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THE NEURONAL MONOCARBOXYLATE TRANSPORTER MCT2: ITS REGULATION BY BDNF AND ITS ROLE IN LEARNING AND MEMORY

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THE NEURONAL MONOCARBOXYLATE TRANSPORTER MCT2: ITS REGULATION BY BDNF AND ITS ROLE IN LEARNING AND MEMORY

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Summary of the thesis

Glucose has been considered the major, if not the exclusive, energy substrate for the brain. But under certain physiological and pathological conditions other substrates, namely monocarboxylates (lactate, pyruvate and ketone bodies), can contribute significantly to satisfy brain energy demands. These monocarboxylates need to be transported across the blood-brain barrier or out of astrocytes into the extracellular space and taken up into neurons. It has been shown that monocarboxylates are transported by a family of proton-linked transporters called monocarboxylate transporters (MCTs). In the central nervous system, MCT2 is the predominant neuronal isoform and little is known about the regulation of its expression. Noradrenaline (NA), insulin and IGF-1 were previously shown to enhance the expression of MCT2 in cultured cortical neurons via a translational mechanism. Here we demonstrate that the well known brain neurotrophic factor BDNF enhances MCT2 protein expression in cultured cortical neurons and in synaptoneurosome preparations in a time- and concentration-dependent manner without affecting MCT2 mRNA levels. We observed that BDNF induced MCT2 expression by activation of MAPK as well as PI3K/Akt/mTOR signaling pathways. Furthermore, we investigated the possible post-transcriptional regulation of MCT2 expression by a neuronal miRNA. Then, we demonstrated that BDNF enhanced MCT2 expression in the hippocampus *in vivo*, in parallel with some post-synaptic proteins such as PSD95 and AMPA receptor GluR2/3 subunits, and two immediate early genes *Arc* and *Zif268* known to be expressed in conditions related to synaptic plasticity. In the last part, we demonstrated *in vivo* that a downregulation of hippocampal MCT2 via silencing with an appropriate lentiviral vector in mice caused an impairment of working memory without reference memory deficit. In conclusion, these results suggest that regulation of neuronal monocarboxylate transporter MCT2 expression could be a key event in the context of synaptic plasticity, allowing an adequate energy substrate supply in situations of altered synaptic efficacy.

Résumé du travail de thèse

Le glucose représente le substrat énergétique majeur pour le cerveau. Cependant, dans certaines conditions physiologiques ou pathologiques, le cerveau a la capacité d'utiliser des substrats énergéiques appartenant à la classe des monocarboxylates (lactate, pyruvate et corps cétoniques) afin de satisfaire ses besoins énergétiques. Ces monocarboxylates doivent être transportés à travers la barrière hématoencéphalique mais aussi hors des astrocytes vers l'espace extracellulaire puis re-captés par les neurones. Leur transport est assuré par une famillle de transporteurs aux monocarboxylates (MCTs). Dans le système nerveux central, les neurones expriment principalement l'isoforme MCT2 mais peu d'informations sont disponibles concernant la régulation de son expression. Il a été montré que la noradrénaline, l'insuline et l'IGF-1 induisent l'expression de MCT2 dans des cultures de neurones corticaux par un mécanisme traductionnel. Dans cette étude nous démontrons dans un premier temps que le facteur neurotrophique BDNF augmente l'expression de MCT2 à la fois dans des cultures de neurones corticaux et dans les préparations synaptoneurosomales selon un décours temporel et une gamme de concentrations propre. Aucun changement n'a été observé concernant les niveaux d'ARNm de MCT2. Nous avons observé que le BDNF induisait l'expression de MCT2 par l'activation simultanée des voies de signalisation MAPK et PI3K/Akt/mTOR. De plus, nous nous sommes intéressés à une potentielle régulation par les micro-ARNs de la synthèse de MCT2. Ensuite, nous avons démontré que le BDNF induit aussi l'expression de MCT2 dans l'hippocampe de la souris en parallèle avec d'autres protéines post-synaptiques telles que PSD95 et GluR2/3 et avec deux « immediate early genes » tels que *Arc* et *Zif268* connus pour être exprimés dans des conditions de plasticité synaptique. Dans un dernier temps, nous avons démontré qu'une diminution d'expression de MCT2 induite par le biais d'un siRNA exprimé via un vecteur lentiviral dans l'hippocampe de souris générait des déficits de mémoire de travail sans affecter la mémoire de référence. En conclusion, ces résultats nous suggèrent que le transporteur aux monocarboxylates neuronal MCT2 serait essentiel pour l'apport énergétique du lactate pour les neurones dans des conditions de haute activité neuronale comme c'est le cas pendant les processus de plasticité synaptique.

Abbreviation list

Akt: serine/threonine protein kinase from AKT virus
ANLSH: astrocyte-neuron lactate shuttle hypothesis
AMPA receptor: alpha-Amino-3-hydroxy-5-methy-4-isoxazol receptor
Arc: activity-regulated cytoskeleton-associated protein
ATP: adenosine triphosphate
BBB: blood brain barrier
BDNF: brain derived neurotrophic factor
αCamKII: acalmodulin kinase II
cDNA: complementary DNA
CNS: central nervous system
DNA: deoxyribonucleic acid
eIF: eukaryotic initiation factor
ERK: extracellular-regultaed kinase
GLAST: glutamate aspartate transporter
GLT1: glutamate transporter 1
GLUT: glucose transporter
GluR, glutamate receptors
IGF-1: insulin-like growth factor-1
kDa: kilodalton
LDH: lactate dehydrogenase
MCTs: monocarboxylate transporters
MEK: mitogen-activated protein kinase kinase
miRNA: micro ribonucleic acid
MNK-1: MAPK-interacting kinase 1
mTOR: mammalian target of rapamycin
NA: noradrenaline
NMDA receptor: N-methyl-D-aspartic acid receptor
NMR: nuclear magnetic resonance
P38 MAPK: p38 mitogen-activated protein kinase
PBS: phosphate buffer saline
PCR: polymerase chain reaction
PET: positron emission tomography
PI3K: phosphoinositide 3-kinase
PSD-95: postsynaptic density-95
RNA: ribonucleic acid
RT: reverse transcription
S6K: S6 kinase
SD: standard deviation
Sem: standard error of the mean
SNAP25: Synaptosomal-associated protein 25
TCA cycle: tricarboxylic acid cycle
TM: transmembrane segment
Zif268: zinc finger protein 268

Chapter 1

Chapter 1

Introduction

The brain is a complex organ which is composed of around 100 billions of cells. The two principal cell types found in the brain are neurons and glial cells. Neurons are highly polarized cells and specialized for information transmission. They are interconnected by synapses. Among glial cells, astrocytes which are up to five times more numerous than neurons (in certain human brain areas) constitute half of the brain volume and are involved in many functions such as synthesis of neuroactive substances, neurotransmitter recycling, synaptic activity modulation, immune roles and trophic as well as energetic support.

This thesis work focused on the regulation of one particular transporter in neurons under conditions purported to reflect synaptic plasticity. This neuronal transporter, called MCT2 (monocarboxylate transporter 2) is involved in the transport of monocarboxylates such as lactate, principally produced by astrocytes from glucose and proposed to be a major energy substrate for active neurons.

In this context, it is essential to describe the processes involved in cerebral energetic metabolism, as well as the specific roles of the monocarboxylate transporter family and their regulations at the cellular level to understand the potential role of MCT2 in synaptic plasticity.

1.1 Brain energy metabolism: an overview

1.1.1 Glucose metabolism in the brain

For more than 50 years, glucose has been considered as the major energetic substrate for the brain (McIlwain, 1956). Sokoloff and colleagues have shown that the adult brain consumes around 50% of the oxygen and 10% of the glucose provided via blood vessels, corresponding respectively to 20% and 25% of the organism total consumption (Sokoloff, 1960). Glucose transport takes place across the BBB via specific transporters expressed by endothelial cells forming capillaries (GLUT1 55KDa), as well as on cerebral cells like astrocytes (GLUT1 45kDa) and neurons (GLUT3) (Maher et al., 1994). After entry into the brain, glucose is almost completely oxidized to CO_2 and H_2O via glycolysis and the TCA cycle (Review Magistretti, 1999).

Glycolysis is the energy producing process by which intracellular glucose is metabolized to pyruvate, resulting in the net formation of two ATP molecules per glucose. Glycolysis account only for a minor part of the ATPs produced from glucose. The largest amount is produced by the mitochondrial oxidative phosphorylation cascade which oxidizes pyruvate into CO_2 and H_2O and leads to the production of about 30 molecules of ATP per molecule of glucose. Because of its higher ATP yield, oxidative phosphorylation represents the source of most of the energy needed for neuronal signalling (Raichle and Mintum 2006). Nevertheless, glycolysis has advantages when energetics needs to be increased rapidly: it is independent of oxygen and can produce cytosolic ATP faster than oxidative phosphorylation. In such case however, pyruvate must be converted into lactate to generate NAD^+ and maintain glycolysis rate, leading to transient lactate accumulation (Raichle and Mintun 2006). Even if glycolysis produces far less energy than oxidative phosphorylation, it can be of strategic importance in particular circumstances as we will see below.

Apart from ATP, another form of energy which is important for cerebral function is carried by nicotinamide adenine dinucleotide phosphate (NADPH). NADPH represents a source of reducing equivalents and is produced predominantly by the processing of glucose through the pentose phosphate pathway. Metabolic energy in the form of reducing equivalents is required for biosynthetic reactions in which the precursors are in a more oxidized state than the products (e.g. the reductive synthesis of fatty acids from acetyl-CoA which are components of

myelin and of neural cell plasma membranes) as well as for scavenging of reactive oxygen species (ROS) which are highly damaging to cells (Magistretti 2003).

1.1.2 Energy consuming processes in the brain

The cerebral processes consuming the major part of the energy are the maintenance and the regeneration of the ionic gradient across the plasma membrane. In particular for Na^+ and K^+ which are important for neuronal excitability. These gradients are predominantly maintained by the Na^+/K^+-ATPase which is located in both neurons and astrocytes and is fuelled by ATP. It was demonstrated that these pumps account for about 50% of basal glucose oxidation in the nervous system (Erecinska and Dagani 1990). The contribution of different energy consuming processes in the rodent brain (grey matter) has been estimated and reviewed by Attwell and Laughlin (Attwell and Laughlin, 2001).

