}\) transients induced by synaptic stimulations can boost the production of NADH, as repetitively shown using NAD(P)H autofluorescence in brain slices (for example (Kann et al., 2003a; 2003b; Brennan et al., 2006; Hall et al., 2012)), mainly using high-frequency stimulation over a short period of time. After an initial dip in NADH due to an increase in energy demand, neurons increase their energy production, as seen in an overshoot of NAD(P)H autofluorescence (for review see (Shuttleworth, 2010)). While the overshoot is frequently explained to be the effect of an increased neuronal mitochondrial energy output (Brennan et al., 2006; Hall et al., 2012), there is evidence that the NADH overshoot is due to increased glycolysis in neighbouring astrocytes (see below) (Kasischke et al., 2004). Nevertheless, the
NADH overshoot is entirely dependent on the presence of Ca$^{2+}$ and, moreover, mitochondrial Ca$^{2+}$ uptake through the MCU (as shown pharmacologically), indicating that it is mitochondrial Ca$^{2+}$ that stimulates the NADH production following synaptic activity, potentially by activating matrix dehydrogenases (Duchen, 1992).

In this thesis, I will try to confirm the role of mitochondrial Ca$^{2+}$ in stimulating NADH production, by using genetic (rather than pharmacological) manipulations of the MCU, and by applying NAD(P)H autofluorescence as well as a novel genetically-encoded imaging tool measuring cytosolic NADH/NAD$^+$ ratios, Peredox.

Nevertheless, alternatively to a possible MCU-dependent induction of matrix dehydrogenases, Ca$^{2+}$ entering neurons during action potential firing can also activate the aspartate-glutamate exchanger aralar.

1.5.6 The neuronal aspartate-glutamate exchanger Aralar

The Ca$^{2+}$-dependent production of NADH can also be mediated by the neuronal aspartate-glutamate exchanger aralar (AGC1/Slc25a12) residing in the IMM (Pardo et al., 2006; Llorente-Folch et al., 2013; 2015). The IMM is not permeable to NADH (Purvis and Lowenstein, 1961), and in order to transfer cytoplasmic NADH into the mitochondrial matrix to supply the ETC with reducing equivalents, cells have developed a shuttle system that does not rely on the direct transport of NADH across the IMM. Instead, NADH in the cytoplasm is reduced to NAD$^+$ in a reaction that creates malate, which can be transported across the IMM, anti-porting α-ketoglutarate. Once malate is inside the mitochondrial matrix, malate is oxidized to oxaloacetate, generating NADH. Oxaloacetate is then further converted into aspartate, which is transported across the IMM using the aspartate-glutamate antiporter aralar (Chappell, 1968; Llorente-Folch et al., 2015). Aralar has a Ca$^{2+}$ binding EF-hand domain in its N-terminal tail facing the mitochondrial intermembrane space, and aralar has been shown to be activated by increasing Ca$^{2+}$ levels (Palmieri et al., 2001).
The genetic deletion of aralar is homozygous lethal and aralar knock-out neurons show a weakened increase in oxidative phosphorylation upon membrane depolarisation (Llorente-Folch et al., 2013) likely as a consequence of a reduced uptake of NADH into mitochondria (Pardo et al., 2006). Since aralar activity increases the availability of NAD$^+$ in the cytoplasm that is needed to drive glycolysis, aralar activity also facilitates pyruvate production, feeding into the NADH producing TCA cycle (Llorente-Folch et al., 2013).

1.5.7 Non-cell autonomous supply of oxidizable substrates

While the energy regulation at the mitochondrial level via IMM-located aralar and possibly matrix dehydrogenase activity are cell autonomous mechanisms, it is thought that astrocytes can confer substrate support to neurons in a non-cell autonomous manner. Astrocytes and neurons are metabolically linked, as astrocytes take up glutamate after it has been released by neurons during synaptic activity (Bergles and Jahr, 1997) by way of the Na$^+$-glutamate co-transporter EAAT2 (excitatory amino acid transporter 2, GLT-1, Slc1a2) (Danbolt, 2001), which is predominantly expressed in astrocytes (Danbolt, 2001; Zhang et al., 2014). Astrocytes will then either convert glutamate into glutamine or use glutamate as a precursor to fuel the TCA cycle (Fig. 1.7).

In the glutamate-glutamine cycle, astrocytes convert glutamate to glutamine using glutamine synthetase, an enzyme not present in neurons (Norenberg and Martinez-Hernandez, 1979), and shuttle it back to neurons, in order to replenish their glutamate pool (Benjamin and Quastel, 1975). However, astrocytes can also convert glutamate into α-ketoglutarate (Hertz et al., 1999), catalysed by glutamate dehydrogenase, a reaction that is actually faster than the conversion of glutamate into glutamine (Yu et al., 1982). Nonetheless, only a small percentage (~15%) of glutamate is used in the TCA cycle (McKenna et al., 1996). With an increase in the extracellular concentration of glutamate, however, astrocytes can more than double the amount of glutamate used in the TCA cycle (McKenna et al., 1996), where it can eventually be completely oxidized into CO$_2$ (Sonnewald et al., 1993). However, instead of being fully oxidized in astrocytes, the...
glutamate-derived α-ketoglutarate can be converted into pyruvate and lactate (via succinate and malate in the TCA cycle) and both can subsequently be shuttled to neurons (Hertz et al., 1999). Hence, when neurons increase their glutamate release from the synapse, astrocytes will take up more glutamate (McKenna et al., 1996) and metabolize it to eventually fuel the increased astrocytic glutamate uptake and potentially the increased neuronal energy demand. A better studied source of lactate, however, is glycolysis within astrocytes (Fig. 1.7).

Glycolysis in astrocytes is stimulated when glutamate taken up via EAAT Na\(^+\)-glutamate co-transporters increases the intracellular Na\(^+\) concentration in astrocytes, which causes the Na\(^+\)/K\(^+\)-ATPase to increase its activity in order to restore the astrocytic Na\(^+\) levels. Since the Na\(^+\)/K\(^+\)-ATPase is fuelled by membrane-bound glycolytic enzymes, this will increase glycolysis in astrocytes and therefore the production of pyruvate and lactate, which in turn will be shuttled to neurons (Pellerin and Magistretti, 1994; Magistretti et al., 1999).

This ‘lactate-shuttle hypothesis’ has been subject to a long standing discussion that has not been resolved as of yet. Strong evidence for the hypothesis comes from studies showing that, as mentioned above, the Na\(^+\)/K\(^+\)-ATPase (Paul et al., 1979; Mercer and Dunham, 1981; Dubinsky et al., 1998), H\(^+\)-ATPase (Lu et al., 2001) and Ca\(^{2+}\)-ATPase (Paul et al., 1989) are closely associated with glycolytic proteins. Furthermore, stimulating astrocytes with glutamate markedly increases levels of lactate and pyruvate in the medium, which is a consequence of increased glycolysis within astrocytes following the activation of the glycolysis driven Na\(^+\)/K\(^+\)-ATPases (see above) (Pellerin and Magistretti, 1994). In addition, replacing glucose with lactate has no effect on synaptic function (evoked population spikes) (Schurr et al., 1988) or synaptic vesicle turn over rates (Morgenthaler et al., 2006), showing that lactate is sufficient to drive synaptic activity and dynamics. Further support comes from in vivo studies using NAD(P)H autofluorescence imaging to show that, following neuronal stimulation, neurons show an increase in oxidative phosphorylation followed by an increase in astrocytic glycolysis (Kasischke et al., 2004).
Figure 1.7: The lactate-shuttle hypothesis.

Astrocytes take up glucose (Glc) from the blood stream, convert it into lactate and shuttle it to neurons by way of the monocarboxylate transporter (MCT), where it is converted into pyruvate and oxidized in mitochondria using oxidative phosphorylation (OP). Glutamate (Glu) spilling from neuronal synapses is taken up by astrocytes where it is converted into glutamine (Gln) and shuttled back to neurons to refresh the neurotransmitter pool. The glutamate-glutamine cycle activates glycolysis in astrocytes and by that supports the lactate shuttle. Image from (Chih and Roberts, 2003).

However, it begs the question whether neurons require lactate and astrocytic glycolysis at all, since neurons can readily take up and oxidize glucose, given they express the neuron-specific glucose transporter GLUT3 (Leino et al., 1997) and hexokinase, the rate-limiting enzyme in glycolysis (Cimino et al., 1998). Furthermore, there is evidence that, at least initially, glycolysis is not necessary to support neuronal activity at all, since blocking the lactate dehydrogenase (LDH), which inter-converts lactate and pyruvate, had no effect on O$_2$ or NADH consumption rates following neuronal stimulations (Hall et al., 2012).

Independent on whether the lactate-shuttle hypothesis eventually holds true or not, it is clear that astrocytes play a major role in supporting and shaping neuronal function, be it via the release of trophic factors, the shuttling of antioxidants and energy.
substrates or the recycling of neurotransmitters. The same holds true vice versa, as neurons and neuronal activity can greatly impact astrocyte behaviour, changing their metabolic activity, morphology and induce astrocytic Ca\(^{2+}\) transients. The next paragraph will introduce the major factors of this bidirectional neuron-astrocyte signalling.

1.6 Neuron-astrocyte interactions

This introduction has already covered several aspects of the astrocytic support of neurons. This includes the protection of neurons from oxidative stress by astrocytic Nrf2 signalling, shuttling of GSH components from astrocytes to neurons to regulate the neuronal redox state, and the shuttling of glutamine and lactate from astrocytes to neurons to support synaptic activity and neuronal bioenergetics. It is clear now, however, that astrocytes also play a role in neuronal circuit formation by contributing to neuronal synapse development, neuronal activity and synaptic transmission, forming a close morphological and functional connection through what has been coined ‘tripartite synapse’ (Fig. 1.8) (Araque et al., 1999). On the other hand, astrocytes express neurotransmitter receptors that allow them to receive synaptic information, inducing complex astrocytic Ca\(^{2+}\) transients (Khakh and McCarthy, 2015). In turn, astrocytes are able to tune synaptic activity by secreting transmitters themselves, in a process termed ‘gliotransmission’ (Araque et al., 2014). This paragraph will introduce these bidirectional neuron-astrocyte interactions.

1.6.1 Regulation of synapse formation and dynamics by astrocytes

Astrocytes were long thought to only play a passive role in the brain, acting as a mere glue to give structural support to their neighbouring neurons. However, astrocytes are intimately connected to neurons, with astrocytes ensheathing somata, dendrites and axons of close-by neurons (Peters and Feldman, 1976; Theodosis et al., 2008). During synaptogenesis, astrocytes refine their intricate morphology and each astrocyte will eventually contact several neurons and up to 100,000 synapses with their thin processes (Bushong et al., 2002). This intimate morphological and
functional connection of the pre- and post-synapse of neurons with astrocytic processes has been termed ‘tripartite synapse’ (Fig. 1.8) (Araque et al., 1999). Furthermore, astrocyte development is closely correlated with the appearance of neuronal synapses (Ullian et al., 2001). It is therefore not surprising that neurons cultured in the presence of astrocytes show an increased number of spontaneous and evoked synaptic events when compared to neurons cultured on their own (Pfrieger and Barres, 1997). It was later shown that astrocytes do so by facilitating the maturation of synapses and that this can be pheno-copied by using astrocyte condition medium (Ullian et al., 2001). This support in synapse formation is mediated by the secretion of several factors from astrocytes, including apolipoprotein E (Goritz et al., 2005), the matrix proteins hevin (SPARCL1) (Kucukdereli et al., 2011) and thrombospondin (Christopherson et al., 2005) as well as the proteoglycans glypican 4 and 6 (Allen et al., 2012). These soluble factors can induce synapse maturation, stability and un-silencing by increasing the synaptic levels of AMPARs (Allen et al., 2012) and NMDARs (Hahn et al., 2015).

This clear role of astrocytes in synapse formation was later extended by showing that astrocytes can engulf and eliminate dendritic spines in an activity-dependent manner, and therefore play a role in synapse dynamics and circuit refinement (Chung et al., 2013). Similarly, synaptic activity was shown to regulate the motility of astrocytic processes enveloping synapses, with an increase in synaptic activity increasing process mobility, which correlated with spine stability (Bernardinelli et al., 2014). Astrocytes can thus respond to neuronal activity, and in fact, show complex and varied Ca$^{2+}$ signals in response to synaptic release of neurotransmitters (Fiacco and McCarthy, 2006).

### 1.6.2 Gliotransmission

When stimulating astrocytes co-cultured with neurons, an astrocytic Ca$^{2+}$ wave can be observed that is closely followed by an increase in neuronal Ca$^{2+}$ (Nedergaard, 1994; Parpura et al., 1994). These were the first two studies showing that an increase in astrocytic Ca$^{2+}$ can modulate neuronal activity and that this is mediated by glutamate released from astrocytes activating neuronal NMDAR (Parpura et al.,
The release of ‘gliotransmitters’ from astrocytes has later been confirmed in slices (Bezzi et al., 1998; Pasti et al., 2001), and was shown to also include ATP (Newman, 2001) and the NMDAR co-agonist D-serine (Mothet et al., 2005). Furthermore, astrocytes express functional vesicular glutamate transporters (VGLUT) as well as components of the SNARE complex, and the release of glutamate from astrocytes was shown to be dependent on intracellular Ca\(^{2+}\) and to be mediated by SNARE proteins (Bezzi et al., 2004; Montana et al., 2004; Crippa et al., 2006). The glutamate released from astrocytes was shown to affect several targets on neurons, including pre-synaptic NMDAR (Jourdain et al., 2007), post-synaptic NMDAR (Fellin et al., 2004) and post-synaptic mGluRs (Navarrete and Araque, 2010).

It is slowly becoming clear how strong the functional interconnection between neurons and astrocytes is. For example, (Jourdain et al., 2007) show that neurons release ATP when stimulated, activating purinergic receptors on astrocytes, which in turn respond with Ca\(^{2+}\) transients and the release of glutamate to stimulate pre-synaptic NMDARs, facilitating neuronal vesicle release and therefore increasing synaptic strength.

### 1.6.3 Astrocytic Ca\(^{2+}\) transients

Based on the finding that astrocytes express functional glutamatergic receptors (Usowicz et al., 1989), astrocytes in culture were shown to respond to glutamate exposure with long-range intracellular Ca\(^{2+}\) waves (Cornell-Bell et al., 1990). Astrocytes responded with an initial increase in Ca\(^{2+}\) that was homogenous throughout the culture but would then turn into waves through the astrocytic syncytium (Cornell-Bell et al., 1990). Similarly, mechanical stimulation of one astrocyte caused Ca\(^{2+}\) transients in the neighbouring astrocytes (Charles et al., 1991).

Later it was shown in slice cultures that the stimulation of neuronal afferents in the hippocampus can induce Ca\(^{2+}\) transients in astrocytes with a lag time of ~2 sec, and that this is mediated by metabotropic glutamatergic receptors (mGluRs) (Dani et al., 1992; Porter and McCarthy, 1996). We now know that astrocytes express a wide variety of receptors that could potentially be used to communicate with neurons,
including metabotropic glutamate receptors and receptors for ATP, acetylcholine and GABA (for review see (Porter and McCarthy, 1997)), and inhibition of either of these receptors can decrease neuronal activity evoked astrocytic Ca\textsuperscript{2+} transients in slices (Haustein et al., 2014; Tang et al., 2015). Although it is well accepted that mGluR5 is a major receptor translating neuronal activity into astrocytic Ca\textsuperscript{2+} transients, there is evidence that this is only the case in young rodents, and that there is a switch in adult rodents from mGluR5 to mGluR3 (Sun et al., 2013).

**Figure 1.8: The tripartite synapse.**

The neuronal pre- and post-synapse are functionally and morphologically linked to astrocytic processes. The close proximity of astrocytic end-feet to the neuronal synapse allows for bidirectional communication between the two cell types. Neurotransmitters released from neurons during action potential firing activate metabotropic G-protein coupled receptors (GPCRs) on astrocytes, inducing complex Ca\textsuperscript{2+} transients. The Ca\textsuperscript{2+} elevation in astrocyte in turn causes the release of gliotransmitters from astrocytes that can regulate synaptic strength and dynamics. Image from (Agulhon et al., 2008).
With the introduction of genetically-encoded Ca\textsuperscript{2+} probes and the improvement of imaging equipment, it became clear that astrocytes not only experience somatic long-ranging Ca\textsuperscript{2+} waves, but also small, local and non-propagating Ca\textsuperscript{2+} elevations present in the thin processes of astrocytes (Shigetomi et al., 2010; Haustein et al., 2014). Depending on the hippocampal region, these highly localized events either occur spontaneously and are not sensitive to TTX (Nett et al., 2002; Haustein et al., 2014), or can be induced by synaptic activity and are therefore TTX sensitive (Di Castro et al., 2011). Additionally, only a subset of the TTX-insensitive Ca\textsuperscript{2+} transients depends on intracellular Ca\textsuperscript{2+} stores (Haustein et al., 2014; Srinivasan et al., 2015). On the other hand, synaptic activity-evoked Ca\textsuperscript{2+} transients strongly depend on intracellular Ca\textsuperscript{2+} stores, as synaptically released neurotransmitters mostly activate metabotropic receptors on astrocytes, releasing intracellular Ca\textsuperscript{2+} via the activation of IP\textsubscript{3}-receptors (Porter and McCarthy, 1996; Srinivasan et al., 2015). Nevertheless, both, spontaneous and evoked astrocytic Ca\textsuperscript{2+} transients have also been shown to occur in vivo following the pharmacological induction of epileptic firing (Hirase et al., 2004) and sensory stimulations (Wang et al., 2006).

In this thesis I will use a novel imaging probe that reports the extracellular exposure to glutamate, iGluSnFR, to investigate whether astrocytes are exposed to glutamate during action potential firing and show the consequences this has on astrocytic Ca\textsuperscript{2+} events.

1.6.4 Effect of neurons on astrocytic morphology and function

It has long been know that neurons can induce dramatic morphological changes in astrocytes (Yang et al., 2013). When co-cultured with neurons, astrocytes change from a simple polygonal into a process-bearing, highly stellate morphology that resembles astrocytes in vivo (Swanson et al., 1997). These morphological changes can be expected to go hand in hand with functional changes and changes in gene expression. However, only three genes are known to be regulated in astrocytes by neurons: Upon co-culture with neurons, astrocytes were shown to increase the expression of glutamate transporters EAAT1 (Slc1a3) and EAAT2 (Slc1a2)
(Swanson et al., 1997) as well as the expression of connexin43 (Gja1) (Koulakoff et al., 2008).

In light of the strong astrocyte-neuron communication described throughout this chapter, it is of utmost interest to develop tools to probe for this astrocyte-neuron interaction. Although there is an extensive amount of literature showing the functional consequences of neuron-astrocyte interactions, including the aforementioned lactate-shuttle, the release of synaptogenic proteins from astrocytes and the induction of Ca\(^{2+}\) transients by neuronally released transmitters, there is almost no knowledge on the effect of neuron-astrocyte interactions at the gene expression level. In this thesis, I will introduce a tool that allows probing for gene expression changes induced in one cell type by another, in order to elucidate the effects neurons have on the astrocytic transcriptome.

### 1.7 Aims of this thesis

This thesis can be split into three parts, with the third and the fourth chapter being based on studying neuronal bioenergetics. Using novel live cell imaging tools I will try to better understand the selective dendritic vulnerability of neurons that is observed in acute and chronic brain diseases. Furthermore, I will investigate how neurons adjust their metabolic output to changes in energy demand. In the fifth chapter, I will introduce a novel tool to study non-cell autonomous changes in gene expression and apply it by studying the intimate interaction between neurons and astrocytes.

Chapter 3:
In Chapter 3, I will validate novel imaging probes to study the redox potential and ATP levels in live cells. I will employ these probes to investigate the potential selective vulnerability of dendrites to excitotoxic insults and redox perturbations.
Chapter 4:
In Chapter 4, I will study how neurons adjust their metabolic output to changes in metabolic demand, in part by validating and applying a novel imaging probe to measure cytosolic NADH/NAD\(^+\) ratios. I will also investigate the role of mitochondrial Ca\(^{2+}\) in regulating neuronal energy production.

Chapter 5:
In Chapter 5, I will introduce a novel tool to determine the cell type origin of a transcript in co-cultures and apply it by investigating whether neurons can induce genome-wide changes in astrocytic gene expression. I will also look into the effect of neuronal activity on astrocytic glutamate exposure, Ca\(^{2+}\) signalling and gene expression.

Most of the work in Chapters 3 and 4 has been published in (Hasel et al., 2015), (Bell et al., 2015), (Baxter et al., 2015) and (Marland et al., 2015), and some of the work in Chapter 5 has been published in (Márkus et al., 2016).
Chapter 2
Material and Methods
Chapter 2: Materials and Methods

2.1 Cell culture

Primary cortical neurons were cultured from embryos taken from E17.5 CD1 mice or E20.5 Sprague Dawley rats. Embryos were decapitated, the brain removed and the cortices dissected in dissociation medium (81.8 mM Na$_2$SO$_4$, 30 mM K$_2$SO$_4$, 5.84 mM MgCl$_2$, 252 µM CaCl$_2$, 1 mM HEPES, 0.1% Phenol Red, 20 mM glucose and 1 mM kyurenic acid). The cortices were then enzymatically digested in dissociation medium containing papain (10 units/mL, Worthington Biochemical Corporation) at 37°C for 40 min, shaken every 10 min and fresh enzyme added after 20 min. Cortices were then washed twice with dissociation medium followed by two washes with Neurobasal-A (NBA) medium containing Anti-Anti (anti-bacterial/anti-mycotic), B27 supplement (all Life Technologies), glutamine (1 mM, Sigma) and 1% rat serum (Harlan SeraLab). Then, cortices were mechanically homogenized using a 2 mL serological pipette and the cell suspension subsequently diluted in Opti-MEM (Life Technologies) supplemented with glucose (20 mM, Sigma) and Anti-Anti, to a concentration of 0.28 cortical hemispheres/2 mL for mouse and 1/7 cortical hemisphere/2 mL for rat. Subsequently, 0.5 mL of cell suspension was used per well of a tissue-culture grade 24-well plate, coated with laminin (13 µg/mL, Roche) and poly-D-lysine (5µg/mL, Sigma). Cells cultured for imaging experiments are grown on cover slips. After 2h in an incubator (37°C, 5% CO$_2$), medium was aspirated and replaced with 1%NBA. At DIV4, cells were fed with 1%NBA containing β-D-arabinofuranoside hydrochloride (araC, Sigma) to inhibit astrocyte proliferation, leading to ~5% of the total cell population being astrocytic. In order to get pure neuronal cultures, araC was added on DIV0, resulting in cultures containing <0.05% GFAP positive cells.