In this review, they simplified the model by treating all neurons as glutamatergic because excitatory neurons are the major class of neurons in the brain and 90% of synapses release glutamate. According to this study, it exists a definite hierarchy of energy usage devoted to signalling. Brain grey matter has an energy usage of 33 to 50 µmol of ATP/g/min when considering the neocortex whereas the energy usage of the whole brain is on average of 21 µmol of ATP/g/min. This phenomenon could be explained by the high signalling-related energy demand in grey matter. Attwell and Laughlin have predicted that the most energy demanding processes were both action potentials and postsynaptic potentials that represent 47% and 34% of brain energy expenditures respectively. However recently these values have been revisited by Alle and colleagues (reviewed by Magistretti 2009). Indeed, the excitatory postsynaptic potentials mediated by glutamate involve six times more charge transfert than that underlying action potentials (96 versus 15pC, respectively) (Alle et al., 2009). Maintenance of resting potentials in both neurons and glial cells (13%) represents a smaller fraction of energy expenses. Presynaptic calcium entry and neurotransmitter recycling (3% each) account for even less. It is noteworthy that, apart from signalling mechanisms, protein synthesis consumes only 2% of the rat brain's total ATP consumption. Similarly, the turnover of oligonucleotides and lipids represents approximately 5% of the total rate for the whole brain (Attwell and Laughlin 2001). It is interesting also to note that earlier reports based on heat production considered action potentials as a very poor energy consuming process (0.3 to 3%) (Creutzfeldt 1975).

Concerning human brain energy consumption, there are still very few data but some studies based upon positron emission tomography (PET) and nuclear magnetic resonance (NMR) spectroscopy are attempting to fill in the gap. These techniques permit to monitor alterations in glucose utilization and metabolism, oxygen consumption and blood flow during activation of specific brain areas. Positron emission tomography (PET) can measure blood flow, oxygen consumption and glucose use associated with neuronal activity; the degree of blood oxygenation is currently thought to contribute to the signal detected with functional magnetic resonance imaging, while magnetic resonance spectroscopy (MRS) identifies the spatio-temporal pattern of the activity-dependent appearance of certain metabolic intermediates such as glucose or lactate (Magistretti and Pellerin, 1999). Energy expenditures in human brain are similar than those from rodent brain. Indeed, several studies using magnetic resonance spectroscopy measurements in a variety of experimental settings have shown that the resting brain accounts for up to 80% of the total energy consumption to support events associated with neuronal activity (energetic consequences of electrical activity associated with cycling of neurotransmitters glutamate and GABA) (Shulman et al., 2004).

1.1.3 Brain energy metabolism at the cellular level: The neuron-astrocyte-capillary metabolic unit

At the cellular level, there are three main partners concerned about brain energy metabolism: The neurons, the astrocytes and the vascular endothelial cells forming capillaries. It is well established that neurons represent, at most, 50% of the cerebral cortical volume and that a ratio of ~ 5-10 astrocytes for 1 neuron is a feature of some brain regions (Tsacopoulos and Magistretti 1996). Astrocytes are particularly well situated to actively participate in the distribution of energy substrates from blood circulation to neurons. Astrocytes are polarized cells with one process contacting the endothelial cells forming capillaries and many processes contacting neurons. Indeed, astrocytes are stellate cells composed of multiple specialized processes, like those that surround brain capillaries called endfeet (Kacem et al., 1998). Thus, astrocytes are ideally positioned to provide coupling between neuronal activity and glucose uptake, even more so as the specific 45kDa glucose transporter GLUT1 is abundantly expressed on endfeet (Morgello et al.,1995). This cytoarchitectural arrangement implies that astrocytes form the first cellular barrier that glucose entering the brain parenchyma encounters, and it makes astrocytes a likely site of primary glucose uptake (Figure1.1)

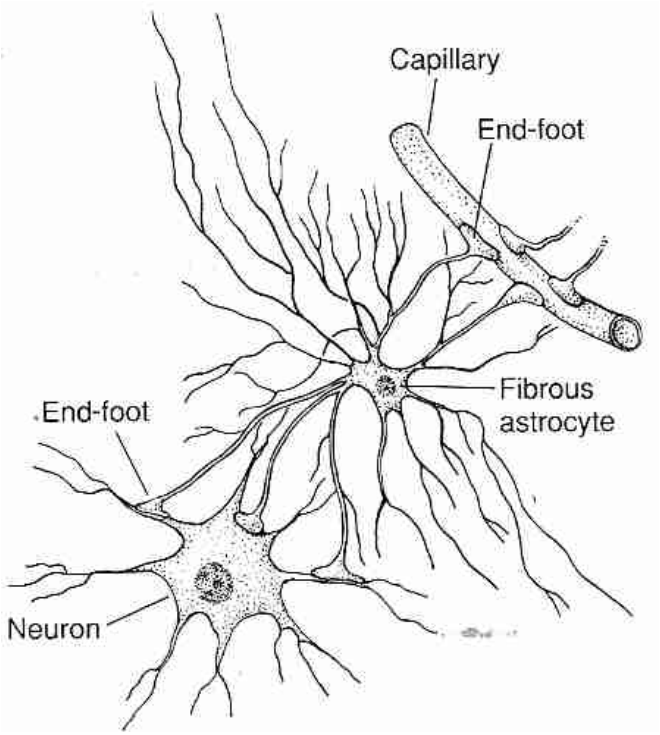

Figure1.1. Schematic representation of the organization and cooperation between neurons, astrocytes and blood vessels (taken from Kuffler and Nicholl; "neuron to brain; A cellular approach to the function of the nervous system", 1976).

In addition to perivascular endfeet, other astrocyte processes are wrapped around synaptic contacts. At these sites, astrocyte processes possess receptor and reuptake sites for a variety of neurotransmitters (e.g. glutamate) (Magistretti and Pellerin 1999). Another important feature of astrocytes is that they form an extensive network interconnected by gap junctions (Benarroch 2005). The astrocytic gap junctions are permeable to small molecules and permit cell-cell communication, allowing astrocytes to form a functional syncytium through which energy metabolites, among others, can circulate (Benarroch 2005) (Figure1.2).

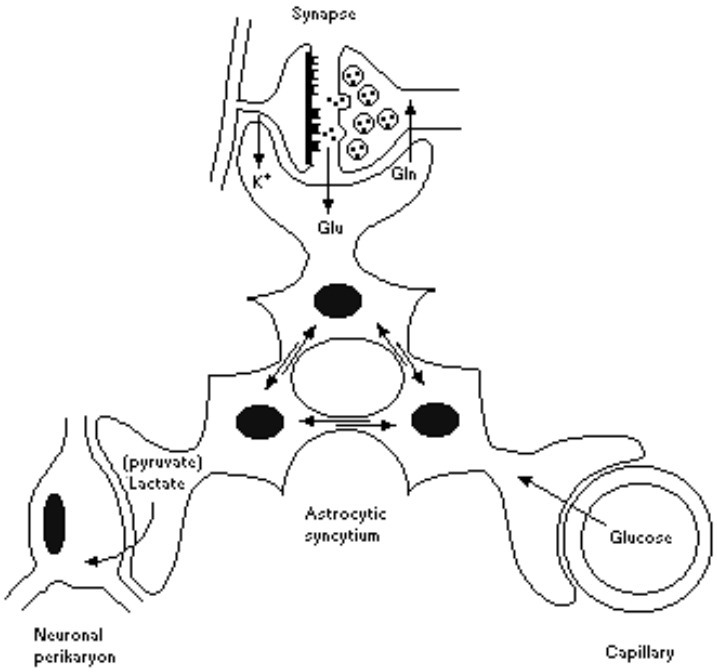

Figure1.2. Metabolic polarity of the astrocytic syncytium. Astrocytes have various processes that make contact either with capillaries, neuronal perikaryon, or synapses. In addition, they are connected with each other at gap junctions; through these junctions small molecules (MW < 1000) can be exchanged. These properties endow the astrocytic syncytium with the capacity to ensure the transfer of metabolic intermediates from areas of production to areas of demand. *Glu*, Glutamate; *Gln*, glutamine. (Picture from Magristretti et al., 1995)

The aforementioned characteristics are of critical importance for astrocytes to exert their main functions. These include the removal of excess glutamate and potassium from the synaptic cleft upon neuronal stimulation and to provide neurons with metabolic support (Magistretti and Pellerin 1996). Astrocytes also possess the largest energy reserve of the brain in the form of glycogen. Glycogen has been considered as a metabolic buffer during physiological activity and its turnover as well as overall levels seem tightly coordinated with synaptic activity (Hevor 1994; Magistretti 2003). Altogether, these features suggest that astrocytes play a key role in regulating the distribution of energy substrates from their site of entry in the brain parenchyma (the capillaries) to their site of utilization (active synapses).

1.2 Monocarboxylates in the CNS

During adulthood, in normal conditions, the entry of energetic substrates other than glucose is low (Stone 1938; Klein and Olsen, 1947; McIlwain, 1953). However, glucose can be partially

substituted by other metabolic intermediates under particular conditions such as starvation (hypoglycaemia), diabetes or breast-feeding in neonates. Indeed, it was shown that these particular conditions lead to increased plasmatic levels of the ketone bodies acetoacetate and D-3-hydroxybutyrate which can be used as metabolic substrates by the brain (Sokoloff 1989) (Figure1.3). Lactate and pyruvate are also possible energetic substrates for the developing and adult brain and can sustain synaptic activity *in vitro* (McIlwain and Bachelard, 1985; Schurr et al., 1988). Ketone bodies, lactate and pyruvate are molecules belonging to the monocarboxylate group which are charaterized by the presence of only one carboxyl group (-COOH) in their structure (Figure1.4).

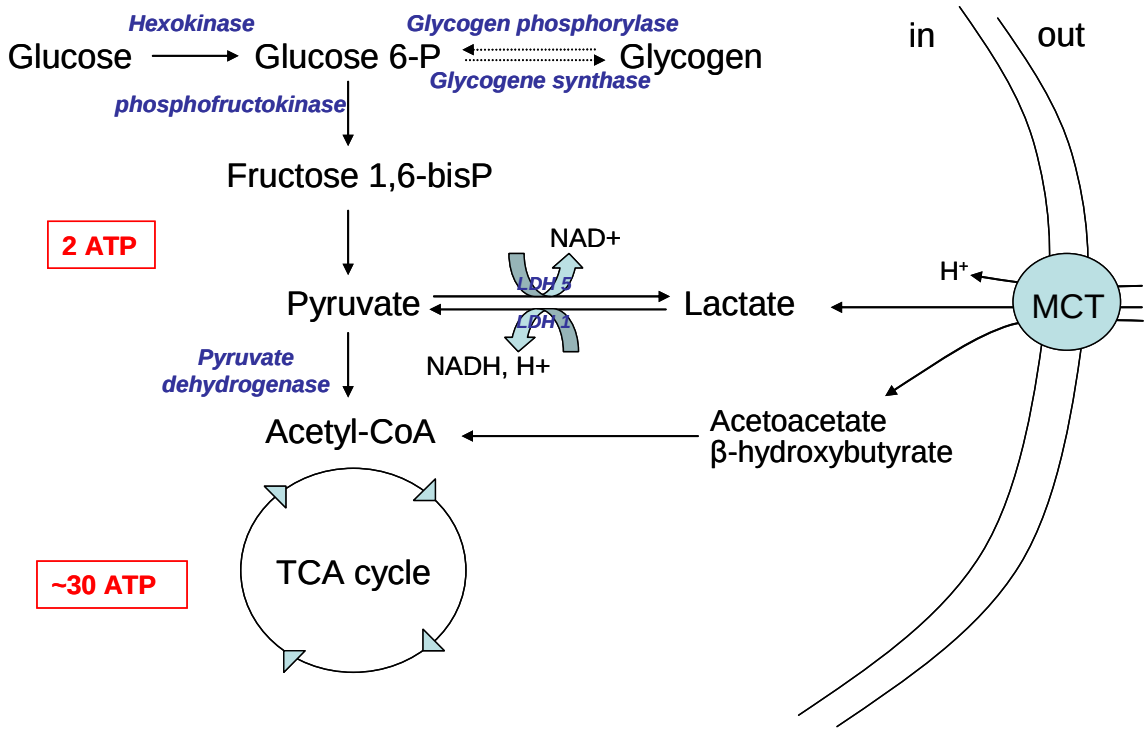

Figure 1.3. Oxidative metabolism of glucose and other substrates in brain cells. LDH: lactate dehydrogenase (Picture adapted from Brain energy metabolism course, Prof. Luc Pellerin, University of Lausanne, 2006)