In case of mixed-species astrocyte-neuron co-cultures, rat neurons were added onto a confluent layer of mouse astrocytes and kept in 0%NBA to reduce proliferation of rat astrocytes. Astrocytes were created by plating down the cell suspension at half the density in DMEM supplemented with 10% Fetal Bovine Serum (both Life Technologies) in a T75 flask (Greiner). Astrocytes were passaged twice before they were plated down on coated 24-well plates. To passage astrocytes, cells were washed...
with PBS and subsequently detached using 0.05% Trypsin-EDTA (both Life Technologies) and cell suspensions spun down at 800 rpm for 4 min. Cells were re-suspended and diluted in 10%DMEM and either added to T75s or 24-well plates. Astrocytes approximately double in number within 24h in 1%DMEM and were split 1:3 two days before plating down neurons, resulting in a confluent layer of astrocytes. Neurons were plated down at 75% density to increase the fraction of RNA deriving from astrocytes.

2.2 Transfection and plasmids

Neurons and astrocytes were transfected using Lipofectamine 2000 (2.33 µl/well, 1µg/mL, Life Technologies) with 0.65 µg DNA/well. For live-cell imaging of neurons, cells were transfected on DIV8 and incubated in the Lipofectamine/DNA mix for 2-5h. Astrocytes were transfected one day before neuron plate down for 45 min. When imaging astrocytes and neurons in a mixed-species astrocyte-neuron co-culture, neurons were subsequently transfected on day 8 post neuron plate down.

Plasmids used were as follows: pCAGGS-GCaMP2 was a gift from Karel Svoboda (Mao et al., 2008), mito-GCaMP2 was a gift from Xianhua Wang (Chen et al., 2011), Grx1-roGFP2 was a gift from Tobias Dick (Gutscher et al., 2008), Peroxid-NLS was a gift from Gerry Yellen (Hung et al., 2011), GCaMP3 (Tian et al., 2009) and iGluSnFR (Marvin et al., 2013) were a gift from Loren Looger. Sequences of MCU knock-down constructs were as follows: shMcu: tagggaataaagggatcttaa, shScr: gtgccaagacgggtagtca, siMcu: cgaccuagagaauaaau, siCnt was siGENOME Non-Targeting siRNA2 (Thermo Fisher). For siRNA-mediated knock-down of MCU, siRNA was transfected at least 2 days prior to imaging.

2.3 Virus generation and infections

AAV1/2 viruses used in this thesis carried plasmids encoding shScr and shMcu as well as Grx1-roGFP2. Target sequences were sub-cloned into an rAAV backbone. shRNA expression was driven by a U6 promoter and the attached mCherry by a CamKII promoter. Grx1-roGFP2 was driven by a CamKII promoter. In order to make the virus, target plasmids were transfected into HEK cells in the presence of plasmids encoding for AAV1/2 packaging proteins, and viral particles purified using heparin columns (Sigma).
Neurons were infected with $10^{11}$ virus particles per µl on DIV4, and cells were used on DIV9-11. Infection efficiency was estimated by counting mCherry or GFP fluorescence. Typically, maximal fluorescence is observed on day 5 post infection, with an infection efficiency of 80-90%.

2.4 General imaging parameters

For live-cell imaging, neurons were transferred into custom imaging chambers and perfused manually or using a fast perfusion system. Cells were kept at 37°C in artificial cerebrospinal fluid (aCSF) consisting of (in mM): NaCl (150), KCl (3), HEPES (10), glycine (0.1), CaCl$_2$ (2), MgCl$_2$ (1) and glucose (10) at pH 7.4. Imaging was performed on a Leica AF6000 LX using a DFC350 FX digital camera. When comparing somatic to dendritic responses, dendrites were defined as structures being at least 2 cell bodies away from the soma and 5x narrower. One ROI was chosen for each of the two compartments, the soma and the according dendrite. When concomitantly imaging transfected astrocytes and neurons, in order to distinguish both cell types, cells were identified by morphology, and one cell type was always co-transfected with mCherry.

2.5 Grx1-roGFP2 imaging, calibration, data analysis

Grx1-roGFP2 is a ratio-metric imaging probe that reports the glutathione redox potential. Grx1-roGFP2 was excited at 387±5 nm and 494±10 nm and emission collected at 530±10 nm. Images were taken every 20 s and the 387/494 ratio was calculated following background subtraction in both channels. In order to induce redox perturbations, H$_2$O$_2$ (Sigma) was applied to the imaging chamber, which causes an increase in the 387/494 ratio. Low levels of H$_2$O$_2$ (10-15 µM) caused changes in the 387/494 ratio that were within the dynamic range of Grx1-roGFP2 and therefore used to compare the capacity of the glutathione redox potential between conditions. At the end of the recording, saturating levels of H$_2$O$_2$ (≥50 µM) were applied to achieve the maximum fluorescence of Grx1-roGFP2. Peak responses were either given as fold change from the baseline or the difference in 387/494 ratio before and after the addition of H$_2$O$_2$. Images were acquired every 20 s.
2.6 AT1.03 imaging, calibration, data analysis

AT1.03 is a genetically-encoded reporter of intracellular ATP levels. The YFP/CFP-based FRET-probe AT1.03 was excited at 427±5 nm (CFP) and emission collected at 472±15 nm (CFP) and 542±13 nm (YFP/FRET). A drop in ATP is reporter as a drop in the YFP/CFP ratio. AT1.03 is calibrated by adding the uncoupler of the mitochondrial membrane potential, FCCP (Sigma, 10 µM), to the cells, giving the minimal YFP/CFP ratio. YFP/CFP ratios measured after the addition of FCCP are subtracted from the baseline YFP/CFP ratio. After background subtraction, peak responses following stimulations are either given as the difference in YFP/CFP before and after the stimulation, or as FCCP-corrected YFP/CFP values at baseline and after the stimulation. Images were acquired every 5 s.

2.7 Peredox imaging, calibration, data analysis

Peredox is a GFP-based imaging probe that reports the cytosolic NADH/NAD$^+$ ratio. It has an mCherry attached to it that allows correcting for different amounts of reporter expressed per cell. Peredox is excited at 387±5 nm and 575±12 nm and emission collected at 530±20 nm and 628±14 nm. Peredox can be calibrated by washing different lactate/pyruvate ratios onto Peredox-expressing cells. This is possible because the conversion of lactate into pyruvate by lactate dehydrogenase (LDH) produces NADH, while conversion of pyruvate into lactate will consume NADH. Therefore, increasing the lactate/pyruvate ratio will increase the NADH/NAD$^+$ ratio and vice versa. Knowing the equilibrium constant (k) of LDH with k=(pyruvate*NADH)/(lactate*NAD$^+$) and k=1.11*10^-4, it is possible to translate the pyruvate/lactate ratio into NADH/NAD$^+$ ratios, since k, [pyruvate] and [lactate] are known. This allows transforming the fluorescence of Peredox at each lactate/pyruvate ratio into NADH/NAD$^+$ levels. Each recording was therefore finished with washing on lactate (10 mM), which gives the maximal fluorescence of Peredox, and changes in fluorescence can be translated into changes in NADH/NAD$^+$. All Peredox values were background subtracted and normalised using the attached mCherry. Images were acquired every 20 s. The Peredox used here is
targeted to the nucleus, since cytoplasmic Peredox is taken up by lysosomes, likely due to the mCherry attached to it (Hung et al., 2011).

2.8 GCaMP2/3 imaging, calibration, data analysis

Ca\(^{2+}\) imaging using cyto-GCaMP2 or mito-GCaMP2 was performed using a standard GFP filter set, with an excitation wavelength of 480±20 nm and emission of 527±15 nm. Changes in Ca\(^{2+}\) are reporter as \((F-F_{\text{min}})/(F_{\text{max}}-F)\) according to the equation \([	ext{Ca}^{2+}] = kd*(F-F_{\text{min}})/(F_{\text{max}}-F)\). In order to obtain \(F_{\text{max}}\), each recording was finished by washing on ionomycin, an ionophore that makes the cytoplasmic and mitochondrial membrane permeable to ions, which causes Ca\(^{2+}\) influx that saturates GCaMP2 (extracellular \([\text{Ca}^{2+}]\) is 2 mM). Subsequently, EGTA-containing medium was washed on, which gives the \(F_{\text{min}}\), since all Ca\(^{2+}\) ions are chelated.

Astrocytic Ca\(^{2+}\) image analysis was performed by selecting active ROIs (i.e. each area of an astrocyte that shows a Ca\(^{2+}\) spike at any point throughout the recording) and each Ca\(^{2+}\) transient was quantified using the Clampfit application of pCLAMP (Molecular Devices).

2.9 Rh123 imaging, calibration, data analysis

Rhodamine-123 (Rh123, Molecular Probes) is a chemical dye that reports the mitochondrial membrane potential (\(\Delta\Psi\)) and is imaged using a standard GFP filter set (ex.: 480±20 nm, em.: 527±15 nm). Rh123 is negatively charged and will accumulate in the positively charged mitochondrial matrix, where it self-quenches. When \(\Delta\Psi\) depolarises, Rh123 leaves the matrix and un-quenches, causing an increase in fluorescence. Cells are incubated in Rh123 (26 µM) for 10 min, after which cells are washed extensively to remove excess dye. Rh123 is calibrated by applying FCCP (5 µM, see above), which causes full \(\Delta\Psi\) depolarization and thus gives the maximal fluorescence of Rh123. A change in \(\Delta\Psi\) will be reported as a change in Rh123 fluorescence relative to the FCCP-induced maximal fluorescence. All recordings undergo background subtraction.
**2.10 NAD(P)H autofluorescence imaging**

NAD(P)H autofluorescence is the combined autofluorescence of the reducing equivalents NADH and NADPH. However, since NADH is in 10 fold excess over NAD(P)H in the mouse brain (Klaidman et al., 1995), it is generally accepted as a tool to measure NADH levels. NAD(P)H autofluorescence was measured by exciting cells at 387±5 nm and collecting the emitted light at 447±30 nm. Images were acquired every 20 s. For each coverslip, 10-20 cell bodies and dendrites were selected. For NMDA stimulations, NAD(P)H autofluorescence was measured until the signal plateaued (~300 s post NMDA application). For BiC/4-AP conditions, recordings lasted between 10-16 min.

**2.11 iGluSnFR imaging, calibration**

iGluSnFR is a reporter of extracellular glutamate that can be imaged using a standard GFP filter set (ex.: 480±20 nm, em.: 527±15 nm). It is targeted to the extracellular side of the plasma membrane where it reports a rise in glutamate with an increase in GFP fluorescence. The probe can be calibrated by adding high concentrations of glutamate to the cells (5 mM), which saturates the probe. Images were taken every second.

**2.12 Measuring oxygen consumption rate**

Oxygen consumption rate (OCR) was measured using a Seahorse XF24 extracellular flux analyser (Seahorse Bioscience). One hour before the recording, cells were placed into medium not containing bicarbonate buffer (modified DMEM, supplemented with 10 mM glucose, pH 7.4). Cells are plated on specialized 24-well plates that allow the bioanalyzer to create a microenvironment that allows it to measure the consumption of O$_2$. Chemical probes sensitive to O$_2$ are lowered onto the cell layer by which they inhibit the influx of external O$_2$ and are able to measure the oxygen consumption of the cells as a decrease in O$_2$ over time. The set-up allows the sequential addition of chemicals. After BiC/4-AP, which increases network bursting and therefore OCR, the following drugs were applied: oligomycin (1 µM) shows the OCR that is due to mitochondrial ATP production, FCCP (0.125 µM)
gives the maximum respiratory capacity and antimycin (2 µM) and rotenone (2 µM) show the non-mitochondrial oxygen consumption.

2.13 Western blotting
Cells were lysed in 30 µl 1.5x sample buffer (1.5 mM Tris, 15% glycerol, 3% SDS, 7.5% β-Mercaptoethanol, 0.0375% bromophenol blue, pH 6.8). Gel electrophoreses and western blots were performed using the Xcell Surelock system (Invitrogen). At least 3 wells of a 24-well plate were pooled for one condition, and 10-20 µl of sample were loaded onto precast gradient gels (4–20%, Life Technologies) and electrophoresis performed using running buffer (50 mM MOPS, 50 mM Tris, 1 mM EDTA, 3.5 mM SDS, pH 7.7) at 120V for 1.5-2h. Subsequently, the protein was transferred onto a PVDF membrane (Millipore) using transfer buffer (96 mM glycine, 12 mM Tris and 20% Methanol) at 45 V for ~ 2 h. Membranes were blocked (20 mM Tris, 137 mM NaCl and 0.1% Tween-20) and incubated with the primary antibody over night at 4°C. Primary antibody concentrations were as follows: b-actin (Abcam, 1:2000), Mcu/ccdc109a (Sigma, 1:500), PDH-E1α (Abcam, 1:10000), PDH-E1α (pSer293) (Calbiochem, 1:10000).

The next day, the membrane was washed in TBS and incubated in HRB-linked secondary antibody for 1 h at room temperature. Bands were visualized on Kodak X-Omat films using LumiGlo reagents and peroxide (Cell Signaling Technology). Blots were digitally scanned and densitometric analysis was performed using ImageJ.

2.14 RNA extractions, qPCR and primers
RNA was collected from pure astrocytic or astrocyte-neuron co-cultures using a High Pure RNA isolation kit (Roche). 4-6 wells of a 24-well plate were collected for astrocytes, and 2-3 wells for astrocyte-neuron co-cultures, and RNA concentrations were measured using a NanoDrop (Thermo Scientific). For qPCR studies, RNA was reverse transcribed into cDNA using Transcriptor First Strand cDNA synthesis kit (Roche). qPCRs were performed using a Mx3000P QPCR system (Stratagene) and FastStart Universal SYBR Green QPCR Master (RoX) (Roche). All qPCR results were normalized to a standard control (H1F0). Primers were as follows:
<table>
<thead>
<tr>
<th>Gene</th>
<th>Fwd/Rev</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dio2</td>
<td>Fwd</td>
<td>CCCTTCTGAGCGAATTGATCCA</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>CACATCGTAAGTATGTATCTGGG</td>
</tr>
<tr>
<td>H1f0</td>
<td>Fwd</td>
<td>GTTTGTCTTCCAGACTTTCTT</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>CTTTGCCCTTTAGACAATGGG</td>
</tr>
<tr>
<td>Slco1c1</td>
<td>Fwd</td>
<td>GATCCAGACCCCTTGCAAACAT</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>GATATCCGACTGTAAAGGATGG</td>
</tr>
<tr>
<td>Sod3</td>
<td>Fwd</td>
<td>CCGCAGCTACAACCCCGATGGAG</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>CGACGCGATGCGCTCCAGA</td>
</tr>
</tbody>
</table>

All primers have been designed to only pick up mouse transcripts and were validated by running them against pure mouse and pure rat samples.

**2.15 RNA-sequencing and analysis**

RNA-sequencing was performed by Edinburgh Genomics. At least 1 µg of RNA was sent for RNA-sequencing and only accepted if the RNA integrity number (RIN) was above 7. RNA-sequencing was performed on a Illumina HiSeq 2500 sequencing system at a sequencing depth of 150 million 50 nucleotide paired-end reads. The data analysis was performed by Dr. Owen Dando, Sam Heron and Dr. Ian Simpson (School of Informatics, University of Edinburgh). Sequenced reads were mapped against a mouse and rat genome using TopHat and the read only accepted if it matched unambiguously to only the mouse genome. Reads were converted into fragments per kilobase of transcript per million mapped reads (FPKM). DESeq2 was used for normalization and calculation of fold changes. GO term analysis was performed using GO enrichment analysis software provided by the Gene Ontology Consortium (http://geneontology.org/page/go-enrichment-analysis). Genes with a fold change ≥ 2 were run for GO terms involved in biological processes, and the top20 enriched GO terms picked for discussion.

**2.16 Quantifying astrocyte complexity**

Astrocytes were transfected with GFP and subsequently either co-cultured with neurons or left as a monoculture. Changes in astrocyte complexity were followed from DIV0-DIV10 by tracing the outline of the cell (Photoshop). The perimeter was calculated and the area used to correct for differences in cell size (ImageJ).
2.17 Immunocytochemistry
Cells were fixed in 1% formaldehyde for 20 min and washed with PBS. Cells were permeabilized for 5 min with NP40 (Life Technologies) and incubated in primary anti-antibody over-night at 4 °C. The next day, cells were washed in PBS and incubated in the appropriate fluorescent secondary anti-body at room temperature for 2 h. After that, cells were covered in vectashield (Vectorlabs) and a cover slip added on top of the cells. Primary anti-body concentrations were as follows: mouse anti-GFAP (1:400, Sigma) and rabbit anti-GFP (1:750, Life Technologies).

2.18 Statistical analysis
Non-independent data was analysed using paired t-tests while Student t-tests were applied on groups that were independent. When comparing multiple groups, one-way ANOVAs with Bonferroni’s or Dunnett’s post-hoc tests were applied. For dependent data, such as the data on somatic and dendritic responses, a repeated measure ANOVA was used. For all tests, significance was reached when p<0.05 and error bars represent standard error of the mean.
Chapter 3
Selective dendritic susceptibility to excitotoxic and oxidative stress
Chapter 3: Selective dendritic susceptibility to excitotoxic and oxidative stress

3.1 Introduction

Chronic and acute neuronal diseases are thought to have common aetiological factors that centre on excitotoxicity, oxidative stress, mitochondrial dysfunction and energy imbalance (Melo et al., 2011; Zündorf and Reiser, 2011; Johri and Beal, 2012; Nakamura et al., 2012; Mehta et al., 2013).

In excitotoxicity, the over-stimulation of NMDARs causes Ca$^{2+}$ influx that eventually leads to neuronal cell death (Choi, 1992; Lipton, 2006). Excitotoxicity is the main contributing factor to neuronal cell death in acute neurological disorders, such as stroke and traumatic brain injury (Lau and Tymianski, 2010) and also plays a major role in neurodegenerative diseases (Lipton and Rosenberg, 1994; Parsons and Raymond, 2014). The mechanism of excitotoxicity in stroke is the spillage of glutamate into the extracellular space as neurons die from oxygen deprivation and the reversed operation of neuronal glutamate transporters. Glutamate then activates glutamatergic receptors on surrounding neurons, causing a bionergetic deficit that abrogates glutamate re-uptake from the extracellular space. However, also chronic neuropathological conditions have been associated with the loss of bioenergetic homeostasis (Khatri and Man, 2013).

At the centre of metabolic and energetic perturbations are dysfunctional mitochondria, which are unable to provide energy in the form of ATP to the cell. Dysfunctional mitochondria can be found in acute and chronic neuronal disorders and can be caused by excitotoxicity-induced mitochondrial Ca$^{2+}$ overload (Duchen, 2012). However, mitochondrial dysfunction can also be caused by oxidative stress, which is generated during periods of excitotoxicity and perturbations of the ETC (Halliwell, 2006).

Neurons have a very polarized morphology, with thin neurites protruding from the cell body. Since the soma, axon and dendrites fulfil specialized roles in the cell, their biochemical make-up differs greatly. Consequently, subcellular neuronal compartments will cope differently well in neuropathological conditions. Dendrites and axons show changes in morphology, such as spine loss or stunted growth, early
in the neurodegenerative disease progression (Luebke et al., 2010), in some cases even before the occurrence of any functional consequences (Rocher et al., 2008).

In recent years, an array of genetically-encoded reporters has been developed that can be targeted to sub-cellular compartments and used to measure neuronal responses to oxidative stress and excitotoxicity in real time. These include reporters for Ca$^{2+}$, including mitochondrial Ca$^{2+}$ (Chen et al., 2011), redox potential (GSH:GSSG ratios) (Gutschner et al., 2009), AMPK activity (Tsou et al., 2011), ATP levels (Imamura et al., 2009) and NADH:NAD$^+$ ratios (Hung et al., 2011).

In this chapter, primary cortical neurons were transfected with reporters for ATP, GSH:GSSG and Ca$^{2+}$, and exposed to either sub-lethal oxidative or lethal excitotoxic stress. The goal of this study was to detect possible differences in the dendritic versus somatic response to these insults. Additionally, the mitochondrial membrane potential $\Psi_m$ was measured using the charged chemical dye rh123 and NADH levels were assessed by NAD(P)H autofluorescence.

In addition to showing the utility of these probes, evidence is provided that dendritic and somatic regions respond differently to oxidative and excitotoxic stress. Dendrites show greater redox fluctuations than somata when exposed to oxidative stress and dendrites show a sharper drop in ATP when undergoing excitotoxicity, while showing a less pronounced depolarization of $\Psi_m$. I also show two possible interventions, Nrf2 over-expression and chronic synaptic activity, that increase the antioxidant capacity of neurons as measured with a genetically-encoded redox reporter.

Most of the data in this chapter has been published in (Baxter et al., 2015; Hasel et al., 2015) and (Bell et al., 2015).
3.2 Results

3.2.1 Measuring the glutathione redox potential of neurons using the genetically-encoded reporter Grx1-roGFP2

Common markers to measure oxidative stress in cell systems are based on chemical dyes that increase in fluorescence upon oxidation. Since this process is irreversible, measuring the redox state of a cell is not possible using these conventional probes. Grx1-roGFP2 is a circularly permuted, genetically encoded reporter of the glutathione redox potential (Gutscher et al., 2008). Grx1-roGFP2 interacts in a reversible fashion with the glutathione system in the cell, and small fluctuations in the cellular redox state will change the excitation ratio at 390/480 nm (see introduction for details). The more oxidized Grx1-roGFP2 becomes, the higher the 390/480 nm ratio and vice versa.

Figure 3.1: Grx1-roGFP2 can be expressed in neurons and responds to H\textsubscript{2}O\textsubscript{2} in a concentration dependent and reversible manner.

A) Grx1-roGFP2 was expressed in neurons via infection with an AAV that contained the plasmid encoding for the probe. Image shows Grx1-roGFP2 fluorescence. Scale bar is 20 μm B) Example trace showing 10 neurons expressing Grx1-roGFP2 responding to increasing concentrations of H\textsubscript{2}O\textsubscript{2} with an increase in the 390/480 nm ratio, followed by a recovery back to baseline. Levels between 10 and 15 μM H\textsubscript{2}O\textsubscript{2} induce reliable, non-saturating changes in the 390/480 nm ratio and were picked for further experiments. Fresh H\textsubscript{2}O\textsubscript{2} was used for each application and applied as a bolus into the imaging chamber.
To test whether Grx1-roGFP2 could be expressed in our primary cortical cultures and whether the dynamic range was suitable for our experiments, the probe was introduced into primary cortical neurons (here by AAV infection with a plasmid containing the reporter, Fig. 3.1A). Oxidative stress was induced by bolus application of increasing amounts of H₂O₂. H₂O₂ levels as little as 3μM were sufficient to induce measurable changes in the Grx1-roGFP2 ratio (Fig. 3.1B). The increase in the 390/480 nm ratio was reversible and saturation occurred at ≥50 μM H₂O₂ (Fig. 3.1B, 3.3E). From these calibration experiments, I picked concentrations between 10-15 μM H₂O₂ as appropriate for further experiments, since they are reliably detectable with Grx1-roGFP2. Concentrations of 50 or 100 μM H₂O₂ were used to achieve the maximum fluorescence and used to calibrate the reporter after each experiment (Fig. 3.3E).

3.2.2 Dendrites suffer greater shifts in the redox potential when exposed to oxidative stress compared to the soma

To investigate sub-cellular changes in the redox state of neurons to oxidative stress, Grx1-roGFP2 was transfected into primary neuronal cultures (Fig. 3.2A). For each individual cell, one somatic and one dendritic ROI were selected, the latter region being defined as 2 cell bodies away from the soma and about 5x narrower.
Figure 3.2: Dendrites are more oxidized than somata and show a more drastic redox shift when exposed to oxidative stress.

A) Example image showing a primary cortical neuron transfected with Grx1-roGFP2. The blue ROI indicates the somatic and the red ROI the dendritic compartment. Scale bar = 10 µm B) Example trace showing that the addition of 10 µM H$_2$O$_2$ increases the 390/480 nm ratio in both, the soma and the dendrite, while 100 µM H$_2$O$_2$ saturates the probe. The dendritic region responds more strongly to the oxidative insult than the somatic one. C) Quantified data showing changes in the 390/480 nm ratio to the strong reducing agent DTT (10 mM) and the addition of H$_2$O$_2$ (10 µM) relative to the untreated control. Addition of DTT only causes a significant drop in the relative 390/480 nm ratio in dendritic but not somatic compartments. *p<0.05 compared to untreated control (two way ANOVA plus Dunnett’s post-hoc test (n=9-23)). Addition of H$_2$O$_2$ causes a significant increase in the relative 390/480 nm ratio in both, dendritic and somatic compartments, but dendrites suffer a greater redox shift than somata. *p<0.05 compared to untreated control (two way ANOVA plus Bonferroni’s post-hoc test (n=9-23)). D,E) bar graphs showing the change in the 390/480 nm ratio for DTT and H$_2$O$_2$. *p<0.05 Student t-test, n=14 (D), n=9 (E). All graphs in this figure have been taken from (Hasel et al., 2015).

The bolus application of 10 µM H$_2$O$_2$ caused a redox shift in the soma and the dendrite, which could be saturated by the application of 100 µM H$_2$O$_2$ (Fig. 3.2B, C). Dendrites responded with a more drastic shift in the glutathione redox potential when compared to the soma of the same cell (Fig. 3.2C, E). Additionally, dendrites appeared to be in a generally more oxidized state than somata, as addition of the strong reducing agent DTT caused a more drastic drop in the 390/480 nm ratio in dendrites compared to somata (Fig. 3.2 C, D). Taken together, these data indicate that dendritic areas of neurons are more vulnerable to oxidative stress when compared to the soma and are in a generally more oxidized state.
3.2.3 Nrf2-overexpression and chronic synaptic activity boost the capacity of neurons to cope with oxidative stress

I subsequently used Grx1-roGFP2 positive neurons to test whether the neuronal capacity to cope with oxidative stress can be improved. Neurons are particularly vulnerable to oxidative insults, which is mainly due to their lower oxidant defence capacity, showing particularly low levels of glutathione (Fernandez Fernandez et al., 2012).

The master-regulator of oxidative stress, Nrf2, is mainly expressed in astrocytes and is epigenetically silenced in neurons early in development (Bell et al., 2015). Downstream targets of Nrf2 include glutamate-cysteine-ligase catalytic subunit (Gclc), which catalyzes the rate-limiting step of glutathione biosynthesis (Dreger et al., 2009; Hayes and Dinkova-Kostova, 2014). I over-expressed Nrf2 in neurons in an effort to boost the neuronal glutathione system, while co-expressing Grx1-roGFP2 to perform live cell imaging of the glutathione redox potential.
Figure 3.3: Over-expression of Nrf2 and chronic synaptic activity increase the antioxidant capacity of the glutathione system.

A) Cells transfected over-night with globin or Nrf2 plus Grx1-roGFP2 were exposed to 10, 20 and 100 μM H₂O₂ and the change from baseline was calculated. Cells over-expressing Nrf2 show a smaller increase in the Grx1-roGFP2 390/480 nm ratio when compared to cells expressing globin when exposed to 10 μM H₂O₂. *p<0.05 (two way ANOVA plus Bonferroni's post-hoc test, n=6 with 30 cells globin and 35 cells Nrf2). Exposure of cells to 20 μM H₂O₂ had no significant effect, which is not surprising since at this level, the probe is often saturated. B) Example traces for quantified data in A).

C) Cells transfected with Grx1-roGFP2 were either treated with BiC/4-AP for 24h to chronically incude synaptic activity or not treated at all. Cells were then exposed to 5, 15 and 50 μM H₂O₂. When treated with 15 μM H₂O₂ cells undergoing elevated synaptic activity show a weaker increase in the Grx1-roGFP2 390/480 nm ratio when compared to non-treated controls. *p<0.05 (two way ANOVA plus Bonferroni's post-hoc test, n=10 cells per condition). 5 μM H₂O₂ induced only small fluctuations that were partly not present at all, and is therefore not a useful read-out. D) Examples traces for quantified data in C).

E) Different levels of H₂O₂ were used to saturate Grx1-roGFP2 in the Nrf2 and BiC/4-AP experiments (100 and 50, respectively). To make sure that both concentrations suffice to saturate...
Grx1-roGFP2, cells were exposed to 50 and then 100 μM H₂O₂, showing that both induce the same changes in the 390/480 nm ratio. Graphs C and D in this figure have been taken from (Bcill et al., 2015).

Similar to previous experiments, a sub-lethal and non-saturating dose of H₂O₂ (10 μM) was applied to neurons either expressing globin or Nrf2. I found that Nrf2 over-expressing neurons show weaker fluctuations in the glutathione redox potential than globin expressing cells (Fig. 3.3A, B), which indicates a Nrf2-induced boost in antioxidant capacity of neurons. The subsequent addition of 20 μM H₂O₂ shows a clear tendency to have similar results to the 10 μM stimulation but does not reach statistical significance (Fig. 3.3A). This is most likely due to the fact that 20 μM H₂O₂ partly saturates Grx1-roGFP2 and therefore underestimates the effect that Nrf2 has on the glutathione redox potential. Applying 100 μM H₂O₂ completely saturates the probe in globin as well as Nrf2 expressing neurons (Fig. 3.3A).

We recently showed that prolonged synaptic activity can increase neuronal survival following oxidative stress and that this is partly due to an increase in gene expression of genes involved in antioxidant defence mechanisms (Papadia et al., 2008). This includes the neuronal cell-autonomous increase in the gene expression of genes of the glutathione system (Baxter et al., 2015). I therefore used a well-established protocol of elevated synaptic activity, which includes the addition of the GABA_A receptor blocker bicuculline (BiC) and 4-aminopyridine (4-AP), a weak potassium channel blocker. While the first will disinhibit the network by reducing the tonic inhibition of excitatory neurons causing network burst firing, the latter will chronically depolarize the neuron. I expressed Grx1-roGFP2 in neurons either undergoing no stimulation or undergoing stimulation with 24h of BiC/4-AP. I found that application of 15 μM H₂O₂ causes a weaker redox perturbation in neurons stimulated with BiC/4-AP compared to un-stimulated controls, similar to what I observed with over-expressing Nrf2 (Fig. 3.3C, D). This supports the idea that chronic synaptic activity can boost the redox defence mechanisms of neurons. I did not observe significant differential changes in the redox potential at 5 μM H₂O₂ between simulated and non-stimulated neurons. This is not surprising, since H₂O₂ concentrations this low are at the limit of the dynamic range of Grx1-roGFP2 and therefore hide possible changes in the redox potential. Similar to before, I added high
concentrations of \( \text{H}_2\text{O}_2 \) (50 \( \mu \text{M} \)) at the end of the recording, which is saturating the sensor (Fig. 3.3E).

### 3.2.4 Measuring intracellular ATP levels in neurons using the genetically-encoded reporter AT1.03

In order to investigate the bioenergetic state of neurons, I employed AT1.03, a genetically encoded reporter of intracellular ATP levels (Imamura et al., 2009). AT1.03 is a FRET-based sensor, which consists of a subunit of the bacterial F\( \text{O} \)F\( \text{F} \)1-ATP synthase and a CFP and YFP moiety (see Materials and Methods for more details). AT1.03 responds to a drop in ATP with a drop in FRET fluorescence (i.e. drop in the YFP/CFP ratio). I initially tested whether AT1.03 expresses in our neuronal cultures and whether I can induce measurable changes in ATP levels.

![Figure 3.4](image)

**Figure 3.4: The ATP reporter AT1.03 can be expressed in neurons and responds to mitochondrial uncoupling.**

A) Example image of a neuron expressing AT1.03 showing the YFP/CFP ratio before and after the mitochondrial uncoupler FCCP was applied. FCCP is an ionophore that acts on mitochondria by uncoupling the proton flow into the mitochondrial matrix from the generation of ATP. B) Example trace from A) showing the drop in ATP following the application of FCCP (10 \( \mu \text{M} \)) expressed as the change in YFP/CFP from the baseline.

Using transfection, a plasmid expressing AT1.03 was introduced into neurons. I observed fluorescence in both, the YFP and CFP channel and could therefore
calculate the YFP/CFP ratio (Fig. 3.4A). Applying an uncoupler of the mitochondrial membrane potential (FCCP) causes a sharp drop in ATP levels and saturates the probe at the lower end of its dynamic range (Fig. 3.4B), allowing me to use it as a way to calibrate the probe. The drop in ATP will be given as a drop in the YFP/CFP ratio at the beginning of the recording \([YFP/CFP]_0\) minus the YFP/CFP ratio over the time course of the recording \([YFP/CFP]_t\).

3.2.5 Excitotoxic levels of NMDA cause a strong drop in intracellular ATP, which is more pronounced in dendrites compared to soma

Excitotoxic Ca\(^{2+}\) influx through NMDARs is associated with acute mitochondrial dysfunction leading to a rapid drop in ATP levels (Abramov and Duchen, 2008). To investigate whether I can visualize this drop in ATP, I transfected AT1.03 into neurons and applied excitotoxic levels of NMDA (100 µM).

Figure 3.5: NMDAR activation causes a drop in ATP that is stronger in dendrites than soma, while cytosolic Ca\(^{2+}\) rises similarly in both compartments.

A) Example trace of an AT1.03 positive neuron experiencing bath application of NMDA (100 µM). For each cell, one dendritic and one somatic region was picked and compared during NMDAR stimulation. Both regions show a sharp drop in ATP, with dendrites experiencing a stronger ATP drop when compared to the soma. B) Quantified levels of the ATP drop from baseline over a time span of 75 s. Dendritic regions show a sharper drop in ATP over the first 45s of NMDA application, after which the probe saturates at the lower end of the dynamic range. \(^*p<0.05\) (two-way ANOVA plus Bonferroni’s post-hoc test, n=8 cells). C) Neurons transfected with a cytosolic version of the Ca\(^{2+}\) indicator GCaMP2 show a sharp increase in Ca\(^{2+}\) following the application of NMDA, with both, dendritic and somatic compartments showing similar Ca\(^{2+}\) before and after stimulation. \(^*p<0.05\) paired t-test comparing pre-
I found that NMDA application causes a rapid drop in ATP, as seen by the strong decrease in the YFP/CFP ratio of AT1.03 (Fig. 3.5A). Moreover, I observed that dendrites suffer a more severe drop of ATP levels when compared to the somata of the same cells (Fig. 3.5A, B). The drop in the dendritic AT1.03 ratio is significantly greater than the somatic one within the first 45 seconds of the NMDA stimulation, after which the probe is starting to saturate (see plateau in Fig. 3.4B and 3.5A). The stronger drop in dendritic ATP compared to somatic ATP following excitotoxic NMDA receptor stimulation could be the due to several factors, including a higher uptake of Ca$^{2+}$ into dendritic compartments. However, using the genetically encoded, cytosolic Ca$^{2+}$ probe GCaMP2, I found that dendrites and somata show a similar Ca$^{2+}$ increase during the excitotoxic insult (Fig. 3.5C).

3.2.6 Mitochondria in dendrites are affected differently by an excitotoxic challenge than somatic mitochondria

Ca$^{2+}$ entering neurons during NMDAR stimulation will quickly accumulate into the mitochondrial matrix, causing the mitochondrial membrane potential ($\Psi_m$) to depolarize (Abramov and Duchen, 2008; Qiu et al., 2013). To understand the faster ATP depletion in dendrites compared to somata, I measured $\Psi_m$ during the excitotoxic insult, and compared somatic to dendritic compartments. As before, 100 µM NMDA was applied to the cells, this time incubated with the chemical dye Rhodamine-123 (rh123), a reporter of $\Psi_m$. Rh123 partitions into mitochondria upon entering the cell, where its fluorescence is quenched. Once mitochondria depolarize, rh123 is released from the organelle and fluoresces (Keelan et al., 1999). Rh123 is calibrated by applying FCCP, which uncouples and therefore depolarizes mitochondria, causing rh123 to fully de-quench and thereby giving the maximum fluorescence.
Figure 3.6: Dendritic mitochondria experience a weaker depolarization and drop in NADH following bath application of NMDA.

A) Example trace of 20 neurons loaded with rh-123, a chemical dye measuring the mitochondrial membrane potential, showing a bi-phasic depolarisation following an excitotoxic insult. Each thin line represents one cell while thick lines represent the average of all cells. Applying the mitochondrial uncoupler FCCP fully depolarizes mitochondria and therefore gives the maximum fluorescence of rh-123. B) Quantification of all rh-123 recordings, showing that the initial depolarization between 50-100 s is comparable between dendrites and soma, whereas the secondary depolarization between 250-350 s is stronger in (or even exclusive to) somatic areas. *p<0.05 t-test comparing primary and secondary depolarization between the dendrite and soma (80 somatic and dendritic areas within an n=4 independent experiments). C) The stronger depolarization in B) is not due to a difference in mitochondrial Ca\(^{2+}\) uptake, as neurons transfected with the mitochondria targeted GCaMP2 Ca\(^{2+}\) reporter show similar NMDA-induced Ca\(^{2+}\) levels in soma and dendrites. *p<0.05 paired t-test comparing pre- to post-NMDA stimulation (n=12). D) shows an example trace of NAD(P)H autofluorescence with 10 somatic and 10 dendritic regions (thick lines show average of all cells in the experiment). NMDA causes a drop in NADH in soma and dendrites. E) bar graph showing the %drop in NAD(P)H autofluorescence between somatic and dendritic areas, with dendrites showing a stronger drop compared to somata. NAD(P)H autofluorescence was measured until the NMDA induced drop reached a plateau (after ~300s). *p<0.05 t-test comparing the maximum drop in NADH between the dendrite and soma (80 somatic and dendritic areas within an n=8 independent experiments). All graphs in this figure have been taken from (Hasel et al., 2015). See Fig. A4, A5 and A6 for example images.
I found that while both, the dendrite and soma showed a similar initial depolarization, the soma showed a much stronger secondary depolarization, that was almost absent in dendrites (Fig. 3.6A, B). To investigate whether the differential secondary depolarization between these two sub-cellular compartments could be caused by mitochondrial Ca$^{2+}$ uptake, I performed Ca$^{2+}$ imaging with GCaMP2 targeted to the mitochondrial matrix (mito-GCaMP2). Dendrites and somata take up similar amounts of Ca$^{2+}$ following NMDAR stimulation, which can explain the initial $\Psi_m$ depolarization that is similar in both compartments (Fig. 3.6C). However, this cannot explain the secondary $\Psi_m$ depolarization that is almost exclusive to somata. Since $\Psi_m$ depolarization can also be caused by NADH depletion (Abramov and Duchen, 2008), I decided to measure dendritic and somatic NADH levels using the autofluorescent properties of NAD(P)H. While using this approach reports both, levels of NADH and NADPH, the first is much more abundant in the cell than the latter, and so NAD(P)H autofluorescence is widely used to assess NADH levels. I found that excitotoxic NMDAR stimulation causes a strong drop in NADH levels in dendrites and somata (Fig. 3.6D, E). However, the relative drop in NADH was stronger in the soma compared to dendrite, which could explain why the soma experiences a much stronger secondary $\Psi_m$ depolarization.

Taken together, these data indicate that, while the cytosolic and mitochondrial Ca$^{2+}$ uptake is similar across the soma and dendrite following excitotoxic NMDAR stimulations, mitochondrial properties change differently between these two compartments.

### 3.3 Discussion

I have shown here that dendrites are more vulnerable to insults from sub-lethal oxidative stress and lethal excitotoxic insults when compared to the soma. This adds to a body of work showing that dendrites are selectively vulnerable when exposed to these kinds of stressors, be it in acute or chronic brain conditions. It begs the
question, however, what the properties are that make dendrites so sensitive to oxidative and excitotoxic insults. I also applied two interventions that are able to boost the capacity of the glutathione system, neuronal Nrf2 over-expression and chronic synaptic activity.

3.3.1 Dendritic vs. somatic responses to oxidative stress

I showed here that dendrites undergo a more drastic fluctuation in the glutathione redox potential \( \text{E}_{\text{GSH}} \) than the soma of the same cell when experiencing low levels of \( \text{H}_2\text{O}_2 \). The \( \text{E}_{\text{GSH}} \) is regulated on several levels and can be influenced by the rate of production of GSH, the conversion of GSSG back into GSH and GSH oxidation by GSH peroxidases to neutralise peroxides. Any difference in these three factors between dendrites and the soma could explain the difference in the \( \text{E}_{\text{GSH}} \) shift observed in this study. However, since the ratio of volume to surface area is greater in dendrites than the soma, dendrites will experience relatively more \( \text{H}_2\text{O}_2 \) per cytoplasmic volume compared to the soma, which could contribute to the larger redox perturbations in dendrites when exposed to \( \text{H}_2\text{O}_2 \).

Neurodegenerative diseases with a component of dendritic pathologies, such as AD, HD, ALS and PD, often also show a deregulation of the glutathione or other antioxidant systems (Luebke et al., 2010; Bell et al., 2011; Johnson et al., 2012). Similarly, many neuropsychiatric disorders, such as schizophrenia, bipolar disorders and autistic spectrum disorders show both, a disturbance of the GSH system and subtle changes in dendritic morphology, including spine alterations (Do et al., 2000; Grima et al., 2003; Gysin et al., 2007; Gawryluk et al., 2010; Frustaci et al., 2012; Ghanizadeh et al., 2012). These disturbances of the GSH system will most likely be affecting dendrites more than the soma if exposed to ROS, be it from endogenous sources such as the ETC, or exogenous sources, such as activated microglia.

Consequently, targeting the GSH system has been suggested as a possible therapeutic approach to confer protection against oxidative stress-induced neuronal damage (Gupta et al., 2011; 2013).
3.3.2 Interventions to boost the $E_{GSH}$

The activation of Nrf2 in neurons has been suggested as a mechanism to boost the neuronal antioxidant capacity. Nrf2 is the master-regulator of antioxidant enzymes (Ma, 2013) and is strongly expressed in astrocytes but not expressed in neurons. Nrf2 in astrocytes regulates the synthesis of GSH, which is eventually shuttled to neurons (Dringen et al., 2000), and over-expression of Nrf2 in neurons can confer protection against oxidative-stress induced cell death (Soriano et al., 2008). I showed here that neurons over-expressing Nrf2 showed smaller fluctuations in $E_{GSH}$ when exposed to small doses of $H_2O_2$. This is most likely due to Nrf2 inducing the transcription of genes that increase glutathione synthesis, including glutamate-cysteine ligase catalytic subunit (Gclc) (Ma, 2013), thereby boosting the neuronal $E_{GSH}$. While the over-expression of Nrf2 seems to be an attractive therapeutic intervention to protect against neuronal oxidative damage, we have recently shown that Nrf2 over-expression can have detrimental effects on neuronal development (Bell et al., 2015). In fact, neurons epigenetically down-regulate Nrf2 expression early in development to create an intracellular redox environment that allows redox-sensitive transcription factors necessary for neurite outgrowth to be activated, and forced Nrf2 over-expression in neurons causes impaired dendritic development (Bell et al., 2015). It will be interesting to see what over-expression of Nrf2 at a later developmental stage does to the neuronal dendritic arbor and whether drugs that target the epigenetic silencing of Nrf2 in neurons can have neuroprotective effects.

It is well established that synaptic activity can have neuroprotective effects against a broad set of insults by regulating a wide variety of down-stream targets, including genes involved in neuroprotection (Hardingham, 2009). For example, chronic elevation of synaptic-activity can confer protection against oxidative stress by upregulating the transcription of enzymes of the thioredoxin-peroxiredoxin system (Papadia et al., 2008). We have shown recently that synaptic activity also increases the transcription of genes involved in GSH synthesis, utilization and recycling (Baxter et al., 2015). I showed here that chronic synaptic activity can boost the
capacity of $E_{GSH}$, as the application of $H_2O_2$ induced smaller fluctuations in $E_{GSH}$ in neurons undergoing elevated synaptic activity compared to non-stimulated controls. This supports the finding that synaptic activity boosts the capacity of the neuronal glutathione system, enabling neurons to better cope with oxidative stress. Unlike the protection of neurons from oxidative stress by astrocytic Nrf2, however, the boost in $E_{GSH}$ by synaptic activity is a cell autonomous process. Interestingly, increasing synaptic activity can have an additive effect on neuroprotection when concomitantly activating astrocytic Nrf2 (Baxter et al., 2015).