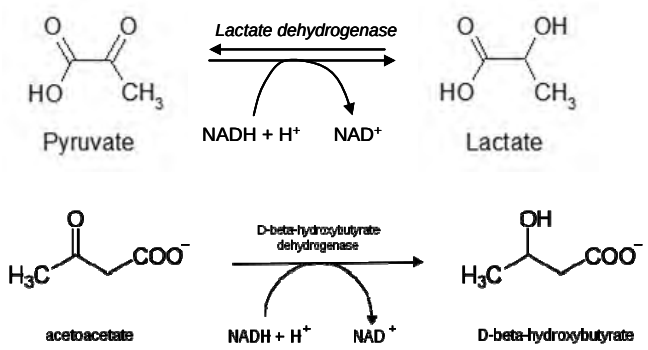

Figure1.4 Chemical structures of the main monocarboxylates: pyruvate, L lactate, acetoacetate and D-hydroxybutyrate.

1.2.1 Perinatal utilization of monocarboxylates

Birth is a period of dramatic changes in body nutrient utilization. During gestation, the foetus is continuously supplied with substrates coming from the placental circulation. This stable situation changes with birth which imposes changes in metabolic environment to the newborn. Studies in the rat showed that the very early neonatal period is always associated with profound hypoglycemia. Indeed, glucose levels are usually between 2.5 and 3.5mM at birth and drop to approximately 1mM at one hour of postnatal life. This low glucose level is accompanied by a low concentration of ketone bodies (around 0.2-0.4mM). Immediately after birth, the principal source of energy for the newborn brain becomes lactate which is present at high concentrations in the blood during the presuckling period (Dombrowski et al., 1989; Vicario et al., 1991). However, the majority of this pool of lactate is consumed during the first two hours after birth, just before the onset of suckling.

It is well known that during the normal development of both human and rodent brain, there is a switch in fuel utilization from a combination of glucose, lactate, and ketone bodies (beta-hydroxybutyrate and acetoacetate) in the immature brain to a total reliance on glucose in the adult (Cremer 1982; Nehlig 1997). With the onset of suckling and the ingestion of the high-fat milk diet, rat pups become hyperketonemic and remain so during the suckling period (Nehlig and Pereira de Vasconcelos, 1993). Whereas cerebral glucose utilization is relatively low in the newborn rat brain (Nehlig et al., 1988; Vannucci et al., 1994), rates of beta-hydroxybutyrate uptake and utilization actually peak during the second and third postnatal weeks before declining to low adult levels after weaning (Cremer et al., 1982; Hawkins et al., 1986; Nehlig et al., 1991). In the rat pup, these two ketone bodies (beta-hydroxybutyrate and acetoacetate) are formed by hepatic oxidation in the liver of fat contained in maternal milk (Hawkins et al., 1971; Cremer 1982) before to be used as significant sources of energy. The high fat content of both human milk and rodent milk generates circulating ketone body concentrations sufficient to contribute to the energetic and synthetic needs of early cerebral maturation (Edmond et al., 1985; Nehlig 1997; Nehlig and Pereira de Vasconcelos 1993).

It was shown that ketone bodies can account for at least 30% of the total energy metabolism balance in suckling rats (Cremer 1982). However, lactate, beta-hydroxybutyrate and acetoacetate do not cross the BBB easily and require a transport system to reach the brain parenchyma. It was demonstrated that these monocarboxylates share a common transport

system: the monocarboxylate transporters (MCTs) (Cremer et al., 1979; Cremer et al., 1982; Pellerin et al., 1998a). Then, during the suckling period, the MCTs as well as the enzymes for ketone body utilization are up-regulated and permit the effective utilization of these alternative substrates by the brain (Pellerin et al. 1998b). There is a 7-fold increase in cerebral beta-hydroxybutyrate permeability during the suckling period and an equivalent decrease after the weaning period (Nehlig 2004). At the end of the suckling period, the high level of ketone bodies in blood tends to decrease and the rate of cerebral glucose utilization concomitantly increases (Hawkins et al., 1971). It was demonstrated that in the 15 days old rat, glucose become the predominant energy substrate (57%) and the beta-hydroxybutyrate and lactate represent respectively 20 and 23% of the total energy supply of the brain.

1.2.2 Adult brain utilization of monocarboxylates

Under specific conditions, monocarboxylates can be used as alternative substrates for adult brain energy metabolism (Sokoloff 1989). For example under starvation, diabetes, hypoglycaemia or intense exercise. All of these situations lead to an increase of ketone bodies acetoacetate and D-3-hydroxybutyrate or lactate in the plasma, all used by the brain as metabolic substrates (Sokoloff 1989). Owen and colleagues (Owen et al. 1967) showed that beta-hydroxybutyrate and acetoacetate replace glucose as brain's primary fuel during starvation in obese patients. Similarly, it was shown by quantitative autoradiography that the brain of starved and diabetic rats increase their capacity to use ketone bodies by up to 50%-60% in such conditions (Hawkins et al.,1986). In the rat under hypoglycemia, cerebral uptake of beta-hydroxybutyrate was shown to be greatly increased as well as the transport capacity of ketone bodies through the BBB (Ghajar et al., 1982; Nehlig 1997). In human adults, during cardiopulmonary resuscitation, arteriovenous differences indicate that lactate is consumed by the brain (Rivers et al., 1991). The same results were observed during exercice in human brain (Ide et al., 1999; Ide et al., 2000; Dalsgaard 2006 ; Dalsgaard and Secher 2007). In the mouse, lactate injection was shown to reduce cerebral glucose utilization, to induce an increase in lactate uptake from plasma and an increase in brain oxidation under insulin-induced hypoglycemia or acute stress conditions (Thurston et al., 1983; Thursten and Hauhart 1989). Measurements made using microdialysis or lactate-sensitive microelectrodes have shown that lactate utilization is enhanced upon electrical or behavioural activation (Fellows et al., 1993; Hu and Wilson 1997), or following brief hypoxic episodes in the rats (Jones et al., 2000). Thanks to NMR spectroscopy, it was further demonstrated that lactate is efficiently

metabolized by the brain. A (^{13}C)MR spectroscopy study using intravenous injection of radiolabelled lactate permitted to confirm in the rat that lactate crosses the BBB and is metabolized in the brain, in a manner analogous to glucose (Qu et al., 2000). Moreover, these studies demonstrated that lactate is prominently metabolized in neurons (Bouzier et al., 2000; Hassel and Brathe 2000; Qu et al., 2000).

In parallel, in human, PET has permitted to monitor alterations in glucose utilization, oxygen consumption and blood flow during the activation of specific brain area. By this technique, Fox and Raichle observed an uncoupling between oxygen consumption on one hand and glucose use or blood flow changes on the other in the visual cortex of human subjects during intense visual stimulation (Figure1.5) (Fox and Raichle 1986; Fox et al., 1988). Indeed, they observed a 30 to 40% increase in glucose utilization whereas oxygen consumption only increased by 6%, indicating that the additional glucose utilized during neuronal activation is processed primarily through glycolysis rather than through the TCA cycle (mitochondrial tricarboxylic acid cycle). In this case, if glucose is processed through glycolysis and does not enter the TCA cycle, lactate will be produced and at least accumulate transiently into the brain.

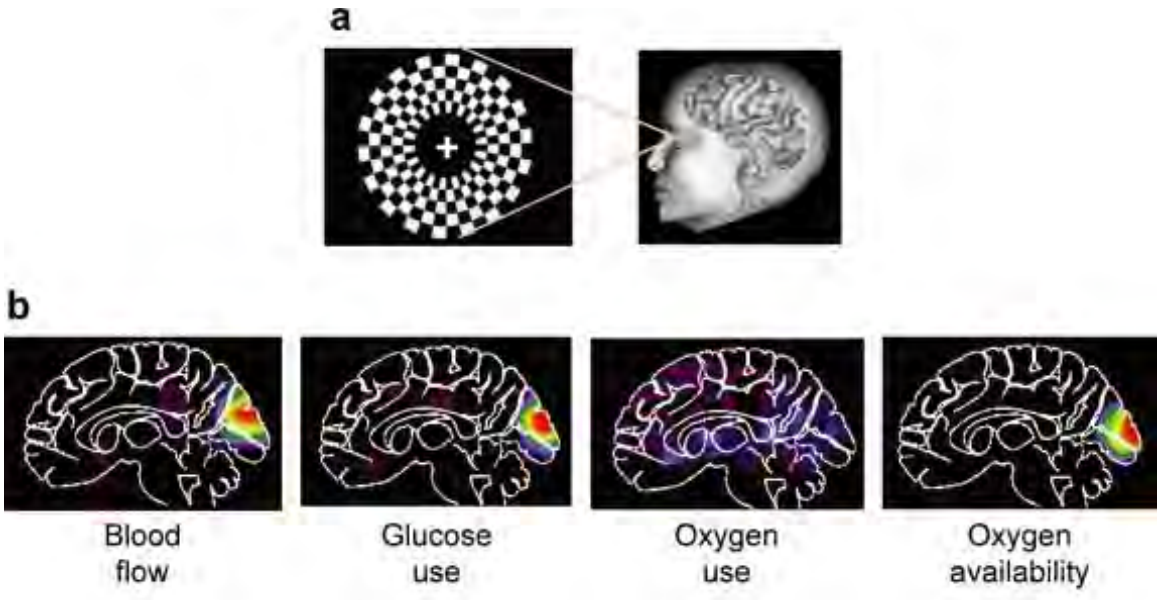

Figure 1.5. Stimulation of the human visual cortex with a reversing annular checkerboard when compared with a simple fixation crosshair (*a*) produces dramatic increases in blood flow and glucose use in the visual cortex that are unaccompanied by similar increases in oxygen use (*b*). The result is an increase in the local oxygen availability (*b*) because the increased supply of oxygen by flowing blood exceeds the increased local demand for oxygen. (picture taken from Raichle and Mintun 2006).