### 3.3.3 Dendritic vs. somatic responses to excitotoxic Ca$^{2+}$ influx

The excessive stimulation of NMDARs causes Ca$^{2+}$ influx into neurons that will eventually lead to cell death, a phenomenon termed excitotoxicity (Olney, 1969). During periods of excitotoxicity, as can be studied by bath-applying NMDA to neuronal cultures, as in this study, neurons experience a steep drop in ATP and NADH levels, depolarisation of the mitochondrial membrane potential ($\Delta\Psi$) and Ca$^{2+}$ deregulation (Abramov and Duchen, 2008). Ca$^{2+}$ entering neurons during an excitotoxic insult will quickly be taken up by mitochondria, following its steep electrochemical gradient, which causes $\Delta\Psi$ depolarisation (Keelan et al., 1999). Pharmacologically inhibiting MCU, the mitochondrial Ca$^{2+}$ uniporter, whose genetic identity has been identified recently (Baughman et al., 2011; De Stefani et al., 2011), prevents glutamate-induced $\Delta\Psi$ depolarisation, identifying mitochondrial Ca$^{2+}$ influx as the main driver of $\Delta\Psi$ depolarisation in excitotoxicity (Abramov and Duchen, 2008). However, other factors have been shown to contribute to $\Delta\Psi$ depolarisation as well, including ROS. Ca$^{2+}$ influx into neurons following NMDAR stimulation will increase ROS production by mitochondria (Duan et al., 2007) and the cytosolic NADPH oxidase (Brennan et al., 2006), which causes damage to DNA that will activate PARP1. PARP1 in turn facilitates the release of pro-apoptotic factors from mitochondria (Yu et al., 2002) and increases its poly(ADP-ribosyl)ation activity, as well as increasing glycolysis (Andrabi et al., 2014), both of which require NAD$^+$. Since NAD$^+$ is needed to sustain H$^+$ transport by the ETC, $\Delta\Psi$ depolarises.
I showed here that bath-application of NMDA caused a sharp increase in cytosolic and mitochondrial Ca\(^{2+}\) that is similar between dendrites and somata. Consequently, the immediate \(\Delta \Psi\) depolarisation is also similar between both compartments, as the initial phase of \(\Delta \Psi\) depolarisation is mediated by the uptake of Ca\(^{2+}\) into the mitochondrial matrix. However, dendrites experience a more drastic drop in ATP, while showing a less severe drop in NADH levels and a weaker secondary \(\Delta \Psi\) depolarisation, demonstrating a disconnect between ATP depletion and \(\Delta \Psi\) depolarisation. A possible explanation for this is that the secondary \(\Delta \Psi\) depolarisation is caused by PARP1 activation, and since PARP1 is activated by ROS induced DNA damage, would cause a more pronounced drop in NADH levels in the soma and therefore a stronger \(\Delta \Psi\) depolarisation when compared to dendrites. The stronger drop of ATP levels in dendrites following the application of NMDA could be a consequence of the higher energy demand of dendrites compared to the soma. It is easy to imagine that dendrites harbour more NMDAR per cytoplasmic volume than the soma, causing a greater energetic burden after NMDAR activation in dendrites than somata, as plasma membrane and ER pumps try to restore the membrane potential. Alternatively, dendrites could have a relatively lower mitochondrial capacity compared to somata. Nonetheless, \(\Delta \Psi\) depolarisation is not the main driving force of ATP depletion, as the secondary \(\Delta \Psi\) depolarisation is observed well after the drop in ATP. However, one possible explanation is that excessive stimulation of NMDAR will lead to a strong Na\(^{+}\) influx, which is counteracted by the Na\(^{+}\)-K\(^{+}\)-ATPase, leading to an unsustainable increase in ATP demand in dendrites, as seen in the sharp drop in ATP (Greenwood et al., 2007). Additionally, the accumulation of Na\(^{+}\) inside neurons causes the uptake of water, leading to changes in the dendritic morphology, such as beading (Greenwood and Connolly, 2007; Mizielsinska et al., 2009) and spine loss (Mizielsinska et al., 2009).

### 3.4 Conclusion

The recent development of genetically-encoded imaging probes for metabolites and second messengers allows the high spatio-temporal study of acute neuronal (patho)-physiological events in real-time. Applying these probes as well as using more
established imaging tools, I showed here that dendrites are particularly vulnerable to oxidative and excitotoxic insults. Since many neurodegenerative diseases show signs of increased ROS production and metabolic perturbations, the selective dendritic vulnerability shown here could, at least in part, explain the dendritic phenotypes observed in these diseases. I also showed that neuroprotective synaptic activity can confer protection against oxidative insults by boosting the glutathione redox capacity of neurons, and confirm the powerful effect that Nrf2 overexpression has on the neuronal antioxidant defence, emphasising Nrf2 as a potential drug target against neurodegeneration.
Chapter 4

The energetic burden of synaptic activity triggers an MCU-dependent increase in energy output that is weaker in dendrites than somata.
Chapter 4: The energetic burden of synaptic activity triggers an MCU-dependent increase in energy output that is weaker in dendrites than somata

4.1 Introduction

While the stimulation of NMDARs by bath application of NMDA causes excitotoxicity, more physiological neuronal Ca\textsuperscript{2+} influx, as in case of synaptic activity, can have neuroprotective effects (Bell and Hardingham, 2011). Synaptic activity, however, is bioenergetically demanding, particularly at the level of the synapse, with most of the energy spent on reversing post-synaptic ion fluxes after action potential firing (Harris et al., 2012). It is therefore not unlikely that dendrites experience a stronger metabolic burden when compared to the soma. Using a recently developed genetically encoded probe for ATP as well as NAD(P)H autofluorescence, I investigated subcellular differences in coping with an increase in metabolic demand.

To cope with rapid changes in synaptic activity, neurons have developed a system to quickly adapt to changes in energy demand (Hall et al., 2012; Harris et al., 2012). Many of these changes happen at the level of mitochondria and are thought to be regulated by mitochondrial Ca\textsuperscript{2+} (Kann and Kovacs, 2006). The recent genetic identification of the gene encoding the mitochondrial Ca\textsuperscript{2+} uniporter MCU (CCDC109A) (Baughman et al., 2011; De Stefani et al., 2015) allowed us to study the effect of mitochondrial Ca\textsuperscript{2+} uptake on neuronal energy output during periods of elevated synaptic activity.

Initially, I wanted to visualize synaptic activity-induced metabolic adaptation mechanisms, then show how dendrites cope with the bioenergetic stress when compared to somata, and lastly show how these adaptive mechanisms are regulated by mitochondrial Ca\textsuperscript{2+}. In the context of this, I used a recently developed reporter of the cytosolic NADH:NAD\textsuperscript{+} ratio, Peredox (Hung et al., 2011), and show how it can be calibrated and applied.

I show here that synaptically active neurons increase their flux through the TCA cycle and the ETC, partly by increasing matrix dehydrogenase activity in a MCU-
dependent manner. I also found that dendrites are less able to adjust to an increase in energy demand by synaptic activity, showing weaker levels of NADH production and a stronger drop in ATP when compared to the soma.

Most of the data in this chapter have been published in (Hasel et al., 2015) and (Marland et al., 2015).

4.2 Results

4.2.1 Measuring the cytosolic NADH/NAD$^+$ ratio using the genetically encoded reporter Peredox

One of the main currencies of cellular energetics is the reducing equivalent NADH, which acts as a proton donor during oxidative phosphorylation to produce ATP (Glancy and Balaban, 2012). NADH is mainly visualized by using the autofluorescent properties of NAD(P)H (Shuttleworth, 2010). While this is a widely accepted approach to measure cellular bioenergetics, NAD(P)H autofluorescence measures both, NADH and NADPH fluorescence as well as being restricted to measuring only the mitochondrial fraction of NADH, since this is where most of the reducing equivalent is present (Shuttleworth, 2010).

I took advantage of the recent development of Peredox, a genetically encoded reporter of the cytosolic NADH/NAD$^+$ ratio (Hung et al., 2011). Peredox is a circularly permuted GFP-based probe. Its bacterial moiety binds NADH and NAD$^+$ and Peredox shows an increase in fluorescence with increasing NADH/NAD$^+$ ratios (Hung et al., 2011). Peredox has a mCherry protein attached to it to allow correcting for differences in the amount of Peredox expressed by each cell.
Figure 4.1: The reporter of the cellular NADH:NAD$^+$ ratio, Peredox, can be calibrated using different ratios of lactate/pyruvate.

A) Example trace of four Peredox expressing neurons that were exposed to different ratios of lactate/pyruvate, showing maximum fluorescence with lactate only and minimum fluorescence with pyruvate only. The NADH/NAD$^+$ sensitive GFP signal was divided by the constant mCherry signal. B) Using the equilibrium constant of LDH \( k=\frac{[\text{pyruvate}][\text{NADH}]}{[\text{lactate}][\text{NAD}^+]} \) and \( k=1.11\times10^{-4} \), the lactate/pyruvate ratio is converted into NADH/NAD$^+$ ratios and plotted as a function of the maximum fluorescence obtained with lactate only (n=6 cover slips, with 3-5 cells each). All graphs in this figure have been taken from (Hasel et al., 2015). See Fig. A8 for example images.

Peredox can be calibrated by perfusing different ratios of lactate/pyruvate onto Peredox-expressing cells (Fig. 4.1A), which allows converting its fluorescence into NADH/NAD$^+$ levels (Fig. 4.1B). This is possible because lactate dehydrogenase (LDH) within neurons interconverts pyruvate and lactate. Addition of pyruvate causes a drop in the NADH/NAD$^+$ ratio, since LDH uses NADH as an electron donor when converting pyruvate into lactate. This reaction is reversible, and adding lactate to neurons will increase the NADH/NAD$^+$ ratio. Using the equilibrium constant (k) of LDH with \( k=\frac{[\text{pyruvate}][\text{NADH}]}{[\text{lactate}][\text{NAD}^+]} \) and \( k=1.11\times10^{-4} \), it is possible to translate the pyruvate/lactate ratio into NADH/NAD$^+$ ratios, since k, [pyruvate] and [lactate] are known, and plot the NADH/NAD$^+$ ratios against the maximal Peredox fluorescence obtained with lactate only (Fig. 4.1B).

For all following experiments, Peredox was calibrated by washing on medium containing lactate only at the end of the recording, giving the maximum fluorescence of Peredox. The NADH/NAD$^+$ sensitive GFP signal was then divided by the non-
responsive signal from the mCherry attached to Peredox. These values were subsequently translated into NADH/NAD$^+$ levels using the function calculated above (Fig. 4.1B). I used Peredox tagged to the nucleus, since non-tagged Peredox is taken up by lysosomes (likely due to the mCherry attached to it) (Hung and Yellen, 2014). Assuming no diffusion barrier between the nucleus and cytoplasm for NADH and NAD$^+$, nuclear Peredox can be used to assess cytoplasmic NADH/NAD$^+$ ratios (Zhang et al., 2002a; Hung et al., 2011).

4.2.2 Neurons adapt to energetically demanding synaptic activity by increasing oxidative phosphorylation and NADH production

To investigate how neurons adapt to an increase in synaptic-activity induced energy demand, I applied Bicuculline/4-Aminopyridine (BiC/4-AP) stimulations, which cause strong action potential bursting. I showed that the acute induction of network bursting can increase the cytosolic NADH/NAD$^+$ ratio, as measured with Peredox (Fig. 4.2A, B). Peredox fluorescence is not saturated by the BiC4 stimulation, as lactate (10 mM) can further increase the signal. Using the established calibration curves for Peredox (Fig. 4.1 A, B), the fluorescence change induced by BiC/4-AP (Fig. 4.2B) can be translated into actual NADH/NAD$^+$ ratios (Fig. 3.2A). This increase in NADH production has been well described using autofluorescence (Kann et al., 2003b) and is thought to be due to an increase in TCA cycle activity (Glancy and Balaban, 2012).
Figure 4.2: Neurons show an increase in oxidative phosphorylation and TCA cycle activity during periods of acutely elevated synaptic activity.

A) Bicuculline (50 μM) plus 4-aminopyridine (250 μM) -induced action potential bursting increases cytosolic NADH/NAD⁺ levels in neurons as measured with Peredox. *p<0.05 t-test (n=19). B) Example trace of 4 Peredox-expressing neurons showing Bicuculline/4-Aminopyridine (BiC4)-induced increase in Peredox fluorescence and calibration with lactate (10 mM). Fluorescence values are converted into NADH/NAD⁺ levels using the function in Fig. 4.1B. C) Example trace of a Seahorse Bioanalyzer run, showing the effect of BiC4 on neuronal energy consumption. BiC4-induced network disinhibition causes an increase in the oxygen consumption rate (OCR), indicative of an increase in oxidative phosphorylation. Subsequent addition of oligomycin (oligo, 1 μM) shows that the OCR is mainly due to mitochondrial ATP production. FCCP (0.125 μM) gives the maximum respiratory capacity and addition
of antimycin (Ant, 2 μM) and rotenone (Rot, 2 μM) shows the non-mitochondrial oxygen consumption. D) Peak of BiC4-induced increase in OCR *p<0.05 t-test (n=17). E) BiC4 induces de-phosphorylation of pyruvate dehydrogenase subunit E1α (PDH-E1α, Ser293). PDH is de-phosphorylated as quickly as 30s post BiC4 application and stays de-phosphorylated as long as BiC4 is present (at least up to 2h). *p<0.05 t-test (n=3). F) When BiC4 stimulations are stopped by silencing network activity with TTX (1 μM), PDH is re-phosphorylated in a time-dependent manner. BiC4-induced de-phosphorylation becomes non-significant to untreated control and significantly different to 5 min BiC4 after 1 min of treatment with TTX. Pi-PDH-E1α (Ser293) was normalised to PDH-E1α. *p<0.05 compared to untreated control, #p<0.05 compared to 5 min BiC4 condition, one-way ANOVA plus Bonferroni’s post-hoc test (n=5). Graphs A-E in this figure have been taken from (Hasel et al., 2015).

To test whether oxidative phosphorylation is regulated by synaptic activity, I employed Seahorse Bioanalyser, a plate-reader system that measures extracellular O2 levels. Inducing synaptic activity with BiC/4-AP increases the oxygen consumption rate (OCR) of neurons, indicative of an increase in oxidative phosphorylation (Fig. 4.2 C, D).

Furthermore, enhancing synaptic activity causes the de-phosphorylation of pyruvate dehydrogenase subunit E1α (PDH-E1α, Ser293) (Fig. 4.2E), a key metabolic enzyme linking glycolysis to the TCA cycle (Rardin et al., 2009). De-phosphorylation of PDH is known to be Ca2+-dependent and causes its NADH producing activity to increase, supplying the elevated oxidative phosphorylation we observed with reducing equivalents needed for ATP production (Rardin et al., 2009; Glancy and Balaban, 2012). The de-phosphorylation is quick (30s post BiC/4-AP addition) and PDH stays de-phosphorylated as long as BiC/4-AP is present (at least for 2h). Additionally, silencing the network firing after an initial increase, by adding TTX following BiC/4-AP stimulations, causes PDH to be re-phosphorylated (Fig. 4.2F). Interestingly, PDH re-phosphorylation is significantly slower than the initial de-phosphorylation (compare Fig. 4.2E and Fig. 4.2F), possibly because even though TTX has stopped the cells from firing, re-establishing the resting state still requires NADH and ATP production, both of which regulate PDH phosphorylation (Glancy and Balaban, 2012). Alternatively, PDH phosphorylation by PDH kinase, which is not regulated by Ca2+ (Sugden and Holness, 2003), could be an inherently slower enzymatic reaction compared to the de-phosphorylation by the PDH phosphatase.
4.2.3 Somata are better equipped to cope with a metabolic challenge than dendrites

The metabolic demand of neurons during action potential firing is mainly due to the high energy levels required to restore the membrane potential by reversing the ion fluxes through post-synaptic channels (Harris et al., 2012). I therefore wanted to see whether the metabolic adaptation I have observed in neurons is equally strong in the soma compared to dendrites.

Due to the fact that Peredox has to be tagged to the nucleus in order not to be degraded, I used NAD(P)H autofluorescence to visualize dendritic and somatic NADH. I found that stimulating neurons with BiC/4-AP causes a stronger NADH fold induction in the soma compared to the dendrite (Fig. 4.3A, B).
**A**

NAD(P)H autofluorescence

**B**

NAD(P)H autofluorescence

**C**

BiC/4-AP

**D**

cyo-GCaMP2

**E**

BiC/4-AP

**F**

mito-GCaMP2

**G**

ATP drop

**H**

AT1.03

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**Legend:**

- Con: Control
- BiC4: Bicain 4
- Soma: Cell body
- Dendrite: Dendrite
Figure 4.3: Dendrites show lower levels of Ca\(^{2+}\) uptake, lower levels of NADH production and a steeper drop in ATP during action potential firing.

A) NAD(P)H autofluorescence example trace of 20 somatic and 20 dendritic areas during BiC4 stimulation, with thin lines representing single cells and thick lines the average of the recording. B) Fold induction of NAD(P)H autofluorescence after 10-16 min BiC4 stimulation shows a stronger increase in soma when compared to dendrites \(^{*}p<0.05\) t-test \((n=8\) independent recordings). C) Example trace of cytosolic Ca\(^{2+}\) uptake in soma and dendrite of a single cell following application of BiC4 as measured with a cytosolic GCaMP2 Ca\(^{2+}\) indicator. D) Dendrites show a smaller increase in cytosolic Ca\(^{2+}\) following BiC4 stimulation when compared to soma with difference of pre- to post-BiC4 stimulation \((60s)\) shown on the right. \(^{*}p<0.05\) t-test for dendrite vs. soma, \(^{*}p<0.05\) t-test \((\text{comparing pre- to post-}\) BiC4 levels, \(n=8\) cells). E) Example trace of mitochondrial Ca\(^{2+}\) uptake following BiC4 stimulations as measured with the mitochondria targeted Ca\(^{2+}\) probe mito-GCaMP2. F) Dendrites show a smaller increase in mitochondrial Ca\(^{2+}\) than somata when comparing the difference of pre-BiC4 to 60s post-BiC4 stimulations \((\text{right})\). \(^{*}p<0.05\) t-test for dendrite vs. soma. \(^{*}p<0.05\) t-test \((\text{comparing pre- to post-}\) BiC4 levels, \(n=12\) cells). G) Examples trace showing AT1.03 positive neurons with one dendritic and one somatic ROI during BiC4 stimulations. H) BiC4 causes a drop in ATP as measured with AT1.03, until AT1.03 YFP/CFP ratio plateaus. Values are YFP/CFP ratios before \(\langle[YFP/CFP]\rangle_{t=0}\) minus YFP/CFP ratios during \(\langle[YFP/CFP]\rangle\) BiC4 stimulations. Dendrites experience a sharper drop in ATP compared to somata of corresponding cells over the first 60s of BiC4 stimulations. \(^{*}p<0.05\) two-way ANOVA plus Bonferroni's post hoc test \((n=7\) cells). All graphs in this figure have been taken from \((\text{Hasel et al., 2015})\). See Fig. A3, A4, A5, A7 for example images.

Given the central role of Ca\(^{2+}\) in regulating NADH production \((\text{Glancy and Balaban, 2012})\), I used a cytoplasmic and mitochondria-targeted GCaMP2 to measure BiC/4-induced Ca\(^{2+}\) transients. BiC/4 increases cytoplasmic Ca\(^{2+}\) in both compartments, the soma and the dendrite \((\text{Fig. 4.3C, D})\). However, the Ca\(^{2+}\) increase is higher in the soma compared to the dendrite, likely due to faster Ca\(^{2+}\) clearance from dendrites \((\text{Hardingham et al., 2001})\). As a probable consequence of this, the increase in mitochondrial Ca\(^{2+}\) is smaller in dendrites compared to the soma as well \((\text{Fig. 4.3E, F})\). Lastly, I looked at ATP levels in dendritic and somatic regions during action potential bursting, using AT1.03. I found that synaptic activity reduces ATP levels in somatic and dendritic regions. However, the drop in ATP is faster in dendrites compared to the soma \((\text{Fig. 4.3G, H})\). This finding was similar to the excitotoxic NMDAR stimulations \((\text{Fig. 3.5A, B})\), but the drop was not as drastic.

Hence, during action potential firing, dendrites take up less Ca\(^{2+}\) into the cytoplasm and into mitochondria compared to somata, with a possible consequence being a smaller fold induction of NADH. Additionally, high energy demands at the post-synapse cause a sharper drop in ATP levels in dendrites compared to the soma.

These data indicate that the coupling between energy production and energy demand is better tuned in the soma than it is in the dendrite.
4.2.4 The mitochondrial Ca\(^{2+}\) uniporter MCU regulates Ca\(^{2+}\) influx into mitochondria following NMDA receptor stimulation

Having established that neurons increase oxidative phosphorylation and TCA cycle activity during periods of action potential firing, I wanted to know what role mitochondrial Ca\(^{2+}\) plays in this and used genetic knock-down approaches of MCU to address this question.

The mitochondrial Ca\(^{2+}\) uniporter (MCU) is the pore-forming subunit of a protein complex that regulates the uptake of Ca\(^{2+}\) into mitochondria (Raffaello et al., 2012). While it is possible to target the MCU pharmacologically with Ruthenium Red, this drug is non-selective and cell-impenetrable (Robert et al., 2001; Hajnóczky et al., 2006). However, very recently, the gene encoding for MCU, CCDC109A, has been identified (Baughman et al., 2011; De Stefani et al., 2011). This allowed us to investigate the role of the MCU in controlling mitochondrial Ca\(^{2+}\) uptake in cortical neurons using knock-down constructs (Fig. A1) (Qiu et al., 2013).