To confirm this hypothesis, studies using MRS technique were performed. For instance, MRS in humans has revealed that a transient lactate peak is observed in the primary visual cortex during physiological activation of the visual system (Prichard et al., 1991). In addition, Mintun and colleagues have shown an increase of the lactate/pyruvate ratio in parallel with blood flow in physiologically activated human brain using a combination of PET and functional MRI (Mintun et al., 2004). These observations support the view that during activation, some brain cells may transiently resort to aerobic glycolysis while others may use lactate as preferential oxidative substrate (Magistretti and Pellerin 1999; Schurr 2006; Smith D, 2003; Wyss et al., 2011).

1.2.3 Monocarboxylate utilization at the cellular level

A) Lactate: another neuronal substrate?

The idea that lactate could be a satisfactory alternative substrate for the brain did not easily gain success as it was considered that substrates other than glucose do not readily enter the brain (Stone 1938; Klein 1947; McIlwain 1953). Several studies have appeared confirming that lactate was efficiently oxidized to CO_2 by brain tissue and particularly by neurons. Indeed, lactate oxidation rate equals or is even greater than glucose utilization and it reduced glucose oxidation to CO_2 in such *in vitro* preparations including cultured neurons (Vicario et al.,1991; Tabernero et al., 1996; McKenna et al.,2001), synaptoneurosomal preparation (McKenna et al.,1993; 1994; 1998) and brain slices (Ide et al.,1969; Fernandez and Medina 1986). In addition, lactate was shown to be able to fully substitute for glucose in order to maintain respiration rate of cultured cortical neurons as measured by mitochondrial dehydrogenase activity (Pellerin et al.,1998a). Other evidences were provided from some structures associated with the peripheral nervous system further suggesting lactate utilization by brain cells. Indeed, lactate conversion to CO_2 was shown to be more rapid than glucose oxidation to CO_2 in the vagus nerve (Vega et al.,1998). Then it was shown that lactate is a better energy substrate than glucose for photoreceptors in the retina (Poitry-Yamate et al.,1995) and that lactate was extensively oxidized to CO_2 in the chick sympathetic ganglia (Larrabee 1996). Further evidence that lactate is a prominent energy substrate in neurons has been provided ultimately by Zielke et al., showing that lactate was readily oxidized by brain cells in vivo including neurons (Zielke et al., 2007).

Another confirmation that lactate was efficiently metabolized by neurons, was made with Nuclear Magnetic Resonance Spectroscopy (NMR). Thanks to its application to biology, some studies on cultured glutamatergic and GABAergic neurons have shown that lactate was metabolized through the TCA cycle in both, leading to labeling of TCA cycle intermediates as well as several amino acids derived from these (Schousboe et al.,1997; Waagepetersen et al., 2000). From these studies, lactate can be considered equivalent to glucose considering its accessibility to the TCA cycle (Waagepetersen et al., 1998a). Note that lactate could serve as a precursor for the synthesis of the neurotransmitters GABA, glutamate and aspartate (Waagepetersen et al., 1998b). In a recent report, Boumezbeur et al. (2010) demonstrated using intravenous infusions of ^{13}C-lactate, combined with in vivo ^{13}C magnetic resonance spectroscopy (MRS), that neurons metabolized 80% of the radiolabeled lactate, i.e., approximately four times more than glial cells in human brain (Boumezbeur et al., 2010; Figley 2011). Furthermore, in presence of both glucose and lactate, as it is the case physiologically, it was shown that lactate is largely preferred as an oxidative energy substrate over glucose by neurons (Itoh et al., 2003; Bouzier-Sore et al., 2003; 2006). Taken together, evidences suggest that lactate can be used by neurons as another efficient energetic substrate in addition to glucose.

B) Functional evidence for the role of lactate as supplementary substrate for neurons

The pioneering work conducted by McIlwain revealed that brain tissue is able to efficiently utilize monocarboxylates (such as lactate) as alternative energy substrates. Indeed, it was demonstrated that lactate could sustain electrical activity of the nervous tissue (McIlwain 1953) and could maintain synaptic activity in hippocampal slices (Schurr et al., 1988). Several studies demonstrated that lactate was not only neuroprotective and preserved morphological integrity of tissue upon glucose deprivation but was also able to maintain synaptic function and allowed the induction of long-term potentiation (Fowler 1993; Izumi et al., 1994; 1997a; 1997b; Schurr et al.,1999a; Cater et al.,2001). Other studies showed that lactate, not glucose, was a necessary substrate for neurons following recovery from an hypoxic episode in hippocampal slices (Schurr et al., 1997a,b,c), supporting its neuroprotective effect.

However, these results must be moderated as some other studies had previously found that evoked granule cell field potentials were only partially preserved when glucose was replaced by lactate (Cox and Bachelard 1988). In contrast to the results of Schurr (Schurr et al.,1999a), it was reported that lactate was unable to sustain NMDA activation and maintain K^+ homeostasis in absence of glucose (Chih et al.,2001). In addition, long-term potentiation as evidenced by the enhancement of population spike was reduced following glucose replacement by lactate. More recent results showing that neuronal activity was diminished in presence of lactate were obtained by the analysis of ^{14}C-labelled energy substrate oxidation using young adult organotypic slice cultures (Gilber and Bergold, 2005). In contrast, a recent study in neonatal hippocampal slices shows that lactate effectively covers energy demands during neuronal network activity (Ivanov et al., 2011). But quite importantly, this last study points out to a possible caveat in previous studies on hippocampal slices that were not able to show the capacity of lactate to sustain neuonal activity. In fact, slices oxygenation seems to play a critical role in the capacity of lactate to sustain neuronal network activity. Thus, despite some divergent results that might find a technical explanation, it seems that most studies on hippocampal slices have highlighted the fact that lactate not only allows to maintain energy status of the tissue as well as insure survival of brain cells, but it can also sustain neuronal activity.

In addition to studies on hippocampal slices mentioned before, several studies suggest that lactate could support neuronal functions. It is the case in the rat optic nerve where it was shown that the evoked compound action potential can be fully supported when replacing glucose by lactate (Brown et al., 2001). More recently, Bliss and colleagues demonstrated that overexpression of MCT2 in hippocampal neuron cultures increased lactate utilization following glutamate treatment and significantly decreased the neurotoxicity of glutamate (Bliss et al., 2004). In cultured cortical neurons, lactate was shown to be equivalent to glucose for sustaining synaptic vesicle turnover during activity (Morgenthaler et al., 2006). Moreover, a study of Aubert et al., based on a mathematical model of brain lactate kinetics, showed that the kinetic properties of monocarboxylate transporter isoforms strongly suggest that neurons represent the most likely compartment for activation-induced lactate uptake. They also demonstrated that neuronal lactate utilization occurring early after activation onset is responsible for the initial dip in brain lactate levels observed in both animals and humans (Aubert et al., 2005). Using measurable levels and kinetic properties of GLUT (Glucose transporter) of capillary endothelial cells, astrocytes, and neurons, along with corresponding

kinetic properties of the MCTs, Simpson and colleagues have successfully modeled brain glucose and lactate levels as well as lactate transients in response to neuronal stimulation (Reviewed by Simpson et al., 2007). However, their conclusions were apparently at odds with the idea of lactate transfer from astrocytes to neurons. In contrast, more recent studies using distinct mathematical approaches and based on *in vitro* and *in vivo* data strongly support the idea of a neuron-glia compartmentalization in energy metabolism (Aubert and Costalat 2007, Aubert et al., 2007, Jolivet et al., 2009, 2010). Authors have shown that a significant part of glucose is taken up by astrocytes while oxygen is mostly consumed within the neuronal population. They found that this bias tends to increase with the level of activation (Jolivet et al., 2009). These results rather support the view of an activity-related transfer of glucose-derived metabolites from astrocytes to neurons.

C) The astrocyte - neuron lactate shuttle

Walz and Mukerji were the first to demonstrate the capacity of astrocytes to produce large amounts of lactate (Walz and Mukerji 1988a,b). Few years later, some reports independently proposed that astrocytes might export lactate as energy substrate for neurons. Two of them suggested that lactate, as a consequence of glycogen breakdown, would be transferred to neurons (Dringen et al., 1993a,b; Magistretti et al.,1993), while a third one simply proposed an exchange between the two cell types based upon their difference in lactate formation vs oxidation (McKenna et al.,1993). Indeed, it was later shown that astrocytic glycogen provide a source of lactate to support axonal function during intense stimulation (Shulman et al., 2001; Brown et al., 2003; 2004; 2005) and during glucose deprivation (Wender et al.,2000).

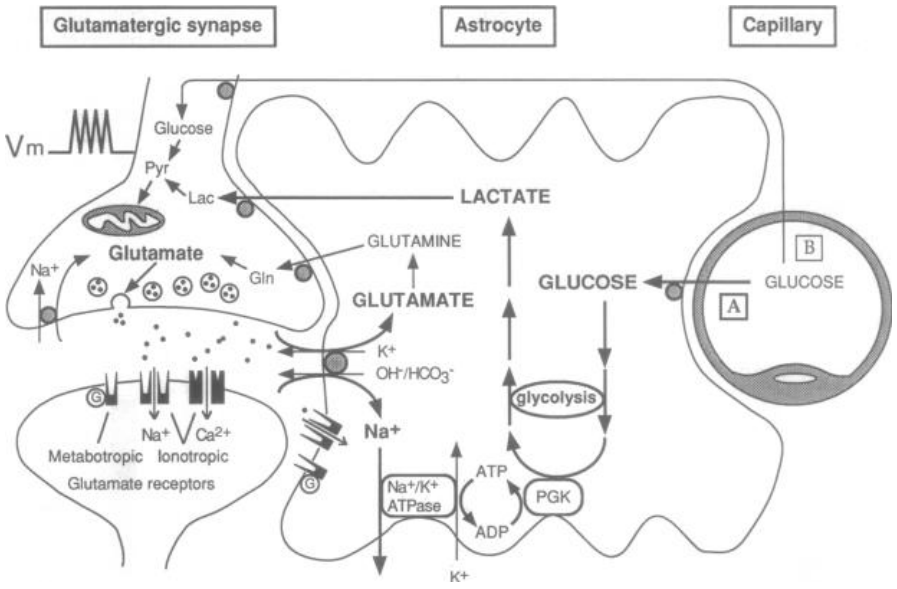

Figure 1.6. Schematic of the mechanism for glutamate-induced glycolysis in astrocytes during physiological activation (taken from Pellerin and Magistretti 1994).

Based on a series of in vitro experiments, Pellerin and Magistretti proposed an original model to explain the coupling between synaptic activity and glucose utilization (Pellerin and Magistretti 1994) (Figure1.6). They challenged the conventional scheme of brain energetics which states that neuronal energy metabolism is fuelled by glucose oxidation and introduced the astrocyte-neuron lactate shuttle hypothesis (ANLSH) (Figure1.6) (Pellerin and Magistretti 1994; 2003a; Magistretti et al., 1999; Bouzier-Sore et al., 2002; Pellerin 2003, 2007, 2008).

In this model, glutamate that is released as a neurotransmitter by neurons is primarily taken up into astrocytes via specific sodium-dependent transporters: glutamate aspartate transporter (GLAST) and glutamate transporter 1 (GLT1) (Danbolt et al., 1998; Gegelashvili and Schousboe 1998). These transporters carry one glutamate, three Na^+, and one H^+ inward while one K^+ is moved out of the cell. Thus, the simultaneous entry of glutamate and sodium increases the intracellular concentration of sodium as measured by cellular imaging (Chatton et al., 2000). As a consequence of raised intracellular Na^+ concentration, Na^+/K^+ ATPase activity will increase to restore ionic balance, while glutamine synthesis from glutamate will take place at the expense of two ATPs per glutamate, leading to enhanced glucose utilization and lactate production (Pellerin and Magistretti 1997; Pellerin et al., 2007; Pellerin and Magistretti 1994). Interestingly, it was shown that a stimulation of the glutamate transporter

GLAST in cultured astrocytes causes a translocation of the Na^+/K^+ ATPase gamma subunit to the plasma membrane that modulates the activity of the Na^+/K^+ ATPase (Gegelashvili et al., 2007; Rose et al., 2009). It was also shown that the α2 subunit of Na^+/K^+ ATPase is responsible for enhanced glycolysis induced by glutamate via its uptake by specific transporters (Pellerin and Magistretti 1997). Further observations at the electron microscopic levels have indicated that the subunit of the α2 Na^+/K^+ ATPase colocalized with glial glutamate transporters in thin astrocytic processes surrounding glutamatergic synapses (Cholet et al., 2002). Some studies showed a decreased metabolic response to synaptic activation (i.e. enhancement of glucose uptake) in both GLAST and GLT1 deficient mice compared to wildtype mice (Voutsinos-Porche et al., 2003, Cholet et al., 2001; Herard et al., 2005). The implication of glial glutamate transporters in this metabolic response was further demonstrated *in vivo* by monitoring lactate production using microdialysis. Indeed, lactate production was measured during specific behavioural task and was prevented by use of glutamate transporter inhibitors (Fray et al., 1996; Demestre et al., 1997, Uehara et al., 2007). Taken together, these data show that glutamate stimulates aerobic glycolysis in astrocyte, i.e. glucose transport and glucose utilization and lactate production in presence of oxygen. This observation was also confirmed by others (Takahashi et al., 1995; Keller et al., 1996; Hamai et al., 1999; Loaiza et al., 2003; Porras et al., 2004, 2008; Bittner et al., 2010).

In the ANLSH, much of the fuel for increased energy demands of neurons is supplied by lactate from surrounding astrocytes. As a result, metabolism of astrocytes is largely glycolytic while that of neurons is largely oxidative. This concept is supported by numerous studies. First, a transcriptome analysis carried out on both astrocytes and neurons isolated from the brain of adult animals confirmed the fact that astrocytes have a prominent expression of glycolytic enzymes as compared with neurons (Lovatt et al., 2007; Rossner et al., 2006; Cahoy et al., 2008). In addition, a differential expression of the two lactate dehydrogenase (LDH) isoforms was found between astrocytes and neurons (Bittar et al., 1996; Laughton et al., 2007; Pellerin et al., 2005; O'Brien et al., 2007). Moreover, glycolytic activity is more important in astrocytes than in neurons (Itoh et al., 2003; Bouzier-Sore et al., 2006) even if neurons do exhibit glycolytic activity (Bittner et al., 2010).

The idea of a metabolic compartmentalization between astrocyte and neurons was further supported by an important finding from Ramos and colleagues. They demonstrated that astrocytes lack an important component in mitochondria for the recycling of NADH to NAD

(Ramos et al., 2003; Xu et al., 2007). In order to maintain the glycolytic rate, the cofactor nicotinamide adenine dinucleotide (NAD) must be regenerated. This could be done in mitochondria, involving the malate / aspartate shuttle. In astrocyte mitochondria, it was recently found that the activity of this shuttle was low, showing a weak activity of aspartate / glutamate transporter (Berkich et al., 2007). In astrocytes, the regeneration of NAD and maintenance of high glycolytic activity can thus be obtained predominantly by conversion of pyruvate into lactate by the LDH.

By the use of a method combining immunocytochemistry with high-resolution microautoradiography, Nehlig and colleagues established *in vivo* that half of all glucose utilization occurs in astrocytes under resting conditions (Nehlig et al., 2004). Furthermore, using a different technique in cerebellar slices, it was recently shown a more important glucose utilization in Bergmann glial cells than in neighboring neurons (Barros et al., 2009). Finally, a critical demonstration of the preferential glucose utilization by astrocytes following synaptic activation was recently provided *in vivo* (Chuquet et al., 2010). It was shown that whisker stimulation leads to enhanced glucose utilization in astrocytes of the somatosensory cortex as revealed by accumulation of the fluorescent deoxyglucose analog 6-NBDG with relatively little changes in neighboring neurons (Chuquet et al., 2010). These observations complement the previous demonstration that astrocytes have a prominent glycolytic activity while neurons are more oxidative.

The ANLSH has also been proposed for other support cell-neuron energetic interactions such as those between Schwann cells and peripheral neurons (Vega et al., 1998), as well as in the retina between Müller glial cells and photoreceptors (Poitry-Yamate et al., 1995; Poitry et al., 2000). Xu and colleagues showed even an absence of the aspartate / glutamate carrier in the retina Müller cells, supporting the idea of a compartmentalized metabolism between Müller cells and photoreceptors (Xu et al., 2007). Thus, as astrocytes exhibit the same type of metabolic characteristics as Müller cells, it reinforces the view that a process such as the ANLS probably exists in various brain regions.

In conclusion, many studies confirmed that monocarboxylates such as lactate can at least partially substitute for glucose. However, these energy substrates do not cross easily the BBB as well as other cell membranes, and thus require a transport system. Indeed, it has been

shown that monocarboxylates are transported by a family of proton-linked transporters called monocarboxylate transporters (MCTs).

1.3 The monocarboxylate transporter (MCTs) family: identification, functional characteristics and distribution

Lactate plays an important role in metabolism, not only in cerebral metabolism but also in most cells. It is produced by cells which have an important glycolytic metabolism, generating two molecules of lactate for one molecule of glucose consumed. Lactate must be exported to maintain the glycolytic rate and an adequate cytosolic pH. Other cells use lactate as an oxidative or a gluconeogenic (liver) substrate. The transport of L-lactate across plasma membranes is of considerable importance to almost all mammalian cells. In most cells, a specific H^+-monocarboxylate cotransporter is largely responsible for this process. In order to support the high rate of production or utilization of L-lactate, the capacity of this carrier must be very high. Evidence for a family of monocarboxylate transporters came initially from kinetic studies of lactate transport in various tissues (Poole and Halestrap 1993) and has been confirmed by the cloning and expression of the first two MCT isoforms, namely, MCT1 and MCT2. L-lactate is the monocarboxylate which has the highest transport rate across membranes, but MCTs are also essential for the transport of pyruvate, as well as ketone bodies such as acetoacetate and beta-hydroxybutyrate. Monocarboxylate transporters have been studied in a variety of cell preparations, e.g. erythrocytes, hepatocytes, tumour cells, cardiac myocytes, skeletal muscle cells, as well as in brain astrocytes and neurons.

Up to now, the monocarboxylate transporter family (part of the solute carrier SLC16 gene family) is composed of 14 members based on sequence homologies, identified as MCT1-9, MCT11-14 and T-Type amino-acid transporter-1 (TAT1) (Halestrap and Meredith 2004). Other proteins called sMCT1 (SLC5A8) and sMCT2 have been identified as sodium-coupled MCT transporter (Ganapathy et al., 2005; Martin et al., 2006; Scrinivas et al., 2005). Among the SLC16 family of monocarboxylate transporters, only four MCTs (MCT1-4) have been functionally characterized. Each isoform possesses unique biochemical properties such as kinetic constants and sensitivity to known MCT inhibitors. In addition, MCT expression is tissue-specific (Reviewed by Pierre and Pellerin, 2005) (table 1.1; Figure1.7; Figure 1.8)

A)

B)

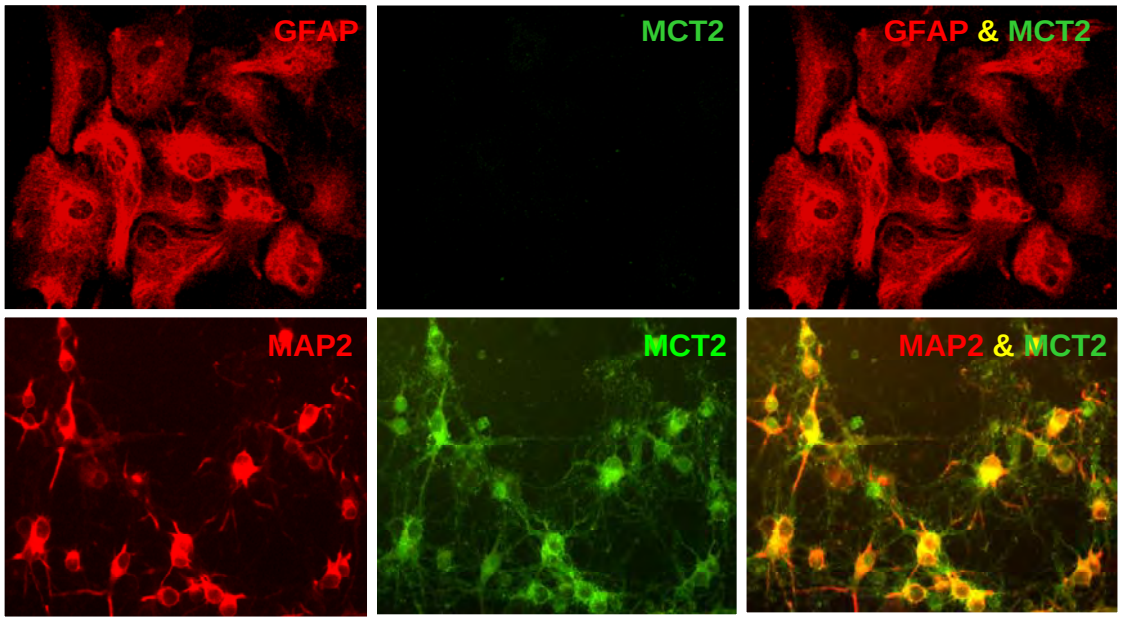

Figure 1.7. MCT2 expression in cultured astrocytes and neurons. A) Double immunolabelling of primary cultures of mouse cortical astrocytes after 14 days in vitro with antibodies directed against MCT2 (green) and GFAP (red). B) Double immunolabelling of primary cultures of mouse cortical neurons after 7 days in vitro with antibodies against MCT2 (green) and microtubule-associated protein 2 (MAP2) (red). (Picture from Pierre K and Pellerin L, unpublished)

One difficulty in the characterization of monocarboxylate transport in cultured cells is the simultaneous expression of multiple isoforms with very similar substrate specificity. Moreover, several changes in MCT expression may be evoked by modifying physiological conditions. The molecular mechanisms underlying the regulation of MCTs are poorly understood and remain to be clarified. In the past few years, our lab has studied monocarboxylate transporter expression in the brain, notably for MCT1, MCT2 and MCT4 (see point 1.3.2).