We found that knocking down MCU in neurons using a siRNA or shRNA targeted against Mcu suppresses mitochondrial Ca\(^{2+}\) uptake following NMDA bath application or the induction of action potential firing (Fig. A1). Jing Qiu and Jamie Marland performed most of the experiments shown in figure A1 (see Appendix for details).

4.2.5 Synaptic activity-induced NADH production and PDH de-phosphorylation are suppressed by MCU knock-down

I then used the established MCU knock-down system to test the hypothesis that mitochondrial Ca\(^{2+}\) plays an important role in regulating neuronal energy production. Ca\(^{2+}\) is known to stimulate NADH producing dehydrogenases in the mitochondrial matrix, including PDH (Denton and McCormack, 1990; Denton, 2009), and inhibition of MCU using Ruthenium Red was shown to suppress the activation of these dehydrogenases in non-neuronal systems (Denton and McCormack, 1990).
used the MCU knock-down approach to investigate the role of mitochondrial Ca\textsuperscript{2+} in regulating the metabolic output of neurons during increased action potential firing induced by BiC/4-AP.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Synaptic-activity induced NADH production and PDH de-phosphorylation are suppressed by knock-down of MCU.}
\end{figure}

A) Cells were transfected with Peredox and either siMcu or siCnt for two days and stimulated with BiC/4-AP. BiC/4-AP-induced action potential bursting increases cytosolic NADH levels, which is suppressed by the siRNA mediated knock-down of MCU (left). Difference in BiC/4-AP induced NADH production shows suppressed NADH induction in siMcu condition compared to siCnt. *p<0.05 comparing siCnt to siMcu and \textsuperscript{a}p<0.05 comparing pre- to post-BiC/4-AP, two-way ANOVA plus Bonferroni's post-hoc test (left) or Student t-test (right) (n=4, with 58 cells siCnt and 55 cells siMcu). B) Example traces of BiC/4-AP-induced increase in Peredox fluorescence for siMcu and siCnt. Fluorescent values were converted to NADH/NAD\textsuperscript{+} levels according to Fig. 3.1B. C) Confirmation of Peredox findings with NAD(P)H autofluorescence, showing that BiC/4-AP-induced increase in NAD(P)H autofluorescence is stronger in the surrounding cells (Cnt) compared to siMcu (+mCherry) transfected cells (left). Difference of BiC/4-AP-induced NAD(P)H autofluorescence when comparing siMcu/mCherry transfected cells with surrounding cells, showing that siMcu suppresses BiC/4-AP-induced increase in NAD(P)H autofluorescence (right). *p<0.05 comparing surrounding cells to siMcu/mCherry transfected cell and \textsuperscript{a}p<0.05 comparing pre- to post-BiC/4-AP, two-way ANOVA plus Bonferroni's post-hoc test (n=11 cover slips for both conditions). D) Neuronal cultures were infected with an AAV either containing plasmids encoding shScr or shMcu. Five days post infection, cells were stimulated with BiC/4-AP for 5 or 30 min and protein collected. Knock-down of MCU suppresses the de-phosphorylation of PDH-E1\textalpha (Ser\textsuperscript{295}) after 5 min of BiC/4-AP stimulation when compared to shScr control. Pi-PDH-E1\textalpha (Ser\textsuperscript{293}) and MCU were normalised to PDH-E1\textalpha. *p<0.05 comparing shScr to shMcu and \textsuperscript{a}p<0.05 comparing pre-
Using Peroxid to measure cytosolic NADH/NAD$^+$ ratios, I found that cells transfected with a siRNA against MCU (siMcu) show a suppressed increase in NADH production following increased synaptic activity when compared to neurons transfected with control siRNA (siCnt) (Fig. 4.4A, B). I confirmed these findings using the more established approach of NAD(P)H autofluorescence (Fig. 4.4C). We then looked at how MCU knock-down affects the de-phosphorylation of PDH during synaptic activity. Using an AAV to introduce the shRNAs into neurons, we were able to knock-down MCU in the whole network (Fig. 4.4E, F). When stimulating neurons with BiC/4-AP for 5 min, we found that shCnt infected cells show a drastic de-phosphorylation of PDH-E1α (Ser$^{293}$), and that this de-phosphorylation is strongly suppressed in shMcu infected cells (Fig. 4.4D, E). After 30 min of BiC/4-AP stimulation, the de-phosphorylation of PDH in the shCnt condition is unchanged. However, cells infected with shMcu showed a PDH de-phosphorylation that resembles that of the shCnt condition at this time point. This could either be the consequence of an imperfect knock-down, or an alternative regulation of PDH de-phosphorylation that is independent of Ca$^{2+}$, which is not unlikely since the phosphorylation of PDH is also regulated by ATP, NADH and acetyl-CoA (Glancy and Balaban, 2012).

These data show that mitochondrial Ca$^{2+}$ is involved in adjusting the neuronal TCA cycle flux to changes in energy demand, and that this is regulated by MCU.

**4.2.6 MCU knock-down has no effect on the activity-induced decrease in ATP levels: indication of a metabolic adaptation**

Since NADH and ATP production in mitochondria are tightly linked by the ETC, I used the ATP reporter AT1.03 to investigate the effect of Mcu knock-down on ATP production during action potential firing. Neurons were transfected with AT1.03 and either siCnt or siMcu.
I found that the drop in ATP following BiC/4-AP stimulation is not different between the two conditions (Fig. 4.5A, B).

A) Neurons were transfected with AT1.03 and either siCnt or siMcu. Cells were exposed to BiC/4-AP and subsequently to FCCP (10 μM). YFP/CFP ratios recorded after the addition of FCCP were used to normalise the recordings. BiC/4-AP-induced action potential firing caused a drop in ATP in both conditions. The difference of the YFP/CFP ratio before and after BiC/4-AP stimulation was not significantly different between siCnt and siMcu (right). siMcu positive cells had in general higher ATP levels when compared to siCnt positive cells (left). *p<0.05 comparing siCnt to siMcu and #p<0.05 comparing pre- to post-BiC/4-AP, two-way ANOVA plus Bonferroni’s post-hoc test (left) or Student t-test (right) (n=3, 10 cells per condition). B) Example traces for AT1.03 positive neurons co-transfected with either siCnt or siMcu shows drop in ATP following application BiC/4-AP and FCCP (10 μM). C) Cells were co-transfected with mito-GCaMP2 and either siCnt or siMcu. Application of BiC/4-AP increased mitochondrial Ca²⁺ uptake equally in both conditions (left), resulting in no difference in the maximal BiC/4-AP induced mitochondrial Ca²⁺ uptake (right). *p<0.05 comparing siCnt to siMcu and #p<0.05 comparing pre- to post-BiC/4-AP, two-way ANOVA plus Bonferroni’s post-hoc test (left) or Student t-test (right) (n=5, with 26 siCnt-positive cells and 21 siMcu-positive cells).

Figure 4.5: MCU knock-down has no effect on the synaptic activity induced drop in ATP or mitochondrial Ca²⁺ uptake.

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81
This could potentially mean that even the reduced levels of NADH produced in the siMcu condition suffice to produce adequate amounts of ATP during action potential firing. Surprisingly, we found that the baseline ATP levels in the siMcu condition are slightly higher compared to the control condition (Fig. 4.5A). This could indicate a metabolic adjustment of neurons to an impaired control of their mitochondrial metabolism by Ca\(^{2+}\), possibly by up-regulating glycolytic activity.

**4.2.7 Ca\(^{2+}\) uptake into mitochondria following network bursting is not suppressed by RNAi mediated knock-down of MCU**

To my surprise, I found that knocking down MCU could not reduce Ca\(^{2+}\) entering the mitochondrial matrix during action potential firing using BiC/4-AP (Fig. 4.5C). This was unexpected, given that Mcu knock-down can suppress mitochondrial Ca\(^{2+}\) uptake following NMDA bath application and the induction of action potential trains using electrodes (Fig. A1). Additionally, siRNA mediated knock-down of Mcu had clear effects on NADH production (Fig. 4.4A, B, C) and (although surprising) effects on baseline ATP levels (Fig. 4.5A). A possibility for this finding is that mito-GCaMP2 is not able to detect the possibly very subtle differences in mitochondrial Ca\(^{2+}\) uptake given the imperfect siRNA mediated knock-down of MCU, and other reporters should be employed. Alternatively, since the nature of NMDAR activation by NMDA bath application vs. synaptic activity differs greatly, this could be reflected in a different Ca\(^{2+}\) uptake mechanism into mitochondria. For example, the release of intracellular Ca\(^{2+}\) stores from the endoplasmic reticulum (ER) following synaptic activity can greatly enhance mitochondrial Ca\(^{2+}\) uptake, since the ER and mitochondria share an efficient Ca\(^{2+}\) transfer system involving MCU (Rowland and Voeltz, 2012). This, however, would not explain the different NADH inductions I observed.

**4.3 Discussion**

Neuronal activity is metabolically challenging and can change drastically over a short period of time. Therefore, neurons have to have systems in place to adjust their
metabolic output to changes in metabolic demand. I showed here that neurons increase their energy production during neuronal network bursting and highlighted the role of mitochondrial Ca\(^{2+}\) therein. Furthermore, I showed that dendrites are not as well equipped to cope with a metabolic burden when compared to the soma, indicating a selective dendritic vulnerability of dendrites to metabolic stress.

4.3.1 Neurons adjust their metabolic output to changes in metabolic demand in a MCU-dependent manner

I showed here that neurons undergoing increased network bursting, as induced by bicuculline and 4-aminopyridine, boost their mitochondrial energy output, as seen by the increase in NADH production (TCA cycle activity) and oxygen consumption (oxidative phosphorylation). The increase in oxidative phosphorylation can be mediated by the cytoplasmic Ca\(^{2+}\)-regulated activity of the aspartate-glutamate antiporter aralar, which increases the availability of NADH reducing equivalents at the ETC (Llorente-Folch et al., 2013) and promotes glycolysis by increasing the cytoplasmic availability of NAD\(^{+}\), which could contribute to the increase in NAD(P)H autofluorescence and oxygen consumption rate I observed. Furthermore, Ca\(^{2+}\) influx into the mitochondrial matrix through MCU can activate matrix dehydrogenases, increasing their NADH output. Indeed, I found that PDH is undergoing activating de-phosphorylation upon synaptic activity, which could explain the increase in NAD(P)H autofluorescence and ultimately the increase in oxygen consumption rate. Interestingly, aralar activity and the matrix dehydrogenases share the substrate α-ketoglutarate, and it has been suggested that the MCU-dependent increase in α-ketoglutarate production will inhibit the NADH shuttle of aralar (Contreras and Satrustegui, 2009). Consequently, low levels of cytoplasmic and mitochondrial Ca\(^{2+}\) uptake would preferentially activate aralar, while high levels of cytoplasmic Ca\(^{2+}\) uptake and therefore high levels of mitochondrial Ca\(^{2+}\) uptake through MCU would activate matrix dehydrogenases (Pardo et al., 2006). Since network bursting induces a strong Ca\(^{2+}\) uptake into mitochondria, it can be expected that the increase in energy production I observed is mainly mediated by matrix dehydrogenases rather than aralar activity. Indeed, I
showed that the production of NADH and the de-phosphorylation of PDH depend on the uptake of mitochondrial Ca\(^{2+}\), since MCU knock-down inhibits both. Interestingly, I found that the knock-down of MCU had no effect on the drop of ATP following network bursting, which I had expected given the weaker induction of NADH and reduced PDH de-phosphorylation in the MCU knock-down condition. Moreover, basal ATP levels were even increased in the MCU knock-down condition, suggesting a possible metabolic adaptation. It would be interesting to probe for a possible up-regulation of glycolysis, for example by replacing glucose with either 2-deoxyglucose to inhibit glycolysis or pyruvate to bypass glycolysis and then look at ATP levels pre- and post-network bursting. The shift from oxidative phosphorylation to glycolysis is called the Warburg effect and is often observed in cancerous cells unable to use oxidative phosphorylation to produce ATP (Vander Heiden et al., 2009).

To my surprise, I was unable to visualize a change in mitochondrial Ca\(^{2+}\) uptake following BiC/4-AP stimulations. This is particularly surprising since I saw changes in the siMcu condition at the level of BiC/4-AP-induced NADH production (with Peredox and NAD(P)H autofluorescence) and a change in baseline ATP levels. We also observed a reduction in mitochondrial Ca\(^{2+}\) uptake following NMDA bath-application when comparing the siMcu to siCnt condition. One possible explanation is that mito-GCaMP2 is not sensitive enough to report possibly subtle changes in mitochondrial Ca\(^{2+}\) uptake, which is not unlikely since siMcu mediated knock-down is relatively weak, and a higher affinity Ca\(^{2+}\) reporter might uncover subtle differences. Alternatively, Ca\(^{2+}\) release from intracellular stores following network bursting could be particularly efficient in increasing mitochondrial Ca\(^{2+}\) uptake, which could very well be possible since the ER and mitochondria are intimately linked (Rowland and Voeltz, 2012). However, this would not explain the phenotypes I observed with NADH and PDH. It would be worth trying the shRNA-mediated approach to knock down MCU, since the knock-down efficiency seems to be higher compared to the siRNA (Qiu et al., 2013). However, the shRNA comes with an attached mCherry, which can interfere with our imaging tools and is therefore not ideal. I will repeat these experiments in a MCU knock-out mouse that will be
available to our lab soon, to find out why we see changes in mitochondrial Ca\textsuperscript{2+} uptake with NMDA bath-applications but not with BiC/4-AP stimulations.

4.3.2 Dendritic vulnerability to metabolic stress

Most of the energy produced in neurons is utilized at the post-synapse to restore ion fluxes following action potential firing (Harris et al., 2012). It is therefore not surprising that I found ATP levels to drop more drastic in dendrites when compared to somata during action potential firing. This could of course also be due to difference in substrate availability between these two regions, which could explain the smaller fold induction of NADH in dendrites following action potential bursting. However, since Ca\textsuperscript{2+} uptake into mitochondria following action potential bursting is smaller in dendrites than the soma, which is partly due to a more efficient Ca\textsuperscript{2+} clearance from dendrites, this could lead to a reduced activation of matrix dehydrogenases, leading to lower levels of NADH production and ultimately ATP synthesis. Alternatively, the higher ATP consumption in dendrites also requires more NADH to fuel oxidative phosphorylation, which could cause the lower fold increase in NAD(P)H autofluorescence in dendrites when compared to the soma.

4.4 Conclusion

I showed here that neurons are able to quickly adapt their energy output to changes in energy demand, and they do so by up-regulating TCA cycle activity and oxidative phosphorylation. I also showed that these events are regulated by mitochondrial Ca\textsuperscript{2+}, which enters mitochondria through the mitochondrial Ca\textsuperscript{2+} uniporter MCU. Using novel as well as more established imaging tools to measure neuronal metabolism, I found that dendrites are less able to boost the energy production following an increase in action potential firing, hinting at a selective dendritic susceptibility to metabolic stress that could potentially play a role in the dendritic phenotypes observed in neurodegeneration.
Chapter 5
RNA-sequencing based on mixed-species co-cultures to probe neuron-astrocyte interactions: Validation and application
Chapter 5: RNA-sequencing based on mixed-species co-cultures to probe neuron-astrocyte interactions: Validation and application

5.1 Introduction

Astrocytes and neurons are intimately linked at the morphological as well as the functional level. The role of astrocytes in supporting neuronal activity and survival is well established, with astrocytes shuttling lactate, glutamine and glutathione to neurons (Chih and Roberts, 2003; Dringen and Hirrlinger, 2003). Moreover, astrocytic processes ensheath the entire neuron, including synapses (Peters and Feldman, 1976; Theodosis et al., 2008), thereby creating the basis for the functional interaction between astrocytes and neurons (Araque et al., 2014). Neurons and astrocytes are both able to release transmitters to communicate with each other, creating what has been coined ‘tripartite synapse’ (Araque et al., 1999). Glutamate released from neurons can elicit Ca\(^{2+}\) transients in astrocytes by activating astrocytic mGluRs, causing the astrocytic release of gliotransmitters, such as ATP, which can in turn regulate synaptic activity (Agulhon et al., 2008). During brain development, astrogenesis happens shortly after neurogenesis, which coincides with the development of synapses, and it is now well established that astrocytes can promote synapse formation by releasing trophic factors (Ullian et al., 2001; Clarke and Barres, 2013).

Similarly, neurons can induce changes in astrocytes. Astrocytes co-cultured with neurons undergo drastic changes in their morphology, showing increased stellation and branching (Swanson et al., 1997). Moreover, neurons induce the expression of Connexin 43 (Koulakoff et al., 2008) as well as the glutamate transporters GLAST (EAAT1) and GLT-1 (EAAT2) in astrocytes (Swanson et al., 1997), and their protein levels were suggested to be regulated by synaptic activity (Perego et al., 2000; Yang et al., 2009). Furthermore, there is evidence that glutamate exposure of pure astrocytic cultures can induce transcriptional changes in astrocytes (Méndez et al., 2004; Habas et al., 2013; Jimenez-Blasco et al., 2015).

Given these drastic non-cell autonomous changes in neurons and astrocytes, it has been of great interest to investigate differences in gene expression between these cell
types. Using immunopanning in combination with RNA-sequencing of neurons and astrocytes purified from brain tissue, the transcriptome of both cell types has been well described and made easily accessible (Zhang et al., 2014). However, these data do not investigate the potential transcriptional changes neurons can induce in astrocytes, be it at baseline or by synaptic activity.

Given the drastic morphological changes astrocytes undergo when co-cultured with neurons, and given that synaptically released neurotransmitters can induce astrocytic Ca$^{2+}$ transients, I hypothesise that astrocytes co-cultured with neurons will also show changes at the level of the transcriptome, at basal levels as well as induced by synaptic activity.

The major obstacle in investigating cell-specific changes in transcription is the inability to distinguish neuronal from astrocytic transcripts. While it is possible to culture both cell types separately and use inserts in order for both cell types to share the same medium, this potential set-up would miss the cell contact-dependent interaction entirely. Additionally, neurotransmitters released from neurons would be too heavily diluted before reaching astrocytes, which is making this culture set-up unsuitable to study my hypotheses. Furthermore, physical cell separation, as performed by (Zhang et al., 2014) and others, has the caveats of inducing aberrant gene expression and is prone to cross contamination (Okaty et al., 2011).

Therefore, in order to distinguish transcripts from astrocytes and neurons in co-culture, we decided to use different species for each of the two cell types. This allowed me to co-culture astrocytes and neurons, collect their combined RNA, and sort the origin of each transcript according to the species-specific sequence. Here, I used RNA-sequencing to establish genome-wide changes in transcription in order to compare the transcriptomes of astrocytes cultured on their own to astrocytes cultured with neurons. Moreover, I show evidence that neuronal activity can induce transcriptional changes in astrocytes.

In this chapter I will show the drastic morphological changes astrocytes undergo when co-cultured with neurons, apply species-specific RNA-sequencing on pure astrocytic cultures and astrocyte-neuron co-cultures to establish the transcriptional changes neurons induce in astrocytes, and show evidence for the validity of this approach. Furthermore, this chapter includes data showing that astrocytes sense
glutamate released from neurons during synaptic activity, that this induces astrocytic Ca\(^{2+}\) transients and that synaptic activity induces transcriptional changes in astrocytes.

5.2 Results

5.2.1 Species-specific RNA-sequencing: Feasibility

In order to distinguish the cell type origin of each transcript in a neuron-astrocyte co-culture, I cultured neurons originating from rat on a confluent layer of astrocytes originating from mouse. While the cells coming from mouse are exclusively astrocytic, the cells added on top of the astrocytic layer consist of neurons and astrocytes. Although cultured in medium not containing any serum, a subset of the rat cells will be astrocytic. Consequently, using this set-up allows us to only study the transcriptome of astrocytes (mouse) in the co-culture and reads allocated to rat are neuronal and astrocytic.

From previous work we learned that astrocytes in a co-culture yield approximately 6 times less RNA than neurons of the same culture. Additionally, we were aware of the high percentage of reads that would need to be discarded due to unambiguous matching to mouse and rat. We therefore decided on a sequencing depth of 150 million paired-end reads, compared to the more standard 50 million reads.

Demanding a perfect match between the sequenced read and the reference genome, and discarding any reads that match to both, mouse and rat, we ended up with 7.71% (or approximately 12 million reads) of the total 150 million reads being unambiguously mouse and therefore astrocytic. Furthermore, 95% of the genes identified with the species-specific RNA-seq lose less then 10% of reads when compared to a standard mapping process (Fig. A2).

Based on this, I investigated whether neurons can induce transcriptional changes in astrocytes, by comparing mouse astrocytes cultured on their own to mouse astrocytes cultured in the presence of rat neurons.
5.2.2 Neurons induce drastic changes in astrocyte morphology

Astrocytes cultured on their own (astrocyte mono-cultures, AM) show a polygonal morphology, with little to no processes. When astrocytes are cultured in the presence of neurons (astrocyte-neuron co-culture, AC), astrocytes undergo a drastic morphological change, becoming more stellate and showing thin processes (Swanson et al., 1997). Indeed, comparing AMs with mixed-species ACs shows a marked increase in cellular complexity, with signs of stellation, thinning of processes and an increase in branch number (Fig.5.1A, left). This is a well-established phenomenon and based on the staining of the astrocytic intermediate filament GFAP (glial fibrillary acidic protein). However, GFAP stainings highly underestimate the astrocytic complexity, since GFAP is only present in the core and main branches of astrocytes. I therefore decided to transfect astrocytes with GFP in order to label the entire astrocyte, including thin processes.
Figure 5.1: Astrocytes co-cultured with neurons undergo drastic morphological changes.

A) Example pictures of GFP positive astrocytes (top) in mono-culture (left) or in co-culture with neurons (right) showing astrocytes undergo stellation and produce spine-like processes upon co-culture with neurons. Neurons were stained with a pan-neuronal marker (Neuro-Chrom). Astrocytes stained for GFAP (bottom) also show the increase in stellation when comparing mono-cultured astrocytes (left) with astrocytes co-cultured with neurons (right). B) Quantification of the change in complexity of GFP-positive astrocytes cultured with or without astrocytes. Astrocytes were traced and the perimeter to area ratio used as a measure for cell complexity. While cells cultured on their own do not change their
morphology, the complexity of astrocytes co-cultured with neurons gradually increases over time, with changes observable as early as 1 day post neuron plate down. At 10 days post neuron plate down, neurons show extensive branching and spine-like processes. *p<0.05 one-way ANOVA plus Bonferroni's post-hoc test, compared to DIV0 (n=14-77 cells per condition).