MCT isoform	Regional distribution	Cellular localization	
		In vivo	*In vitro*
MCT1	Widespread. Neuropil labelling with a few entirely labelled cells	Endothelial cells forming blood vessels, ependymocytes, astrocytes. Some neurons in rat	Astrocytes, weak in a few neurons
MCT2	Widespread but strong expression in cortex, hippocampus, cerebellum. Neuropil labelling and negative somata except Purkinje cells	Neurons, some astrocytes in rat (white matter)	Neurons. Astrocytes also from rat
MCT4	Widespread but strong cellular expression in cortex, hippocampus, striatum, cerebellum	Astrocytes	Not determined

Table 1.1 Regional distribution and cellular localization of monocarboxylate transporters (MCTs) in the adult rodent central nervous system (Table taken from Pierre and Pellerin 2005)

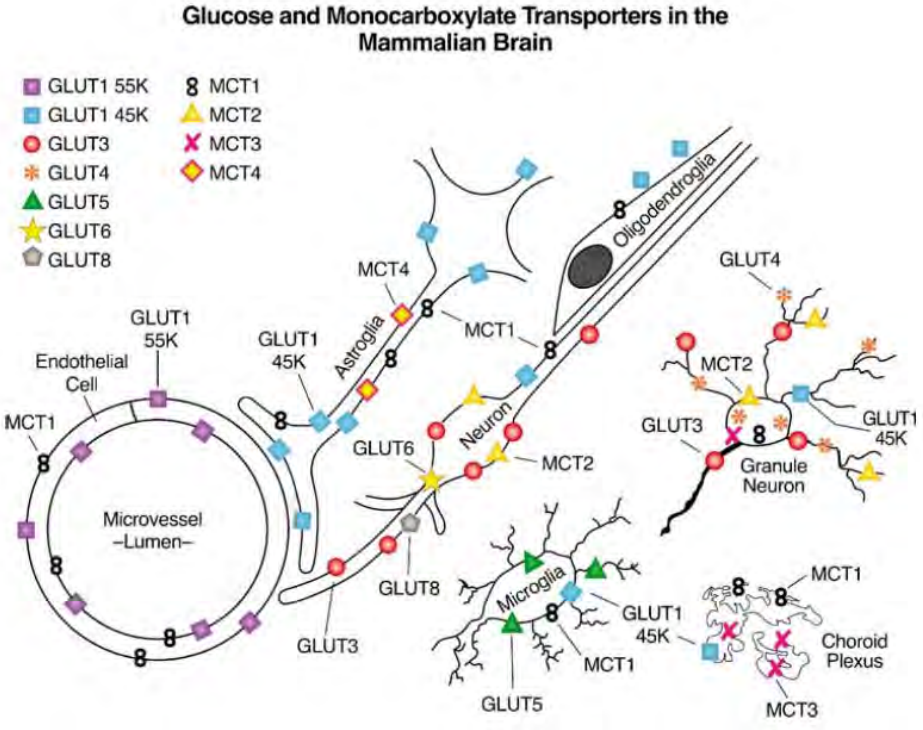

Figure 1.8 A schematic representation of the cellular localization of glucose transporter (GLUTs) and monocarboxylate (Taken from Simpson et al., 2007)

1.3.1 Structure and functional characteristics of MCTs

The secondary structure of MCTs was determined based on hydrophobicity by standard algorithms, predicting the number of transmembrane segments (TM). By this technique, it was found that MCT1, MCT2, MCT3, MCT7 and MCT8 possess 12 TM while between 10 and 12 were predicted for the other MCTs. Thus, it seems probable that there are 12 TM domains with the N- and C- termini located within the cytoplasm for most MCTs, as illustrated in Figure 1.9 for MCT1. A study using proteolytic digestion of the MCT1 protein led to a topological model of the transporter with these 12 segments separated between TM 6 and TM 7 by a large hydrophilic loop and an extended hydrophilic C-terminal tail (Figure 1.9). The 12-TM helix topology is shared by many other plasma-membrane transporters such as the GLUT family (Baldwin 1993; Gould and Holman 1993). It has been proposed that the N-terminal domain is important for energy coupling, membrane insertion and correct structure maintenance, whereas the C-terminal domain may be more important for the determination of substrate specificity.

For expression at the membrane, MCTs require ancillary proteins. CD147 (also known as basigin), a widely distributed cell surface glycoprotein, enables the proper expression and function of MCT1 and MCT4 at the cell surface. Without CD147, proteins accumulate in the endoplasmic reticulum or the Golgi apparatus (Kirk et al.,2000). Similarly, gp70 (also known as embigin) is required for the translocation and catalytic activity of MCT2 (Wilson et al., 2005)

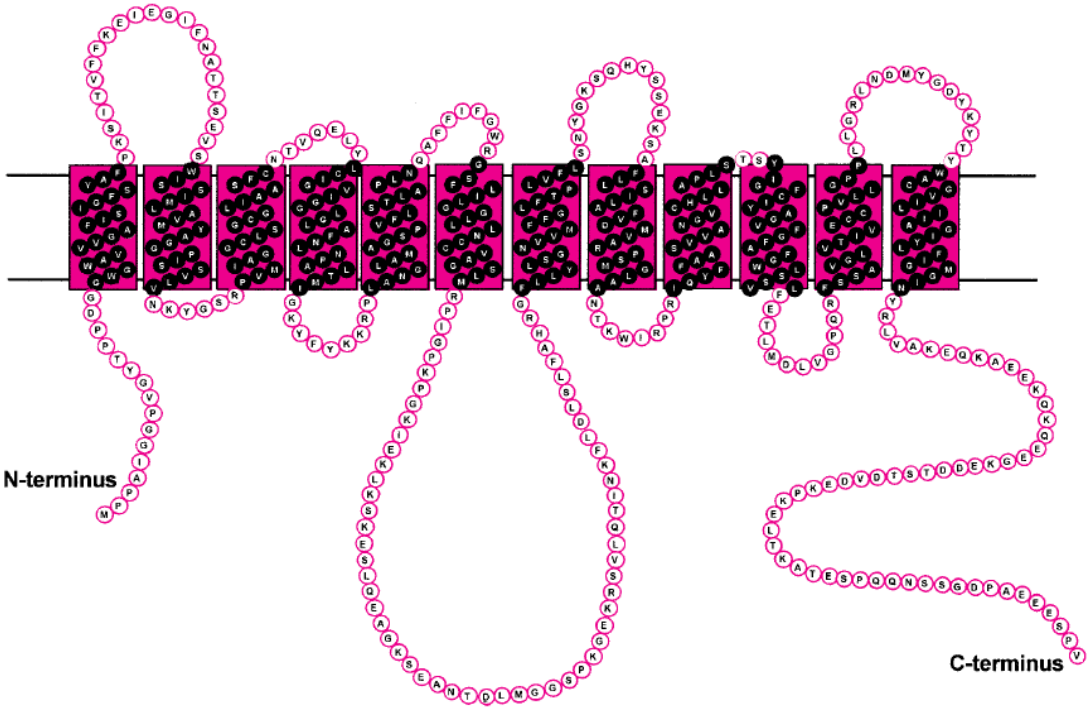

Figure1.9. Proposed membrane topology of the MCT family. The sequence shown is that of human MCT1. (Figure taken and modified from Halestrap and Price 1999)

1.3.2 Distribution of the MCTs in the periphery and in the CNS

The existence of several different MCT isoforms must bear a physiological significance that is related either to their unique properties or to their regulation. In either case, their tissue distribution should give some clues as to their role. The mRNA distribution of different MCT isoforms was achieved by Northern-blot analysis. But it was also important to directly detect MCT proteins. Several techniques such as Immunocytochemistry, immunogold electron microscopy and Western-blotting allowed to detect MCT1-4 protein expression. As mentioned before, each MCT has been shown to be expressed in specific tissues depending on the species studied (Table 1.1).

In the periphery, MCT1, in humans and rodents is present in almost all tissues including muscle, kidney, heart, liver, and brain, in many cases with specific locations within each tissue (Fishbein et al., 2002; Bonen et al., 2006). In contrast, it was originally thought that MCT2 expression was more limited, with high level of expression in tissues such as testis, liver, stomach and kidney, and lower levels in lung and brain. It was also considered that MCT2 distribution was much more species specific than MCT1 and important differences

in tissue distribution was observed between rat, mouse and hamster (Jackson et al., 1997; Merezhinskaya and Fishbein 2009). In mouse tissues, MCT2 was expressed both in kidney and in liver (Pierre and Pellerin 2005). MCT3 is found exclusively in the retinal pigment epithelium and choroid plexus epithelia in both chicken and rat (Yoon et al.,1997; Philp et al.,1998). MCT4 is expressed particularly strongly in skeletal muscle, and is also the major MCT isoform in white blood cells and some mammalian cell lines. In addition, MCT4 is expressed in cancer-associated fibroblasts, and its expression is induced by oxidative stress (Whitaker-Menezes et al., 2011). This has led to propose that it may be of particular importance in tissues that rely on high levels of glycolysis to meet their energy needs, and hence require rapid lactic acid efflux (Wilson et al., 1998). In addition, MCT4 is expressed on various mammalian cell lines (COS, ETL, and NBL-1 cells), in the rat neonatal heart and in the placenta (Halestrap and Meredith 2004).

In the central nervous system, the MCTs that are expressed in the brain are: MCT1, MCT2 and MCT4. They exhibit a cell-specific distribution throughout the brain.

a) MCT1

MCT1 is expressed in the whole rodent brain, both at the mRNA and protein levels. In situ hybridization studies demonstrated that MCT1 mRNA is abundant in the hippocampus, in the cortex and in the cerebellum of young (15 days-old) and adult rodent brain (Pellerin et al., 1998a). It was shown that during the suckling period, the endothelial cells forming capillaries expressed MCT1 mRNA and expression decreased at the adult stage. The same observations were obtained in rat brain. Indeed, the MCT1 mRNA was strongly expressed from postnatal day 7 to 21 and was gone at day 27 (Vannucci and Simpson 2003). MCT1 protein is highly expressed in endothelial cells forming microvessels and ependymocytes lining the four brain ventricles in both young and adult rodent brain (Gerhart et al., 1997, 1998; Hanu et al., 2000; Pierre et al., 2000; Baud et al., 2003). Experiments using light and electron microscopy revealed the presence of MCT1-positive glial end-feet surrounding capillaries and of MCT1 on glial-like processes (Gerhart et al., 1997; Leino et al., 1999). Confocal microscopy using double labellings with glial markers such as glial fibrillary acidic protein (GFAP) or S100β confirmed the association *in vitro* and *in vivo* between astrocytic processes and MCT1 immunoreactivity (Hanu et al., 2000; Pierre et al., 2000; Ainscow et al., 2002; Debernardi et al., 2003). In cultured astrocytes, MCT1 protein expression was observed in both soma and processes of astrocytes (Broer et al., 1997; Hanu et al., 2000; Debernardi et al., 2003).

Experiments based on chronic administration of nicotine in rat brain have shown an overexpression of MCT1 within endothelial cells and astrocytes of treated animals (Canis et al., 2009a).

A low MCT1 expression level was found in some cultured neurons (Debernardi et al., 2003). In addition, MCT1 expression was enhanced by 6 weeks of high fat diet in mouse brain neurons (Pierre et al., 2007) and after chronic hyperglycemia in rat brain (Canis et al., 2009b). Some subpopulations of neurons *in vivo* expressed MCT1 protein; it is the case for CA1 pyramidal neurons of the hippocampus (Tseng et al., 2003) and for specific neuronal populations in the hypothalamus (Ainscow et al., 2002). MCT1 was even found in the cerebral cortex (Leino et al., 1999). In addition it was shown that neurons express transiently MCT1 during specific embryonic stages both in rat and in human (Baud et al., 2003; Fayol et al., 2004).

Other cell types in which MCT1 protein were detected are microglial cells and oligodendrocytes. Indeed, in a rat ischemia model, MCT1 expression was increased in microglial cells after 48h of reperfusion (Moreira et al., 2009). Moreover, in cultured slices of developing cerebral cortex from mice MCT1 is expressed in both oligodendrocytes and myelin (Rinholm et al., 2011). Authors have demonstrated that L-lactate can support oligodendrocyte development and myelination through MCT1.

b) MCT4

In the rodent brain, MCT4 was shown to be expressed exclusively in astrocytes. Immunogold electron microscopy revealed that MCT4 is localized in the membrane of astrocytes in adult cerebellum (Bergersen et al., 2001, 2002). The same localization was observed in the developing rat brain. Indeed, MCT4 expression is very low at birth and is enhanced at P14 in the hippocampus, in the cerebellum and in the spinal cord (Rafiki et al., 2003). Finally, in the adult rat brain, MCT4 protein expression is intense in various regions of the brain, such as the cerebral cortex, the hippocampus, the paraventricular nucleus in the hypothalamus, and the capsula internalis (Pellerin et al., 2005). Very few data on its regulation are available. It was described that mice fed with a high fat diet during 6 weeks display an increased expression of MCT4 in neurons (Pierre et al., 2007). In cultures of cortical astrocytes, MCT4 is expressed at very low levels, but a recent study shows that MCT4 mRNA and MCT4 protein were induced

in astrocytes by NO donors (Marcillac et al., personal communication). However, up to now, specific mechanisms of regulation as well as specific modulators of MCT4 expression remain to be identified.

c) MCT2

In 1995, Garcia et al., screened a cDNA library from hamster liver and found a cDNA encoding MCT2, a monocarboxylate transporter 60% identical to hamster MCT1. The functional properties of the two MCTs were compared by expression in Sf9 insect cells using recombinant baculovirus vectors (Garcia et al., 1995). MCT2 is predicted to contain 484 amino acids plus 5'- and 3'-UTRs (of 118 and 496 base pairs respectively) and has a molecular weight of 52,839 Da. However, on blotted polyacrylamide gel, MCT2 has an apparent molecular weight of about 43,000 Da. MCT2 has now been cloned and sequenced from human, rat and mouse.

MCT2 catalyses the proton-linked transport of a variety of monocarboxylates as shown by expression of rat MCT2 in *Xenopus laevis oocytes*, but with a considerably higher affinity than other MCTs. The K_m value for L-lactate is about 0.7mM and the V_{max} value of MCT2 is as much as 20 times lower than MCT1 (Broer et al., 1999) (Table 1.2). It was also demonstrated by expression in *Xenopus laevis oocytes* that the human MCT2 is a high affinity pyruvate transporter with an apparent K_m value of 25µM. Thus, MCT2 has been considered a primary pyruvate transporter in humans (Lin et al., 1998). Functional studies were also performed in Sf9 insect cells in which the hamster MCT2 was expressed. The K_m values found were 0.8mM and 8.7mM for pyruvate and lactate respectively (Garcia et al., 1995). MCT2 can be inhibited by CHC (alpha-cyano-4-hydroxycinnamate), anion-channel inhibitors and flavonoids (Broer et al., 1999).

Substrate	K_m or K_i (mM) MCTI	MCT2
Lactate	3.5	0.74 ± 0.07
DL-β-Hydroxybutyrate	12.5	1.2 ± 0.2*
Pyruvate	1.0	0.08 ± 0.01*
2-Oxoisovalerate	1.25	0.30 ± 0.02*
2-Oxoisohexanoate	0.67	0.10 ± 0.01*
Acetoacetate	5.5	0.8 ± 0.1*

* K_i values were determined by assuming competitive inhibition.

Table 1.2. Table 1 Comparison of kinetic constants of MCT1 and MCT2 (taken from Broer et al., 1999)

As previously mentioned, in the periphery MCT2 is only expressed in kidney and in liver (Pierre et al., 2005). More specifically, MCT2 is localized to basolateral membranes of epithelial cells lining the nephron (Becker HM et al., 2010). Moreover MCT2 was also found in the small intestine, in the epithelium of gastrointestinal tract reindeer (Koho N et al., 2005). In the central nervous system MCT2 is widely expressed and is considered as the predominant neuronal monocaroboxylate transporter. Considering its putative importance for neuronal energetics, we will devote the next section to describe in details what is known about MCT2 and the central nervous system.

1.3.3 The predominant neuronal monocarboxylate transporter MCT2

a) Distribution of MCT2

MCT2 is considered as the major neuronal monocarboxylate transporter. MCT2 mRNA is abundant in the cortex, the hippocampus and in the cerebellum of mouse brain as shown by northern blot and *in situ* hybridization studies (Broer et al., 1997; Koehler-Stec et al., 1998; Pellerin et al., 1998a; Debernardi et al., 2003; Vannucci and Simpson 2003). At the cellular level, the MCT2 mRNA had a predominantly neuronal pattern of expression in young as well as in adult rodent brain (Pellerin et al., 1998a; Vanucci and Simpson 2003). Northern blot analysis on primary cultures of mouse cortical astrocytes and neurons indicated a prominent expression of MCT2 mRNA in neuronal cultures (Broer et al., 1997; Debernardi et al., 2003). The demonstration that MCT2 is the major neuronal monocarboxylate transporter was finally

obtained by immunohistochemistry in adult mouse brain (Pierre et al., 2002). Expression of the MCT2 protein follows the distribution pattern of its mRNA, as it is strongly expressed in the cortex, the hippocampus and the cerebellum of rodent brain (Bergersen et al., 2001; Bergersen et al., 2002; Pierre et al., 2002; Rafiki et al., 2003). MCT2 expression was found to be particularly abundant in the neuropil as well as on several fiber tracts. It was usually absent from neuronal cell bodies with the exception of Purkinje cells in the cerebellum. Cortical neurons in culture exhibited MCT2 immunoreactivity at the surface and in the cytoplasm of their cell bodies and neurites (Debernardi et al., 2003; figure 1.7). MCT2 immunoreactivity *in vivo* revealed that MCT2 labeling occurred predominantly in the neuropil of most structures including the neocortex, caudate-putamen, and hippocampus (Pierre et al., 2002). Authors have shown that MCT2-positive elements in the neuropil were associated with neuronal cell processes. Thus, MCT2 appears to be present on many axonal projections, supporting a putative role in lactate transfer into axons to fuel action potential propagation (Brown et al., 2003). It was particularly visible on sensory fibers in the brainstem as well as on facial and trigeminal nerves (Pierre et al., 2002). Except in some astrocytes of the white matter, MCT2 protein expression was never detected in glial-like cells of the mouse brain parenchyma, whereas it could be visible in some endothelial cells forming brain capillaries (Pierre et al., 2000; Pierre et al., 2002). In contrast, in the rat brain, MCT2 was present in astrocytes both in vitro (Hanu et al., 2000) and in vivo (Gerhart et al., 1998).

Observations that MCT2 is expressed on membrane of dendrites *in vitro* and *in vivo* suggested that part of MCT2 expression is associated with synapses. Thus, double immunofluorescent labellings with the presynaptic marker synaptophysin and MCT2 revealed a parallel distribution of these proteins in developing cultured neurons (Debernardi et al., 2003). A close apposition without colocalization between MCT2 and presynaptic elements was observed, suggesting a postsynaptic localization for MCT2. Similarly, in the mouse brain, double immunostaining for MCT2 and PSD95 (postsynaptic density protein) indicated a partial colocalization (Pierre et al., 2002). By electron microscopy, Bergersen et al., showed that MCT2 was associated with postsynaptic δ2-glutamate receptors in the cerebellum (Bergersen et al., 2001, 2002). Several years later, using immunogold staining, Bergersen and colleagues demonstrated that MCT2 and AMPA receptor GluR2/3 subunits have a similar postsynaptic distribution at glutamatergic synapses with high levels expressed within the postsynaptic density (Bergersen et al., 2005; Pierre et al., 2009). The MCT2 protein is not only present in dendrites but it is also specifically associated with spines, where it was found to be expressed

in the postsynaptic density as well as on vesicle-like structures forming an intracellular pool (Bergersen et al, 2005). MCT2 was recently found to interact with a specific subset of postsynaptic proteins (Pierre et al, 2009). This is particularly the case with GluR2, a subunit of the glutamatergic AMPA receptor subtype. It was shown that MCT2 seems not only to determine the subcellular localization of GluR2 in neural cells but also to regulate its expression levels (Maekawa et al, 2009). In addition, it was observed that MCT2 together with GluR2 undergoes a trafficking process between the plasma membrane and an intracellular pool under conditions inducing synaptic plasticity (Pierre et al, 2009).

b) Regulation of MCT2 expression in the brain

The brain is capable of adjusting its supply of monocarboxylates to meet specific energy requirements, under physiological (Cremer 1982; Dombrowski et al., 1989; Vicario et al., 1991) as well as pathological conditions (Hawkins et al., 1986; Schurr et al., 1999b, 2001a). For example, during the early postnatal development, monocarboxylate uptake increases during this period (Cremer et al., 1976; Cornford et al., 1982; Edmond et al., 1985; Cornford and Cornford 1986; Nehlig et al., 1991; Nehlig and Pereira de Vasconcelos 1993). Furthermore, it was shown that during development, a peak in the expression of both MCT2 and MCT1 mRNA occurs during the suckling period around postnatal day 15, followed by a rapid decline after weaning to reach at postnatal day 30 levels observed in adults (Pellerin et al., 1998a). MCT2 expression was shown to be also induced by 6 weeks of high fat diet in mouse brain neurons as MCT1 and MCT4 (Pierre et al., 2007).