Using lipofectamine, a plasmid encoding GFP was transfected into mouse astrocytes, which were subsequently either cultured on their own or co-cultured with rat neurons (Fig. 5.1A). In order to measure astrocytic complexity, I traced the outline of the cell (perimeter) and normalised it to cell size (area) (Fig. 5.1B). I found that the astrocyte complexity in AMs stays the same over time, while the complexity of astrocytes in AC increased gradually after the addition of neurons. The change in morphology is immediate, showing signs of stellation already on day 1 post neuron plate down and increasing throughout the culture period until 10 days post neuron plate down. On day 10, AC astrocytes have developed several branches and sub-branches that show spine-like processes, a phenotype that strongly resembles in vivo astrocytes (Reichenbach et al., 2010).

Based on this, I then went on to compare the transcriptome of AM astrocytes to that of AC astrocytes using species-specific RNA-sequencing.

**5.2.3 Co-culturing neurons with astrocytes changes the astrocyclic transcriptome**

Total RNA of mouse astrocytes and mouse astrocyte/rat neuron co-cultures was collected on DIV9 post-neuron plate down and three biological replicates were sent for RNA-sequencing at a sequencing depth of 150 million reads. After species-unmixing, only reads were accepted that had zero mismatches and were allocated to only one species. Reads were normalised, transformed into FPKM (fragments per kilobase of transcript per million mapped reads) values and the differential gene expression calculated using DESeq2 (Fig. 5.2). To avoid noise that is generated by low numbers of reads in the following analysis, only genes were included that have an FPKM count of >2.
Figure 5.2: Mixed species RNA-seq uncovers neuronally-induced astrocytic gene regulation.

A) Neurons promote widespread gene expression changes in astrocytes. RNA from mixed species mouse astrocyte/rat neuron co-cultures (n=3) was subjected to RNA-seq, followed by SSS-workflow to identify reads that were unambiguously mouse (i.e. astrocytic) in origin. The same workflow was applied to mouse astrocytic mono-cultures. Expression of genes (FPKM) in astrocytes in the presence or absence of neurons is plotted for all 11,352 genes expressed >2 FPKM in either mono- or co-culture. Red crosses indicate the astrocytic genes induced or repressed >1.5-fold by neurons (DESeq2 P_adj<0.05). Highlighted are the 3 astrocytic genes previously identified as being induced by neurons (Slc1a2, Slc1a3 and Gja1), plus 3 classical astrocytic markers (Gfap, Aldh1l1 and S100b) which are not influenced by the presence of neurons.

Neurons induced a widespread change in gene expression in astrocytes, including three genes that were previously described as upregulated in astrocytes in the presence of neurons (Slc1a2, Slc2a3 and Gja1). Importantly, the expression of the classical astrocytic markers Gfap, Aldh1l1 and S100b were unchanged.
I found that 367 genes showed a significant fold-increase ≥2 and 356 genes showed a fold-decrease of ≥2 (Fig. 4.3 shows Top50 genes that are either up- or down-regulated in astrocytes upon co-culture with neurons).

I then went on to perform gene ontology (GO) term enrichment analysis on the data set, which clusters genes into groups according to their functional characteristics. The analysis compares the frequency of a GO term in the provided gene list with the expected frequency of that GO term.
Top 20 GO terms for genes UP

<table>
<thead>
<tr>
<th>axon choice point recognition</th>
<th>Robo1, Gap43, Elnb3, Robo2</th>
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<tbody>
<tr>
<td>neuron recognition</td>
<td>Cntnap2, Robo1, Ntm, Celsr3, Gap43, Dscam, Fz32, Elnb3, Robo2</td>
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<td>regulation of astrocyte</td>
<td>Ntrk3, Lif, Gpr37, Dab1, Id4, Hes5</td>
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<tr>
<td>differentiation</td>
<td>Dcx, Shank3, Dscam, Dok1, Klf7, Fz32, Dab1, Grinda, Prx2, Ephb1</td>
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<td>learning</td>
<td>Atp1a2, Cntnap2, Ntrk2, Shank3, Scl12a5, Atp8a1, Grm5, Nrx1, Sld6a1, Fgfl3, Jph4, Atp1a3, Pln</td>
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<tr>
<td>cell recognition</td>
<td>Cntnap2, Robo1, Ntm, Celsr3, Vcan, Gap43, Dscam, Mge68, Fz32, Elnb3, Robo2, Ephb1</td>
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<tr>
<td>regulation of axogenesis</td>
<td>Om9, Robo1, Ntrk2, Ntrk3, Lrc4c, Bcl11a, Dscam, Spp1, Dab1, Fgfl3, Robo2</td>
</tr>
<tr>
<td>CNS neuron differentiation</td>
<td>Dcx, Rora, Robo1, Ntrk2, Shank3, Sletb2, Nrx1, Dok1, Fz32, Chd5, Dlx1, Id4, Robo2, Ephb1, Unc5d</td>
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<tr>
<td>modulation of synaptic</td>
<td>Atp1a2, Egr1, Ntrk2, Shank3, Cspg5, Syp, Bcan, Grm5, Nrx1, Sld6a1, Adra1a, Agt, Jph4, Cell4, Sncap, Ptn, Scl1a3, Glu</td>
</tr>
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Selected GO terms with genes UP

Of the 367 genes up-regulated ≥2 upon co-culture, 324 could be mapped by the software and fell within 116 GO terms that were significantly enriched. I found that the top 20 GO terms of genes up-regulated upon co-culture mostly describe glial and
neuronal development as well as basic CNS functions (Fig 4.4). These include genes involved in dendro-and axogenesis (e.g. Robo1, Dscam, Shank3), astrocyte differentiation (e.g. Hes5, Lif) and synaptic function (e.g. Syp, Grm5). Given that these GO terms fall into categories that one would expect to be up-regulated in this set-up, this was the first confirmation that in the mixed-species co-culture, rat neurons can induce meaningful changes in mouse astrocytes.

Top 20 GO terms for genes DOWN

<table>
<thead>
<tr>
<th>GO term</th>
<th>Genes</th>
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<tr>
<td>microtubule cytoskeleton organization involved in mitosis</td>
<td>Cenpe, Ndc80, Aurkb, Pina, Birc5, Ki67</td>
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<tr>
<td>spindle checkpoint</td>
<td>Ube2c, Aurkb, Birc5, Bub1, Cdc20, Pkl1, Bub1b, Cenpf</td>
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<tr>
<td>regulation of mitotic metaphase/anaphase transition</td>
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<tr>
<td>platelet aggregation</td>
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<tr>
<td>regulation of metaphase/anaphase transition of cell cycle</td>
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<tr>
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<td>collagen metabolic process</td>
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<td>collagen catabolic process</td>
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<td>multicellular organismal catabolic process</td>
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<td>negative regulation of proteasomal ubiquitin-dependent protein catabolic process</td>
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<td>regulation of chromosome segregation</td>
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<td>platelet degranulation</td>
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Selected GO terms with genes DOWN

<table>
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<th>GO term</th>
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<td>microtubule cytoskeleton organization involved in mitosis</td>
<td>Cenpe, Ndc80, Aurkb, Pina, Birc5, Ki67</td>
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<td>spindle checkpoint</td>
<td>Ube2c, Aurkb, Birc5, Bub1, Cdc20, Pkl1, Bub1b, Cenpf</td>
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<tr>
<td>negative regulation of cell division</td>
<td>E2f7, Bmp4, Ube2c, Aurkb, Bub1, Cdc20, Pkl1, Bub1b, Cenpf, E2f9</td>
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<tr>
<td>extracellular matrix disassembly</td>
<td>Col3a1, Col11a1, Col4a1, Col18a1, Col4a3, Col4a2, Col8a1, Col1a2, Mmp3, Col1a1, Col6a5, Don, Timp1, A2m, Lamic2, Lama5, Lamb1</td>
</tr>
</tbody>
</table>
Figure 5.5: GO terms for genes down-regulated in astrocyte upon co-culture with neurons.

Top: 317 of the 356 genes down-regulated in astrocyte when co-cultured with neurons underwent GO annotations analysis and top20 enriched GO annotations are listed. GO annotations include the regulation of cell division, extracellular matric disassembly and collagen metabolism. Bottom: Selected GO annotations from top20 with according genes.

Furthermore, prominent in the top20 of GO terms for genes that are down-regulated in astrocytes upon co-culture are GO terms involved in the regulation of cell division (Fig. 5.5). These include genes involved in the negative regulation of cell division (e.g. Aurkb, Bub1b) as well as extracellular matrix disassembly (e.g. Col3a1, Mmp3). This again is something that can be expected, as astrocytes will create an established connection with neurons that potentially requires the reorganization of the extracellular matrix, rather than undergoing cell division. Of the 356 genes down-regulated, 317 could be mapped by the software and were categorized into 176 significantly enriched GO terms.

5.2.4 Neurons push astrocytes into a more in vivo like state

Next, I compared the changes in gene expression I observed with the differential gene enrichment of two data sets comparing in vitro to in vivo astrocytes (Cahoy et al., 2008) (Fig. 5.6A, B).
Our data on the genes listed in Cahoy et al. (2008) as being enriched in *in vivo* astrocytes compared to culture.

A) Our data on 75 of the genes enriched in *in vivo* astrocytes when compared to astrocytes in culture as found in (Cahoy et al., 2008). Of the 75 genes, 72 are up on average 3.6 fold in astrocytes upon co-culture with neurons, with 63 showing a fold induction of >1.5. B) Our data on 77 of the genes enriched in cultured astrocytes when compared to astrocytes *in vivo* shows 67 genes down-regulated on average 1.8 fold, with 50 genes down >1.5 fold.
The Cahoy micro-array data set reports 80 genes enriched in in vivo astrocytes compared to cultured astrocytes, 75 of which I used in our analysis, with the 5 remaining genes having been un-or re-annotated. Of these 75 genes, I found 72 up-regulated on average 3.6 fold in AC astrocytes when compared to AM astrocytes, with 63 genes being up-regulated >1.5 fold (Fig. 5.6A).

Of the 80 genes enriched in cultured astrocytes vs. in vivo astrocytes in the Cahoy data set, I have data on 77. Of these 77 genes, 67 were down-regulated on average 1.8 fold in AC astrocytes compared to AM astrocytes, with 50 genes being down-regulated >1.5 fold (Fig. 5.6B).

Next, I compared our RNA-seq data sets to RNA-seq data from FACS-purified astrocytes (Zhang et al., 2014) (Fig. 5.7). Only genes were used with >0.5 FPKM and present in both data sets.

Figure 5.7: The transcriptome of co-cultured astrocytes shows a stronger correlation to the transcriptome of in vivo astrocytes when compared to that of mono-cultured astrocytes.

Gene expression (FPKM) of astrocytes in mono-culture (A) and astrocytes in co-culture (B) was compared to the gene expression profile of FACS-purified cortical astrocytes (Zhang et al., 2014) using the Pearson correlation coefficient r. Co-cultured astrocytes show a stronger correlation (r=0.66, 0.65-0.67 95% CI) than mono-cultured astrocytes (r=0.51, 0.5-0.53 95% CI) when compared to in vivo astrocytes. The 11005 genes in these graphs had to be >0.5 FPKM and present in both, our and the (Zhang et al., 2014) data set to be included.
Astrocytes co-cultured with neurons show a stronger \((r=0.66, \text{ 95\% CI: 0.65-0.67})\) correlation than astrocytes cultured on their own \((r=0.51, \text{ 95\% CI: 0.5-0.53})\) when compared to \textit{in vivo} astrocytes, confirming that neurons drive astrocytes into a more mature phenotype.

These data clearly show that neurons induce meaningful changes in the astrocytic transcriptome and, moreover, that neurons push astrocytes into a more \textit{in vivo} like state, which agrees with the changes in astrocyte morphology I observed (Fig. 5.1). This also indicates that the fact that I used rat neurons on mouse astrocytes does not prevent this from happening.

\textbf{5.2.5 Neurons induce glutamate metabolism and receptor expression in astrocytes}

The number of genes that are up-and down-regulated in astrocytes by the presence of neurons is too large to discuss in detail. However, because of the well-described role of astrocytes in glutamate uptake and metabolism and the role of glutamate as an inducer of astrocytic \(\text{Ca}^{2+}\) transients, I will highlight genes involved in these processes that are regulated by neurons in astrocytes here.

The most pronounced induction is seen with Slc1a2, a ‘glial high affinity glutamate transporter’ also known as Glt-1 or Eaat2 (Table 1). Its expression is up 11 fold in the co-culture, making it the second strongest induced gene (after the glial transcription factor Hes5, see Fig. 5.3). Another glutamate transporter, Slc1a3 (also known as Glast1 or Eaat1), is also strongly up-regulated in astrocyte upon co-culture with neurons, supporting results that the astrocytic surface expression of both transporters can be induced by neurons (Swanson et al., 1997).
Table 1: Glutamate metabolism and receptor genes up-regulated in astrocytes upon co-culture with neurons.

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene</th>
<th>Fold</th>
<th>Product</th>
<th>Description/Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate metabolism</td>
<td>Slc1a3 (Glast1, Eaat1)</td>
<td>4.0</td>
<td>Glutamate transporter</td>
<td>‘Glial’ glutamate transporter, glutamate uptake</td>
</tr>
<tr>
<td></td>
<td>Slc1a2 (Glt-1, Eaat2)</td>
<td>11.3</td>
<td>Glutamate transporter</td>
<td>Glutamate uptake, together with Slc1a3 supports findings in (Swanson et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>Glul</td>
<td>5.4</td>
<td>Glutamine synthetase</td>
<td>Converts glutamate into glutamine, central in glutamate-glutamine cycle supplying neurons with glutamate</td>
</tr>
<tr>
<td></td>
<td>Glud1</td>
<td>1.8</td>
<td>Glutamate dehydrogenase</td>
<td>Converts glutamate into α-ketoglutarate, connecting glutamate uptake to astrocyte metabolism</td>
</tr>
<tr>
<td>Glutamate receptors (GluR)</td>
<td>Grm5</td>
<td>5.3</td>
<td>Metabotropic glutamate receptor 5 (mGluR5)</td>
<td>Glutamate binding on astrocytic mGluR5 induces Ca^{2+} release from intracellular stores in astrocytes (Panatier and Robitaille, 2015)</td>
</tr>
<tr>
<td></td>
<td>Grid1</td>
<td>2.9</td>
<td>GluR subunit delta-1</td>
<td>Ionotropic GluR</td>
</tr>
<tr>
<td></td>
<td>Grin3a</td>
<td>2.5</td>
<td>NMDAR subunit 3</td>
<td>Ionotropic GluR subunit, cation permeable</td>
</tr>
<tr>
<td></td>
<td>Gria1</td>
<td>1.9</td>
<td>AMPAR subunit 1</td>
<td>Ionotropic GluR subunit, cation permeable</td>
</tr>
</tbody>
</table>

Glutamate taken up by astrocyte can either be converted into glutamine and subsequently shuttled back to neurons, or converted into a TCA cycle substrate to be used to fuel the energetically expensive glutamate uptake. I found that the gene expression of both, the glutamine synthetase (Glul) converting glutamate to glutamine, and the glutamate dehydrogenase (Glud1), converting glutamate into α-ketoglutarate, are significantly up-regulated in astrocytes in the presence of neurons. All in all, these data indicate that astrocytes increase their capacity to take up glutamate coming from neurons, increase the activity of the glutamate-glutamine cycle and increase glutamate catabolism to fuel the uptake of glutamate.

Furthermore, in the presence of neurons, astrocytes increase the expression of several glutamatergic receptor subunits (Table 1). These include subunits of the NMDA- and AMPA-type glutamate receptor, the less well-studied delta-type and the metabotropic mGluR5 receptor. While there is little knowledge on the role of NMDARs and AMPARs in glutamate-induced signalling in astrocytes, the effect of
mGluR5 activation is well studied, indicating that glutamate binding activates the release of internal Ca\(^{2+}\) stores via IP\(_3\), which induces the subsequent release of gliotransmitters (Panatier and Robitaille, 2015).

In order to better understand how astrocytes in our culture experience glutamate, and to find an appropriate stimulation paradigm to probe the effect of synaptic activity on astrocytic gene expression, I decided to employ a fluorescent reporter of extracellular glutamate, iGluSnFR (Marvin et al., 2013).

### 5.2.6 Astrocytes experience synaptically released glutamate

Glutamate released from neurons is thought to activate glutamatergic receptors on astrocytes, and it is therefore not unlikely that glutamate released during action potential firing can regulate astrocytic gene expression. To test whether astrocytes in our culture experience synaptically released glutamate, I transfected the genetically encoded reporter of extracellular glutamate, iGluSnFR (Marvin et al., 2013) into our mouse astrocytes. iGluSnFR is a GFP-based, mono-chromatic, genetically encoded reporter that expresses at the extracellular surface of the target cell, where it reports an increase in extracellular glutamate levels with an increase in GFP fluorescence. I then added rat neurons on top of the mouse astrocytes, which were in turn transfected with the cytosolic Ca\(^{2+}\) indicator GCaMP3. This allowed us to simultaneously measure neuronal Ca\(^{2+}\) transients and astrocytic glutamate exposure, as both probes are imaged using a standard GFP filter set. GCaMP3 was co-transfected with mCherry in order to distinguish GCaMP3-positive from iGluSnFR-positive cells. However, since astrocytes and neurons can be easily distinguished by their morphology, and because the signals obtained from both probes are very distinct, it was easy to select the appropriate cells.

While I used the well-established BiC/4-AP stimulation paradigm in prior experiments in this thesis, I decided to chose a different approach, since the main current across the astrocytic plasma membrane is mediated by K\(^{+}\), and inhibiting 4-AP sensitive channels can reduce this K\(^{+}\) current in astrocytes (Bekar et al., 2005). Stimulation with BiC alone is not creating sufficiently strong action potential bursting, since neurons cultured on a confluent layer of astrocytes already show a
high spontaneous activity. I therefore decided to exploit a homeostatic plasticity mechanism based on the prolonged silencing of network activity with TTX (100 nM) and the subsequent wash-out of TTX, which causes a re-bound of neuronal activity in the form of strong network bursting (Fig. 5.8A). To increase the extracellular concentration of glutamate as well as the dwell time of glutamate on the astrocytic surface, I decided to subsequently apply DL-threo-β-Benzylxoyaspartic acid (TBOA, 50 µM), which inhibits the glutamate transporters EAAT1-5.
Figure 5.8: Astrocytes experience synaptically released glutamate after TTX re-bound.

A) Example traces of an iGluSnFR-positive astrocyte (top, red trace) and a GCaMP3-positive neuron (bottom, blue trace) in the same field of view during re-bound network bursting after overnight TTX (100 nM) treatment. Neurons silenced for a prolonged time will undergo a homeostatic plasticity mechanism that causes them to strongly fire when un-silenced. Both probes are excited using a standard GFP filter set. There are no glutamate transients observed on astrocytes in the presence of TTX. When neurons start firing, neuronal Ca\(^{2+}\) transients and extracellular astrocytic glutamate...
transients coincide. The addition of TBOA (50 μM) greatly increases the amplitude of the iGluSnFR signal as glutamate re-uptake is inhibited. iGluSnFR is calibrated by adding high concentrations of glutamate (5 mM) on to the cells, saturating the sensor and giving the maximum fluorescence. B) Example traces showing that TBOA also increases the duration of the glutamate transient on astrocytes. iGluSnFR baseline fluorescence is set to 0 and peak fluorescence to 1 to show the increase in the duration of the iGluSnFR transient with TBOA. C) Example traces of single GCaMP3-positive neurons showing that even 16h after TTX wash-out, neurons show strong network bursting. The presence of TBOA increases the duration and amplitude of neuronal Ca^{2+} transients.

Co-cultures with iGluSnFR-positive astrocytes and GCaMP3-positive neurons were silenced over night and then transferred into the imaging chamber. iGluSnFR-positive astrocytes as well as GCaMP3-positive neurons were brought into one field of view, and using a fast perfusion set-up, the cells were washed with TTX free medium until neurons started firing (Fig. 5.8A). While there were no glutamate transients on astrocytes when TTX is present, I found that after TTX wash-out, Ca^{2+} transients in neurons occurred simultaneously with iGluSnFR transients on astrocytes, which indicates that astrocytes experience glutamate that is released by synaptic activity. iGluSnFR is calibrated by adding high concentrations of glutamate (5 mM) at the end of the recording, which gives the maximum fluorescence. Moreover, when adding TBOA to the imaging chamber, iGluSnFR transients become higher in amplitude (Fig. 5.8A) and longer in duration (Fig. 5.8B). Incidentally, TBOA also increases the duration of neuronal Ca^{2+} transients (Fig. 5.8B, C).

I then used the data on which genes are regulated in astrocytes by co-culture with neurons to screen candidate genes that could possibly be regulated by synaptic activity using qPCR. Based on these screens, I decided to use a long stimulation paradigm rather than a short one, as I could not detect changes in astrocytic gene expression at stimulations of 4h, but did so after 16h. To confirm that neurons still fire 16h after TTX re-bound, I imaged GCaMP3-positive neurons undergoing 22h TTX silencing and subsequent TTX wash-out. I found that even after 16h post-TTX re-bound, neurons are still undergoing network bursting, unlike cells washed with TTX, which are silent (Fig. 5.8C).

To investigate the downstream consequences of astrocytic glutamate exposure following action potential bursting, I decided to monitor astrocytic Ca^{2+} transients.
5.2.7 Synaptic activity induces astrocytic Ca\textsuperscript{2+} transients

Similar to the prior set-up, a confluent layer of astrocytes was transfected, this time with cytosolic GCaMP3. Neurons were added on top of the astrocytic layer and subsequently transfected with GCaMP3 as well. This allowed me to image astrocytic and neuronal Ca\textsuperscript{2+} transients simultaneously. Cells were kept in TTX over night and washed with TTX free medium to induce network bursting.
Figure 5.9: Neuronal activity induces astrocytic Ca\(^{2+}\) transients.

A) Example trace showing concomitant recording of neuronal (red trace) and astrocytic (blue trace) Ca\(^{2+}\). Image shows one neuron and one astrocyte with 6 active ROIs located on branches and processes. Cells are kept in TTX (300 nM) over night and TTX is washed out to induce neuronal firing. When neurons are active, astrocytes experience small Ca\(^{2+}\) fluctuations, mainly in their thin processes and branches. The addition of TBOA (50 μM) causes Ca\(^{2+}\) waves that can span the whole astrocyte. B) Right: Magnification of indicated area within the trace above, showing astrocytic Ca\(^{2+}\) transients follow neuronal Ca\(^{2+}\) transients. Left: Quantification of the latency of astrocytic Ca\(^{2+}\) transients in respect to neuronal Ca\(^{2+}\) transients show a lag time of about 2 seconds (n=10 cells). C) Quantification of astrocytic Ca\(^{2+}\) transients shows a statistically non-significant (p=0.11) increase in frequency when TTX is washed out (0.44±0.15 to 1.07±0.24 events per minute, average±SEM) and a significant increase in the presence of TBOA (2.93±0.42 events per minute). Astrocytic Ca\(^{2+}\) transients show no difference in...
amplitude or duration in the presence or absence of TTX or TBOA. *p<0.05 paired one-way ANOVA plus Dunnett’s post-hoc test (n=27 cells). See Fig. A9 for example images.

I found that astrocytes show spontaneous, TTX-insensitive Ca\(^{2+}\) transients lasting on average approximately 10 seconds that were located on astrocytic branches and endfeet (Fig. 5.9A, C). When TTX is washed out and neurons start firing, the frequency of astrocytic Ca\(^{2+}\) events more than doubles (although this is not yet statistically significant), indicating that synaptic activity can indeed induce astrocytic Ca\(^{2+}\) events (Fig. 5.9A, C). While these synaptic activity-induced events had a tendency to have a higher amplitude and duration, the values never reached statistical significance (Fig. 5.9C). Furthermore, the addition of TBOA increased the frequency of astrocytic Ca\(^{2+}\) events 7 fold, while showing no statistically significant change in duration or amplitude (Fig. 5.9A, C). Interestingly, while TBOA increased the frequency of Ca\(^{2+}\) events mostly localized to endfeet and branches, TBOA also induced Ca\(^{2+}\) waves through the entire cell, including the soma, and spanning several cells (Fig. 5.9A). These Ca\(^{2+}\) waves in astrocytes closely followed the Ca\(^{2+}\) transients in neurons and occurred with a latency of about 2 seconds, strongly suggesting that they are mediated by metabotropic receptors (Fig. 5.9B).

Based on these findings I decided to use this stimulation paradigm to probe for genes in astrocytes that are regulated by synaptic activity. However, in order to support the neuronal firing, we decided to add BiC (50 µM) when washing out TTX.

### 5.2.8 Synaptic activity regulates gene expression in astrocytes

To test whether synaptic activity can indeed regulate gene expression in astrocytes, I co-cultured mouse astrocytes with rat neurons. Based on the previous findings that an over night incubation in TTX followed by TTX wash-out can induce strong action potential bursting in neurons, and that this causes glutamate exposure of astrocytes and astrocytic Ca\(^{2+}\) transients, I decided to apply this stimulation paradigm for our gene expression study (Fig 5.10A). Furthermore, given the strong effect of TBOA on astrocytic glutamate exposure and the induction of Ca\(^{2+}\) waves, TBOA was included in one of the conditions (Fig 5.10B).
A) Mixed species mouse astrocyte/rat neuron co-cultures were transferred into TTX-containing medium on DIV8 for 22h to inhibit neuronal action potential firing. Subsequently, cells were washed with medium free of TTX and then kept in medium containing bicuculline (50 μM) for 16 h. A control co-culture was treated identically except that TTX was present at all times during the 'washes' and remained in the medium with the bicuculline. RNA from both conditions was extracted, subjected to RNA-seq, followed by species read sorting to identify reads that were unambiguously mouse (i.e. astrocytic) in origin. Expression of genes (FPKM) in astrocytes in the presence or absence of neuronal synaptic activity is plotted for the 11,872 genes expressed >2 FPKM. Red crosses indicate the astrocytic genes induced or repressed >1.3-fold by synaptic activity (DESeq2 \( P_{\text{adj}}<0.05 \), \( n=4 \) independent biological replicates).

B) Impairing glutamate re-uptake enhances activity-dependent astrocytic gene regulation. Experiment performed exactly as in (A) except co-cultures were treated with TBOA (50 µM) at the time of bicuculline addition. Red crosses indicate the astrocytic genes induced or repressed >1.3-fold by synaptic activity (DESeq2 \( P_{\text{adj}}<0.05 \), \( n=4 \) independent biological replicates).

Cells were either kept in TTX for 38h (control), or for 22h followed by two washes with TTX free medium in the presence of BiC or BiC and TBOA to induce network bursting for 16h. Additionally, two conditions were included that were kept in TTX for 22h and then washed with BiC or BiC and TBOA and left in the presence of TTX for 16h to control for direct drug effects that are independent of synaptic activity, and to control for possible artefacts introduced by washing the cells. Lastly, one condition did not undergo any treatment to look at the effect of network silencing by TTX on gene expression levels. RNA was collected at day 10 post neuron plate down and subsequently sent for RNA-seq.
Table 5.11: Top50 genes UP- or DOWN-regulated in astrocytes following neuronal action potential firing

<table>
<thead>
<tr>
<th>UP</th>
<th>DOWN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scg2</td>
<td>Cited1</td>
</tr>
<tr>
<td>1700018L02Rik</td>
<td>Pde10a</td>
</tr>
<tr>
<td>Dio2</td>
<td>Ncan</td>
</tr>
<tr>
<td>Tmem100</td>
<td>Serpine1</td>
</tr>
<tr>
<td>Sod3</td>
<td>Papss2</td>
</tr>
<tr>
<td>Nfr4a3</td>
<td>Nupr1</td>
</tr>
<tr>
<td>Slco1c1</td>
<td>Rgs7</td>
</tr>
<tr>
<td>Msfd2a</td>
<td>Cdo1</td>
</tr>
<tr>
<td>3930402G23Rik</td>
<td>Nfil3</td>
</tr>
<tr>
<td>Pdeo4d</td>
<td>Rorb</td>
</tr>
<tr>
<td>Ttc39c</td>
<td>Kcnk1</td>
</tr>
<tr>
<td>Actr3b</td>
<td>Csrp2</td>
</tr>
<tr>
<td>Cnn1</td>
<td>March3</td>
</tr>
<tr>
<td>Angptl2</td>
<td>Vwa1</td>
</tr>
<tr>
<td>Slc7a5</td>
<td>Rasl10b</td>
</tr>
<tr>
<td>Hmgb3</td>
<td>Ptp4a1</td>
</tr>
<tr>
<td>Rab27b</td>
<td>Hdac4</td>
</tr>
<tr>
<td>Bmper</td>
<td>Adm</td>
</tr>
<tr>
<td>Pmeapa1</td>
<td>Npnt</td>
</tr>
<tr>
<td>Reit</td>
<td>Slc43a3</td>
</tr>
<tr>
<td>Slc39a14</td>
<td>Ndnf</td>
</tr>
<tr>
<td>Ifo2</td>
<td>Baalc</td>
</tr>
<tr>
<td>Mcam</td>
<td>Slc44a3</td>
</tr>
<tr>
<td>Popr1c</td>
<td>Dusp1</td>
</tr>
<tr>
<td>Slc6a17</td>
<td>Csd2</td>
</tr>
</tbody>
</table>

I found that 268 genes are up-regulated and 102 down-regulated in astrocytes following synaptic activity, and that 635 are up and 434 down when glutamate re-uptake is inhibited with TBOA (FPKM >2, >1.3fold, Fig. 5.10 and Fig. 5.11 for Top50 genes).

Because a GO term analysis only identified a very limited number of relevant GO terms, gene clusters were identified manually.

A striking group belongs to genes involved in the uptake and metabolism of oxidizable substrates, strongly supporting the lactate-shuttle hypothesis. Synaptic activity boosts the expression of genes regulating the uptake of glucose (Slc2a1,
Slc2a13), glycolysis (Hk2, Gpi1, Pfk, Aldoa, Tpi1, Gapdh, Pgk1, Pgam1, Eno1/2, Pkm2, Pfkb3 and Ppp1r3c), the conversion of the glycolysis product pyruvate into lactate (Ldha) and the shuttle of lactate to neurons (Slc16a3). Furthermore, synaptic activity boosts the expression of genes involved in thyroid hormone signalling (Dio2, Slco1c1 and Slc7a5), extra-cellular antioxidant defence (Sod3, Gpx3) and cyclic nucleotide metabolism (Pdes 4d, 7b, 10a, 9a, 3a, 4b, 3b and 7a).

I then went on to confirm a subset of gene inductions using qPCR (Fig. 5.12). In order to pick up only astrocytic (mouse) transcripts using qPCR in a mouse-rat co-culture, species-specific primers were designed. The primers were validated by running them on pure mouse and pure rat samples and only accepted when they were not amplifying any rat transcripts.
Figure 5.12: Synaptic activity up-regulates the expression of Dio2, Slco1c1 and Sod3 in astrocytes: confirmation using qPCR.

Mouse astrocyte-rat neuron co-cultures were kept in TTX for 22h and either left in TTX for another 16h (control) or TTX was washed out and cells were left in BiC (50 μM) or BiC and TBOA (50 μM) for 16h. To control for effects of the washing and the drugs added, controls were included in which cells were washed in the presence of TTX and either BiC or BiC and TBOA. One condition was included with no treatment, to compare the effect of TTX on astrocytic gene expression. Using qPCR with mouse-specific primers, Dio2, Slco1c1 and Sod3 were confirmed to be up-regulated when TTX is washed out in the presence of either BiC or BiC and TBOA. In the presence of TTX, none of the three genes is induced. *p<0.05 paired one-way ANOVA plus Dunnett’s post-hoc test compared to the 38h TTX condition (n=4).

I could confirm the induction of Sod3, Dio2 and Slco1c1 in astrocytes by synaptic activity using qPCR and mouse-specific primers.
Interestingly, the two genes identified as being up-regulated in astrocytes by synaptic activity, Dio2 and Slco1c1, were also up-regulated upon co-culture with neurons,
while the expression of Sod3 is induced by synaptic activity, but unaltered by the presence of neurons. This suggests that the gene expression changes induced by co-culturing astrocytes with neurons is via a different mechanism than the ones induced by synaptic activity, but that they can be additive.

Incidentally, Dio2 and Slco1c1 belong to the group of genes that are enriched in *in vivo* astrocytes when compared to cultured astrocytes (Fig. 5.6A) and, moreover, are functionally linked. Both genes are involved in the activation of the thyroid hormone, which is known to take place in astrocytes (Morte and Bernal, 2014).

Sod3 encodes the enzyme superoxide dismutase 3 (SOD3), which converts $O_2^-$ into $H_2O_2$. Intriguingly, SOD3 is secreted into the extracellular space to detoxify $O_2^-$, and increasing its expression in astrocytes during periods of prolonged synaptic activity could potentially be a mechanism to protect neurons from synaptic activity-induced oxidative stress. Additionally, since SOD3 decreases the concentration of $O_2^-$, which inactivates the vasodilator NO, it has been suggested to be involved in the regulation of blood pressure (Demchenko et al., 2010), which could be a mechanism to increase blood flow to active brain areas.

Furthermore, the expression level of all three genes is boosted by the presence of TBOA, indicating that the gene induction is mediated by glutamate. None of the gene levels are changed by TTX alone when compared to the spontaneously active condition.

All in all, I show here that prolonged synaptic activity can induce drastic changes in astrocytic gene expression. These changes include neuron-astrocyte metabolic coupling, thyroid hormone signalling and antioxidant defences and can be expected to have substantial functional consequences.

### 5.3 Discussion

I showed here that neurons can induce drastic changes in astrocytes, be it at the morphological or physiological level. These changes in astrocytes are accompanied by changes in the astrocytic transcriptome, which we identified using a novel tool
that allowed us to extract cell-type specific transcripts from neuron-astrocyte co-cultures.

5.3.1 RNA-sequencing on astrocyte-neuron mixed-species co-cultures

The development of next generation sequencing technologies has allowed the study of differences in genome wide gene expression profiles between cells, which has been of particular interest for neuroscientists, as the brain consists of many different, highly interacting cell types (Wang et al., 2009; Zhang et al., 2014). RNA-sequencing has recently been used to establish the transcriptome of neurons, astrocytes, oligodendrocytes and vascular cells in rodents and humans (Zhang et al., 2014; Darmanis et al., 2015; Zeisel et al., 2015). However, studying how their interaction can change cell type specific gene expression profiles has not been possible so far.

Since it is not possible to distinguish neuronal from astrocytic RNA in a co-culture or slice, most of the data on non-cell autonomous regulation of gene expression between astrocytes and neurons is based on immunohistochemistry and western blotting (Swanson et al., 1997; Perego et al., 2000; Mamczur et al., 2014). The only study known to me that looked at non-cell autonomous changes at the transcriptional level in astrocyte-neuron co-cultures used a cold-jet approach to physically remove neurons after co-culturing them with astrocytes (Goudriaan et al., 2014). However, this study failed to report the major changes in gene expression profiles that have been observed by other labs (such as glutamate metabolism and gliogenesis), and I was not able to find many common regulated genes between this and our study. One reason for this could be the fact that the study used very low neuron densities in order to completely remove them with the cold jet, resulting in sparse neuron-astrocyte interactions. Additionally, the study used micro-arrays to investigate changes in gene expression, which greatly limits the number of genes that can be studied.

Here, I provided a novel tool that allows the study of non-cell autonomous changes in gene expression in a co-culture set-up. I show that co-culturing cells derived from different species allows to distinguish their transcripts. I cultured neurons derived
from rat on top of a confluent layer of mouse astrocytes to study the effect that neurons have on astrocytic gene expression.

It is crucial in this set-up to establish that reads picked up as mouse are indeed mouse and therefore derive from astrocytes only. Each read needs therefore to be matched against the genome of both species. Using a non-stringent analysis to map the reads to both reference genomes can cause reads to be called as mouse, while they are actually rat, or possibly map unambiguously to the genomes of both species. This prompted us to use an ultra-stringent approach, where we demanded a perfect match of the mouse reads to only the mouse genome.

Furthermore, I found that only about 1/6 of the RNA collected from a neuron-astrocyte co-culture is astrocytic, while there are more astrocytes in the co-culture than there are neurons.

Thus, expecting to lose sequencing depth from unambiguous mapping of the sequenced reads and knowing the poor RNA yield from astrocytes prompted us to increase the sequencing depth from the more standard 50 million to 150 million paired-end reads. We found that only 7.71% (or approximately 12 million reads) of the total 150 million reads were unambiguously mouse and therefore astrocytic. This translates into 11352 genes with an FPKM >2 that can be used to study differential gene expression. Although increasing the sequencing depth could help covering more genes than we do now, it is also the cost-defining factor in RNA sequencing, and one should think carefully about the cost-benefit relationship. Even with less than 8% of the 150 million reads being unambiguously mouse (astrocytic), we were able to identify more than 1200 genes differentially regulated (fold change ≥1.5) in astrocytes when cultured together with neurons.

One could argue that neurons derived from rat might not be able to induce meaningful changes in astrocytes derived from mouse. However, I showed evidence that rat neurons create a transcriptional landscape in mouse astrocytes that strongly resembles that of *in vivo* astrocytes, which means that the fact we used different species does not prevent this from happening. Of course, this does not completely rule out that species-mixing has consequences on the astrocytic transcriptome, and it is important to confirm gene induction in single-species co-cultures or even *in vivo*. This obviously requires genes that are only present in astrocytes and are additionally
induced in astrocytes by neurons. One such an example is Dio2, which is not present in neurons or any other brain cell type other than astrocytes (Zhang et al., 2014), and I showed that it is also induced by neurons and synaptic activity.

5.3.2 Effect of neuron-astroocyte co-culture on the astrocytic transcriptome

Co-culturing astrocytes with neurons has drastic consequences on astrocyte morphology (Swanson et al., 1997). Astrocytes transform from a polygonal shape with almost no branches into a stellate and highly branched morphology, a phenomenon often mimicked using the cAMP inducer forskolin and relying on actin polymerization (Murk et al., 2013). Using GFP-positive astrocytes, I showed that the change in morphology already occurs in the first few days after neuron plate down, and astrocytes increase in complexity over time to eventually develop spine-like processes. The highly complex morphology I observed in astrocytes co-cultured with neurons resembles the morphology of astrocyte in vivo (Reichenbach et al., 2010). Agreeing with this, I found that neurons induce a transcriptome in astrocytes that resembles the gene expression profile of astrocytes in vivo. This is based on a data set from (Cahoy et al., 2008) comparing cultured astrocytes to astrocytes in vivo, the latter having been purified by immunopanning. Similarly, the transcriptome of our co-cultured astrocytes showed a greater correlation to in vivo astrocytes than monocultured astrocytes. This begs the question how physiologically relevant experiments on pure astrocytic cultures are, given the drastic changes in morphology and gene expression I reported. With the transcript-sorting approach I showed here, we introduce a system that allows to work on astrocytes co-cultured with neurons that can be easily applied in a lab, and using species-specific primers and qPCR or RNA-sequencing, allows gene expression studies on astrocytes that are closer to in vivo astrocytes. Nevertheless, the genes that are up-regulated in astrocytes upon co-culture with neurons fall into categories including neuro- and axogenesis, gliogenesis and the regulation of synaptic activity. This suggests that astrocytes up-regulate genes to support neurite out-growth and establish synaptic connections. Indeed, neurons
cultured in the presence of astrocytes show an increase in frequency and amplitude of synaptic events (Allen et al., 2012; Hahn et al., 2015), which was suggested to be mediated by astrocyte-derived soluble factors in the medium. In fact, two of the synaptogenic proteins secreted by astrocytes, hevin (SPARCL-1) and apolipoprotein E, are up-regulated upon co-culture. Moreover, astrocyte-secreted hevin has very recently been found to be necessary for the refinement of thalamo-cortical synapses (Risher et al., 2014) and we find it to be down-regulated upon synaptic activity. Interestingly, two other well-known synaptogenic proteins secreted by astrocytes, glypican 4 and thrombospondin 1 (Christopherson et al., 2005; Allen et al., 2012) are actually significantly down-regulated and glypican 4 is further suppressed by synaptic activity. Furthermore, genes involved in glutamate metabolism are up-regulated in astrocytes when co-cultured with neurons. These include the glutamate transporters, GLT-1 and GLAST, as well as the glutamine synthetase and the glutamate dehydrogenase. This indicates that neurons induce a gene expression profile in astrocytes that allows astrocytes to take up glutamate and either use it to convert it into glutamine in order to shuttle it back to neurons, or use it to fuel the bioenergetically expensive glutamate uptake by feeding glutamate into the TCA cycle. Additionally, astrocytes increase the expression of mGluR5, enabling astrocytes to respond to synaptically released glutamate with Ca^{2+} transients and possibly gliotransmitter release. Genes down-regulated in astrocytes upon co-culture with neurons mainly belong to gene clusters involved in the organization of the extracellular matrix and cell division. This is in accordance with the more in vivo transcriptome and morphology of astrocytes, indicating that astrocytes undergo a maturation process that allows them to establish contacts with neurons and possibly renders them post-mitotic. Furthermore, the extracellular matrix plays a major role in neurite growth and synaptic function, and its components are secreted by neurons and astrocytes (Dityatev and Schachner, 2003; Barros et al., 2011). Therefore, it is not difficult to imagine that astrocytes regulate (tri-partite-) synapse formation by regulating the composition of the extracellular matrix. Intriguingly, the proteoglycan brevican (Bcan), which is not expressed in neurons, is strongly up-regulated in astrocytes upon co-culture with neurons and was recently shown to be necessary for the
generation of long term potentiation (Brakebusch et al., 2002). Similar to glypican 4 and hevin, brevican is down-regulated by synaptic activity. Interestingly, genes down-regulated in astrocytes upon co-culture with neurons also include actin as well as the actin nucleating factor ARP3, the over-expression of which has recently been shown to cause astrocyte stellation (Murk et al., 2013). The discrepancy between these findings begs the question what exactly the forskolin-induced astrocyte stellation means in terms of astrocyte maturation.

5.3.3 Genes in astrocytes regulated by neuronal activity

Glutamate released during neuronal activity is thought to bind to metabotropic glutamatergic receptors on astrocytes and induce astrocytic Ca\(^{2+}\) transients (Khakh and McCarthy, 2015). While the function of this is not yet known, it has been suggested to trigger transmitter release from astrocytes that will in turn tune synaptic activity (Araque et al., 2014). Unlike the all-or-nothing responses seen in neuronal action potential firing, astrocytic Ca\(^{2+}\) transients differ greatly in their spatio-temporal properties, ranging from local non-propagating Ca\(^{2+}\) ‘sparkles’ on endfeet and branches to waves through the astrocytic syncytium (Khakh and McCarthy, 2015). Furthermore, Ca\(^{2+}\) transients can only be induced with strong neuronal stimulations (Wallach et al., 2014). Because of the slow response-time of metabotropic receptor activation from glutamate binding to intracellular Ca\(^{2+}\) release (~2 sec), it is unlikely that astrocytes integrate information from short-term changes in action potential firing. Much rather, astrocytes could tune the overall excitability and synchrony of a neuronal network (Fellin et al., 2004).

Nevertheless, I showed here that astrocytes sense the glutamate released from neurons during action potential firing, and that I can increase the dwell time of glutamate on astrocytes by using the glutamate transporter inhibitor TBOA. Action potential firing in turn caused an increase in astrocytic Ca\(^{2+}\) transients, which can be boosted by applying TBOA, indicating that the Ca\(^{2+}\) transients are indeed mediated by glutamate. I will need to perform more experiments to identify which glutamatergic receptor is responsible for the astrocytic Ca\(^{2+}\) transients. Initial experiments will involve the pharmacological inhibition and activation of mGluR5,
since this is the most likely candidate (Panatier and Robitaille, 2015) and is up-regulated in astrocytes by the presence of neurons.

Nonetheless, I found that action potential firing-induced Ca$^{2+}$ transients are mostly small and located on endfeet, while Ca$^{2+}$ transients in the presence of TBOA can include somatic regions and encompass several astrocytes. This potentially suggests that Ca$^{2+}$ released in the presence of TBOA passes a threshold and induces Ca$^{2+}$ release in neighbouring cells, potentially by Ca$^{2+}$-dependent Ca$^{2+}$ release and IP$_3$ activation, both of which can cross astrocytic gap junctions (Giaume and Venance, 1998).

In order to find out whether neuronal activity can also induce changes in astrocytic gene expression, I created four biological replicates of mixed-species neuron-astrocyte co-cultures undergoing network firing for 16h.

The most outstanding gene cluster regulated in astrocytes by neuronal activity is involved in astrocyte-neuron metabolic coupling, and includes the uptake (Slc2a1, Slc2a13) and metabolism (Hk2, Gpi1, Pfk, Aldoa, Tpi1, Gapdh, Pgk1, Pgam1, Eno1/2, Pkm2, Pfkfb3 and Ppp1r3c) of glucose, as well as the production of lactate (Ldha) and its transport to neurons (Slc16a3). This strongly supports the lactate shuttle hypothesis, reiterating the central role astrocytes play in supplying oxidizable substrates to neurons, and provides evidence that electrically active neurons signal to astrocytes to increase this metabolic support.

Other gene clusters involve thyroid hormone signalling. Dio2 encodes the type 2 deiodinase (D2), which converts the inactive thyroid pre-hormone thyroxine (T4) into the active hormone triiodothyronine (T3). Slco1c1 encodes a thyroid hormone transporter and mediates the uptake of T4 into the cell (Morte and Bernal, 2014). Dio2 and Slco1c1 are strongly expressed in astrocytes and not present in neurons (Zhang et al., 2014), and it is thought that astrocyte take up T4, convert it into T3 and subsequently shuttle it to neurons (Morte and Bernal, 2014). T3 itself induces strong changes in gene expression, including a wide array of mitochondrial genes such as uncoupling protein 2 (ucp2), cytochrome oxidase c1 (cycs) and mitochondrial transcription factor A (mtTFA) (Wrutniak-Cabello et al., 2001).
I showed here that astrocytes co-cultured with neurons increase the expression of Slco1c1 and Dio2 and that this can be further boosted by synaptic activity. This indicates that active neurons increase thyroid hormone signalling via astrocytes, and as a consequence increase the expression of genes in neurons involved in mitochondrial function, potentially boosting their capacity to generate energy.

One way to test whether thyroid signalling via astrocytes is indeed increased with synaptic activity is by using pure neuronal cultures and astrocyte-neuron co-cultures and exposing them to T4. As neurons should not be able to convert T4 to the transcriptionally active T3, there should be no change in gene expression of T3 target genes in pure neuronal cultures. In an astrocyte-neuron co-culture, however, the T4 is taken up by astrocytes, converted into T3 and shuttled to neurons where it should induce transcription of T3 target genes, which should be boosted by synaptically active neurons. However, this requires distinguishing the neuronal transcripts from astrocytic ones. In order to do this, an anti-mitotic needs to be added when the neuronal cell suspension is added to the layer of astrocytes, inhibiting the proliferation of astrocyte present in the ‘neuronal’ cell suspension. This new co-culture set-up is currently being validated.

As a second functional read out, we are going to exploit a recent finding showing that Slco1c1 is responsible for the uptake of sulforhodamine 101 (SR101) into astrocytes (Schnell et al., 2013), a red fluorescent dye often used to mark astrocytes for in vivo live-cell imaging in order to distinguish them from neuronal populations. If Slco1c1 does indeed mediate SR101 uptake, astrocytes cultured on their own will not take up the red fluorescent SR101, but astrocytes co-cultured with neurons will, and will do so particularly well when undergoing prolonged synaptic activity.

Another gene cluster identified as being regulated by synaptic activity in astrocytes is involved in antioxidant defences and consists of Sod3 and Gpx3. Interestingly, both genes encode for antioxidant enzyme that are secreted into the extracellular space (Zelko et al., 2002; Brigelius-Flohé and Maiorino, 2013). Sod3, which encodes superoxide dismutase 3 (SOD3), is an antioxidant enzyme that converts $\text{O}_2^{-}$ into $\text{H}_2\text{O}_2$, while GPX3 is a glutathione peroxidase that in turn detoxifies $\text{H}_2\text{O}_2$. SOD3 is one of three SODs, with SOD1 being expressed in the cytoplasm, SOD2 in mitochondria and SOD3 being secreted into the extracellular space (Zelko et al., 2002; Brigelius-Flohé and Maiorino, 2013).
Interestingly, unlike Dio2 and Slco1c1, Sod3 expression in astrocytes is not induced by co-culturing astrocyte with neurons, but is increased by synaptic activity. This suggests that the induction of gene expression upon co-culture works via a different mechanism than the induction by synaptic activity.

One possible candidate for inducing gene expression in astrocytes by neurons is Notch signalling, which is known to play a central role in astrogliogenesis (Chenn, 2009). Furthermore, two major Notch target genes, Hey2 and Hes5, and the less well-studied Notch target gene Nrarp, are strongly up-regulated in astrocyte upon coculture with neurons.

On the other hand, transcription induced by synaptic activity has to be regulated via a different pathway. Given that synaptic activity increases the frequency of Ca$^{2+}$ events in astrocytes, it could be a pathway sensitive to Ca$^{2+}$ fluctuations, and since it is most likely due to activation of mGluR5 receptors, the Protein Kinase C pathway is a probable candidate. However, active neurons will also secrete soluble factors into the extracellular space, such as BDNF, and any change in gene expression observed in astrocytes could be mediated by these factors, too. Interestingly, a large number of genes regulated in astrocytes by neuronal activity belongs to the family of phosphodiesterases (Pdes 4d, 7b, 10a, 9a, 3a, 4b, 3b and 7a), which convert cAMP into AMP, suggesting a role for PKA.

Additionally, as could be the case with the increased expression of Sod3 and Gpx3, ROS or NOS produced during synaptic activity in neurons could induce gene transcription in astrocytes. This is particularly intriguing, since this could be a pathway by which active neurons signal to astrocyte to confer protection from synaptic activity-induced oxidative stress, by up-regulating the secretion of SOD3 and GPX3 into the extracellular space. I will test the functional consequences of this induction in astrocyte by synaptic activity by measuring the levels of O$_2$•⁻ in the medium of synaptically active or silent astrocyte-neuron co-cultures and pure neuronal cultures. This can be done using a simple colorimetric assay based on the O$_2$•⁻-induced reduction of a formazan dye. Alternatively, one can assay the amount of cell death induced by O$_2$•⁻ and compare active to silent astrocyte-neuron co-cultures and pure neuronal cultures.
Furthermore, an increase in extracellular SOD3 will also decrease extracellular concentrations of $O_2^{•−}$, which will decrease its reaction with, and inactivation of, the potent vasodilator NO into peroxynitrite, ultimately leading to vasodilation (Demchenko et al., 2010). Hence, this could be a mechanism to increase blood flow to brain regions with high neuronal activity.

Surprisingly, neither the glutamine synthetase (Glul) nor the glutamate transporters EAAT2 (Slc1a2) or EAAT3 (Slc1a1) were regulated by synaptic activity. The protein levels of EAAT2 have been shown to be reduced in cultures (Perego et al., 2000) and slices (Yang et al., 2009) when silenced with TTX. However, both set-ups used TTX silencing protocols of several days (6 and 7, respectively), raising the question of whether this does not impact on cell viability. Should there indeed be an increase in (neuronal) cell death, the drop of EAAT2 is possibly induced by the absence of neuron-astrocyte contact rather than synaptic activity, since we see a strong induction of Eaat2 in astrocytes when co-cultured with neurons, but not with synaptic activity.

All in all, the genes identified here as being regulated in astrocytes by synaptic activity show the tight coupling of neurons and astrocytes, indicating that active neurons signal to astrocyte to boost their lactate shuttling capacity as well as thyroid hormone signalling and antioxidant defences.

5.3.4 Confirming the mixed-species RNA-sequencing results in vivo: Future experiments

All the data presented in this chapter are based on primary cultures and I therefore plan to confirm them with experiments in in vivo set-ups. To do this, I am going to utilize two mouse models that affect the innervation of astrocytes in the striatum and/or cortex.

First, in collaboration with Prof. Gillian Bates (King’s College London), I am going to use the R6/2 mouse model of Huntington’s Disease (HD). The R6/2 mouse expresses exon 1 of the human huntingtin gene that includes 150 CAG repeats, leading to an abnormally long poly-glutamine stretch in the huntingtin protein (Li et
This mouse shows huntingtin inclusions in striatal and cortical neurons associated with signs of cell death and cell atrophy of neurons, which is ultimately leading to atrophy of the striatum and cortex. Incidentally, glial cells were reported to not show any huntingtin inclusions (Li et al., 2005) and astrocytes have recently been implicated to play a role in the HD pathology of R6/2 mice (Tong et al., 2014). I hypothesise that the neuronal atrophy accompanied by a decrease in neuronal activity in the R6/2 mouse will affect the gene expression profile of astrocytes in the striatum and cortex. I am going to probe for gene expression levels of genes we know are regulated by neurons and neuronal activity in astrocytes, and hypothesise that genes up-regulated in astrocytes upon co-culture and/or synaptic activity will be reduced in the striatum and cortex of the R6/2 mouse. This requires probing for genes that are only expressed in astrocytes and regulated by neurons and/or neuronal activity, as for example Dio2, and I hope we will identify more genes that fall into this category once the RNA-sequencing data comes back. Intriguingly, going through micro-array data on the R6/2 mouse from (Mielcarek et al., 2013), we identified Dio2 as strongly down-regulated in the R6/2 cortex when compared to WT. Similarly, other genes up-regulated in astrocyte upon co-culture with neurons or synaptic activity, such as Gja1, Slc1a3, Slco1c1 and Sod3, are down-regulated in the R6/2 cortex. However, while these genes are not expressed in neurons, the cell type origin of these transcripts is not known, since they are expressed in other cell types of the brain as well (Zhang et al., 2014).

As a second animal model I am going to use mice with cortical ablations, which will be created in the lab of Prof. Tom Gillingwater (University of Edinburgh). Removing one hemisphere of the cortex for several days will strongly reduce the afferent synaptic input into the ipsilateral striatum (Gillingwater et al., 2006). This will allow me to study the consequences of a decrease of synaptic input on astrocytic gene expression in vivo, and the approach will be similar as with the HD mouse.

Finally, experiments are on the way extracting live astrocyte from the whole brain using immuno-panning and comparing their transcriptional profile shortly after extraction and several days post-culture. We expect the genes we identified to be boosted by the presence of neurons to decline over time and be re-induced when co-cultured with neurons.
5.4 Conclusion

The development of next generation sequencing over the past 10 years has opened up the way to study the transcriptome of a wide array of cell types, as well as to investigate genome-wide changes in gene expression. The tool I have introduced here allows studying changes in gene expression that are induced non-cell autonomously. By using different species for different cell types, I was able to allocate a transcript to a cell type according to the sequence of the transcript. I was applying this tool on astrocyte-neuron co-cultures to investigate the effect that neurons have on the astrocytic transcriptome, showing that neurons push astrocyte into a more in vivo like phenotype, which agrees with the changes in astrocyte morphology I observed. Additionally, I identified a large number of genes that are regulated in astrocyte by neuronal activity that will have major functional implications, especially at the level of astrocyte-neuron metabolic coupling.

Furthermore, this tool can be applied in a wide set of experiments, and it will be interesting to see whether rodent neurons can induce similar changes in human astrocytes. If this would be the case, one could study patient-derived astrocytes with a glial disease component and study the effect of/on neurons. Similarly, any wild-type cell-type can be mixed with a transgene cell-type of a different species to study non-cell autonomous effects of the disease phenotype.

All in all, I showed here how powerful this tool is to study non-cell autonomous changes in gene expression and think it can find major application in the general field of biological cell sciences, as it is easy to set up, and yet addresses questions on inter-cell communication that could not have been answered before.
Chapter 6
Concluding remarks
6 Concluding remarks

Due to the post-mitotic nature of neurons, it is crucial to have homeostatic mechanisms in place that protect neurons from damage, such as induced by oxidative stress, Ca^{2+} dyshomeostasis and energy imbalance. These mechanisms can be found within neurons, or can be conferred non-cell autonomously by surrounding astrocytes in the tightly coupled neuro-glia unit. If these homeostatic mechanisms fail, however, neurons undergo damage that can render them dysfunctional. In neurodegenerative and neurological disorders that show signs of redox imbalances, aberrant Ca^{2+} levels and mitochondrial dysfunction, neuronal axons and dendrites are often found to be dysmorphic and dysfunctional.

In this thesis I provided evidence that dendrites show a selective vulnerability to excitotoxic, oxidative and metabolic stress by applying genetically-encoded imaging probes, providing a possible explanation for the dendritic phenotypes found in neurodegeneration. One possible explanation for this is that the homeostatic coupling between neurons and astrocytes is better tuned at the level of the soma than the dendrite. Alternatively, cell-autonomous mechanisms within neurons to confer protection against stress might work more efficiently in the soma. Whatever the case, failure to adjust to a disturbance of homeostasis has detrimental consequences on dendritic integrity, leading to the impairment of neuronal physiological activity and ultimately death. As a possible intervention to improve neuronal health by boosting the redox buffer capacity of neurons and making its regulation independent of the neuro-glial homeostatic unit, I over-expressed Nrf2 in neurons. This master antioxidant regulator that is usually not expressed in neurons can boost the neuronal redox capacity, protecting neurons from oxidative insults and emphasizing Nrf2 as a potential drug target against oxidative stress in the brain. Similarly, I showed that chronic synaptic activity can increase the glutathione redox potential of neurons, showing that the neuronal redox buffer capacity can be boosted independently of astrocytes. However, neuronal activity is also metabolically demanding, and in order to avoid energy dyshomeostasis, neurons can adjust their energy production when facing changes in energy demand.

I showed here that during an acute increase in synaptic activity, neurons increase their metabolic output by increasing TCA cycle activity and oxidative
phosphorylation, and that this is regulated by mitochondrial Ca\textsuperscript{2+} and matrix dehydrogenases in a cell-autonomous fashion. This is in contrast to the non-cell autonomous lactate supply conferred by astrocytes, one of many ways in which homeostasis within the neuro-glial unit is maintained.

In order to study this tight coupling between astrocytes and neurons, we developed a tool that allows studying non-cell autonomous changes in gene transcription in a co-culture set-up. This is based on using different species for different cell types and the post-hoc un-mixing of the sequenced reads following RNA-sequencing. By applying this tool on astrocyte-neuron co-cultures, I could show that neurons induce drastic changes in the astrocytic transcriptome and push them into a more \textit{in vivo}-like state, which is also reflected by the changes in morphology I presented. Furthermore, I show that neuronal activity boosts the expression of genes involved in lactate shuttle, antioxidant defences and thyroid hormone signalling in astrocytes, uncovering a novel form of homeostatic mechanisms in the neuro-glial unit.

All these data combined show that the neuro-glial unit as well as neurons themselves have a wide range of tools to guarantee homeostasis when facing oxidative, excitotoxic and metabolic challenges, that these homeostatic mechanisms can be regulated by neuronal activity and that the failure of these homeostatic responses can have detrimental effects on neuronal function and health.
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Figure A1: Knock-down of MCU suppresses mitochondrial Ca\textsuperscript{2+} uptake following application of NMDA or induction of synaptic activity.
A) Neurons were nucleofected with either siCnt or siMcu. Example western blot showing siMcu mediated knock-down of the MCU protein compared to siCnt. Protein levels were normalised to β-actin.
B) qPCR showing knock-down efficiency of siRNA against the Mcu mRNA compared to siCnt. *p<0.05 paired t-test (n=4).
C) Example traces showing mitochondrial Ca\(^{2+}\) uptake after bath application of NMDA (30 μM) in siCnt (left) and siMcu (right) conditions. Thin lines represent single cells, thick lines average of all cells in the recording.
D) Cells were transfected with mito-GCaMP2 and either siMcu or siCnt and exposed to NMDA (30 μM), siMcu suppresses Ca\(^{2+}\) uptake into mitochondria following NMDAR stimulation compared to siCnt. "p<0.05 paired t-test (n=5).
E) Example trace of Rhod2 imaging confirming that siRNA-mediated knock-down of MCU suppresses mitochondrial Ca\(^{2+}\) uptake following NMDA (20, 30 μM) application. Red line represents siMcu-positive cell, black line the average of surrounding cells.
F) Quantification of Rhod2 data. #p<0.05 paired two-tailed t-test, comparing siMcu-positive cells to surrounding cells.

Experiments A-F in this figure were performed by Jing Qiu and is published in (Qiu et al., 2013).

G,H) Similar to Fig. 3.4 E,F. Western blots showing efficiency of shMcu-mediated knock-down of MCU compared to shScr. ***p<0.001, student t-test.

I) shMcu positive cells show a weaker uptake of mitochondrial Ca\(^{2+}\) following an action potential train (10 Hz, 30s) when compared to shScr positive cells, as measured with mito-GCaMP2. Graph shows average traces from shScr n=3, shMcu1 n=4 and shMcu2 n=5.

J) Action potential firing causes a drop in mitochondrial pH that takes longer to restore in shMcu positive cells compared to shScr positive cells, possibly explaining the drop in mito-GCaMP2 signal in the shMcu knock-out conditions following action potential firing. shMcu1: ***p<0.001, **p<0.01. shMcu2: ##p<0.01, #p<0.05, two-way anova, Holm-Sidak post-hoc test, compared to shScr controls.

Experiments in I and J were performed by Jamie Marland and together with my data in G and H are published in (Marland et al., 2015). Figures A-F are directly taken from Qiu et al. 2013 and figures G-J from Marland et al. 2015.
Figure A2: The species-specific sorting (SSS) workflow has little impact on single-species RNA-seq analysis.

A) A small proportion of RNA-seq reads is lost due to species-specific sorting (SSS). RNA-seq reads were generated from RNA extracted from a pure mouse neuronal culture (n=3). The paired-end reads were subjected to both the SSS-workflow and conventional STAR mapping. The % of reads lost due to SSS (compared to conventional STAR mapping) was calculated on a gene-by-gene basis, for all genes (except predicted genes, 20,409 in total) and a frequency distribution histogram (5% bins) generated. B,C) Gene fold-change is little-altered by SSS of RNA-seq reads. Pure neuronal mouse cultures were treated ± BiC/4-AP for 4h, RNA extracted and RNA-seq performed (n=3). Read mapping was performed using both SSS-workflow and normal STAR mapping, and in each case the DESeq2 fold-change in gene expression calculated for all genes expressed in either of the two conditions (11,059 genes). The fold-changes derived from the different mapping methods were plotted against each other. C) For the genes studied in (B), the ratio of fold changes derived from the different mapping methods (calculated in (B)) was calculated and plotted against the % of reads lost due to the SSS-workflow. D) Read losses due to SSS have little impact on P-value significance. 4631 genes were called as being significantly up- or down regulated by BiC/4-AP treatment (DESeq2 P_adj<0.05) using the normal STAR mapping method. For those same genes, the DESeq2 P_adj was calculated after the SSS-workflow, and the increase in P-value calculated. This increase was plotted against the % of reads lost due to the SSS-workflow. The red crosses indicate the 33 genes for which P_adj>0.05 when calculated after the SSS-workflow. The bioinformatical analysis presented in this figure was carried out by Giles Hardingham, Owen Dando and Samuel Heron.
Figure A3: Example images showing cytoplasmic GCaMP2 before and after the addition of NMDA.

Cytoplasmic GCaMP2 was expressed in primary cortical neurons and imaged using a standard GFP filter set. Pink arrows indicate GCaMP2-positive neurons. The turquoise circle and magenta rectangle indicate typical somatic and dendritic areas that were picked for measuring sub-cellular Ca\(^{2+}\) uptake.
Figure A4: Example images of mito-GCaMP2 pre- and post-NMDA.

Mito-GCaMP2 shows the expected punctate expression in primary cortical neurons. Pink arrows indicate mito-GCaMP2 positive neurons. See Fig.A3 for how dendritic vs. somatic areas were identified.
Figure A5: Example images of NAD(P)H autofluorescence pre-and post-BiC/4-AP.

Example images of NAD(P)H autofluorescence before and after network bursting. Primary cortical neurons were excited at 387±5 nm and excitation was measured at 447±30 nm. Images represent baseline (top) and maximal response (bottom) to network bursting induced by BiC/4-AP. Yellow circles indicate typical somatic and magenta rectangles indicate typical dendritic areas.
Figure A6: Example images of rh123 to measure the mitochondrial membrane potential.

Example images of neurons loaded with rh123. Primary cortical neurons were incubated with rh123 and subsequently imaged using a standard GFP filter set. Top image represents baseline and bottom image shows mitochondrial membrane depolarization by NMDA bath application resulting in an increase in rh123 fluorescence.
Figure A7: FRET ratio of AT1.03 expressed in primary cortical cultures.

Example images of the change in AT1.03 FRET ratios. AT1.03 expressing neurons were exposed to BiC/4-AP and FCCP resulting in a drop in the AT1.03 YFP/CFP FRET ratio. Pink represents high, blue represents low AT1.03 FRET ratios. White arrows indicate AT1.03-positive neurons.
Cortical neurons expressing Perox show accumulation of the sensor in the nucleus due to the NLS sequence attached to it. Images on the left show NADH/NAD⁺ dependent fluorescence and images on the right show NADH/NAD⁺ independent mCherry fluorescence. Addition of BiC/4-AP causes Perox fluorescence to increase. White arrows indicate single Perox-positive cells.
Figure A9: Example images and trace of astrocytic Ca$^{2+}$ imaging during increased network bursting.

A) Example image showing GCaMP2-positive astrocytes (white arrow) and a GCaMP2-positive neuron (magenta arrow). B,C) Subcellular regions of interest (ROIs) were selected when showing signs of Ca$^{2+}$ fluctuations. Network bursting increases Ca$^{2+}$ transients in astrocytic processes but not in soma (see orange ROI and trace). However, the addition of the glutamate reuptake inhibitor TBOA can cause an increase in somatic Ca$^{2+}$ in processes as well as the soma.