MCT2 was shown to be expressed by mouse cultured cortical neurons. It was found that the level of MCT2 expression correlated with increased expression of synaptophysin, a presynaptic protein along days *in vitro*, suggesting a relationship between synaptic development and energy substrate requirements (Debernardi et al., 2003). Recent observations have shown that MCT2 expression can be regulated in primary neuronal cultures. Indeed, it was found that noradrenaline, insulin, and insulin-like growth factor-1 (IGF-1) increase MCT2 protein expression in neurons through a translational mechanism (Pierre et al., 2003; Chenal and Pellerin, 2007; Chenal et al, 2008). Interestingly, each of these neuroactive substances is known to induce long-term changes in synaptic transmission (Kobayashi and Yasoshima, 2001; Trejo et al, 2007; van der Heide et al, 2006). Thus, evidences suggest that changes of MCT2 expression could participate in the process of synaptic plasticity induced by

noradrenaline, insulin, or IGF-1. Up to now, no direct demonstration was provided of a putative link between the regulation of MCT2 and synaptic plasticity processes.

1.4 Metabolic plasticity in the brain

1.4.1 BDNF: synaptic plasticity and learning/memory

Brain derived neurotrophic factor is a neurotrophin whose status as a regulator of survival, growth, and differentiation of neurons during development (Barde et al., 1994; Wang et al., 1995) has matured to include the adult nervous system. Indeed, it is now known that BDNF functions to translate activity into synaptic and cognitive plasticity in the adult animal. BDNF is able to modulate the efficacy of neurotransmitter release (Kang and Schuman, 1995; Bolton et al., 2000), stimulate the synthesis of vesicle-associated proteins (Lu and Chow, 1999; Schinder and Poo, 2000), and regulate transcription factors (Finkbeiner et al., 1997; Tully, 1997). In the hippocampus, BDNF is capable of inducing a rapid potentiation of glutamate-mediated synaptic transmission (Lessmann and Heumann, 1998) and a long-lasting potentiation of perforant path-dentate gyrus connections *in vivo* (Messaoudi et al., 1998). Learning and memory (Falkenberg et al., 1992) and long-term potentiation (LTP), considered an electrophysiological correlate of learning and memory (Patterson et al., 1992, 2001), selectively increase BDNF mRNA levels in the hippocampus. Transgenic animals with diminished BDNF expression lose their ability to induce LTP (Patterson et al., 1996) and are impaired in learning a spatial memory task (Linnarsson et al., 1997). Quenching endogenous BDNF with function-blocking anti-BDNF antibodies has been shown to impair learning and memory in rats in both the water maze and in an inhibitory avoidance task (Ma et al., 1998; Mu et al., 1999). Similarly, blocking endogenous BDNF was able to significantly reduce LTP (Ma et al., 1998). Moreover, replenishing the depleted hippocampus with exogenous BDNF seems to ameliorate these deficits. Exogenous BDNF application (Patterson et al., 1996) or transfection of hippocampal slices with a BDNF expressing adenovirus (Korte et al., 1995) has been shown to restore the ability to induce LTP. BDNF, but not NGF or NT-3, seems to play a role in consolidating short-term memories into long-term memories (Johnston and Rose, 2001). Clinical studies support the importance of BDNF in learning and memory in humans (Egan et al., 2003; Hariri et al., 2003). A study conducted by Egan et al. (2003) has found that individuals expressing a specific polymorphism in the BDNF gene exhibit learning impairments.

1.4.2 BDNF and its intracellular signaling pathways in memory

The binding of BDNF to its receptor tyrosine kinase, TrkB, leads to the dimerization and autophosphorylation of tyrosine residues in the intracellular domain of the receptor and subsequent activation of cytoplasmic signaling pathways including the Akt and phosphatidylinositol-3 kinase (PI3K), the mitogen-activated protein kinase (MAPK), and the phospholipase-γ (PLC- γ) pathways (Figure 1.10) (Kaplan and Miller 2000). Akt, a serine-threonine protein kinase which is a downstream target of PI3K has been demonstrated to phosphorylate the mammalian target of rapamycin (mTOR). The PI3K/Akt/mTOR signaling pathway plays an important role in the regulation of mRNA translation. BDNF increases protein synthesis by enhancing translation initiation via multiple signaling pathways including PI3K and Akt (Takei et al., 2001). Regarding the cellular mechanisms of BDNF-induced synaptic plasticity *in vitro*, activation of MAPK and PI3K is required to mediate the BDNF-induced modulation of high-frequency synaptic transmission. It was demonstrated that BDNF triggers LTP in the hippocampus *in vivo* through MAPK and selective induction of the dendritic mRNA species Arc (Ying et al., 2002). In addition, it was shown that the radial arm maze training for spatial reference and working memory activates the BDNF/TrkB/PI3K/Akt signal pathway in the hippocampus of well-trained rats (Mizuno et al., 2000). Activation of the BDNF/TrkB/PI3K/Akt signal pathway in the hippocampus of well-trained animals is associated with an increase in phosphorylated 4E-BP1 and a decrease in phosphorylated eEF-2, indicating an increase in activity to translate mRNA into protein. These findings suggest that activation of TrkB /PI3K and protein synthesis signaling pathway by BDNF in the hippocampus is important for spatial memory (Mizuno et al., 2003).

In contrast to the activation of TrkB/PI3K signaling for spatial memory in the positively motivated radial arm maze test, the distinct signaling molecule MAPK appears to be activated by BDNF in the hippocampus for acquisition of fear memory in the negatively motivated passive avoidance test (Tyler et al., 2002, Alonso et al., 2002). MAPK/Erk also regulates protein synthesis dependent plasticity by increasing phosphorylation of eukaryotic initiation factor 4E (eIF4E). The 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 (Kelleher et al., 2004; Klann and Dever, 2004). Thus, MAPK/Erk plays a critical role in protein synthesis dependent plasticity as well.

Another signaling pathway activated by BDNF is the PLC-gamma pathway. The phosphorylation of Tyr785 recruits and activates PLC-gamma which in turn hydrolyzes phosphatidylinositol 4,5-bisphosphate, to produce diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (Huang and Reichardt, 2003; Reichardt, 2006). DAG activates protein kinase C (PKC), and IP3 releases Ca^{2+} from intracellular stores. In developing hippocampal neurons, focal application of BDNF results in fast calcium transients at postsynaptic sites (Lang et al., 2007). Some studies link the PLC-γ pathways underlying BDNF induced Ca^{2+} transients directly to synaptic plasticity. The Ca^{2+} elevation triggered by PLCc increases Ca^{2+}-sensitive adenyl cyclase (AC) activity that is necessary for the formation of synaptic PSD-95-TrkB complexes (Ji et al., 2005) and that is involved in cyclic AMP responsive element binding (CREB)-dependent transcription (Nguyen et al., 1994; Shaywitz and Greenberg, 1999).

Although the reasons why distinct signaling pathways are activated are unclear, it is likely that these three signaling pathways are involved in BDNF-dependent memory formation.

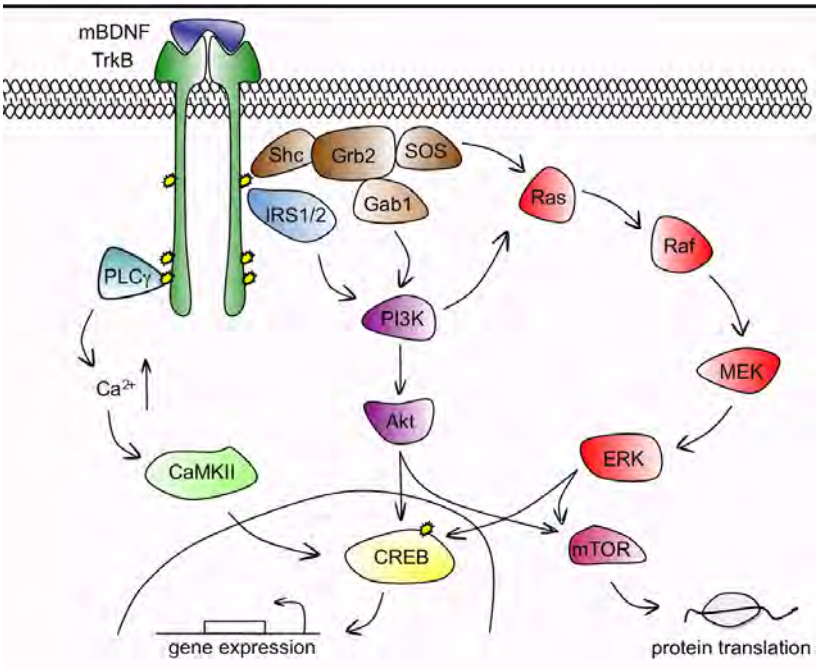

Figure 1.10. BDNF–TrkB signalling pathways. BDNF binds TrkB with high affinity to induce its dimerization and autophosphorylation of tyrosine residues in the cytoplasmic kinase domain that serve as docking sites for effector molecules and trigger the activation of three main signalling pathways: PLCγ, PI3K and ERK cascades, which ultimately lead to the phosphorylation and activation of the transcription factor CREB that mediates transcription of genes essential for the survival and differentiation of neurons. The recruitment of PLCγ increases intracellular Ca^{2+} levels and leads to the activation of CaMKII to phosphorylate CREB. PI3K can be activated via the Shc/Grb2/SOS complex through Gab1 and by IRS1/2. Lipid products generated by the activated PI3K, the phosphatidylinositides, bind and activate protein kinase Akt, upstream of CREB. The ERK cascade can be activated both by the Shc/Grb2/SOS complex and by PI3K. ERK phosphorylation leads directly to CREB phosphorylation. Both Akt and ERK activate mTOR, responsible for enhanced translation initiation. PLCγ, phospholipase Cγ; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; CaMKII, calcium-calmodulin dependent kinase; Shc, src homology domain containing; Grb2, growth factor receptor-bound protein 2; SOS, son of sevenless; Gab1, Grb-associated binder 1; IRS1/2, insulin receptor substrates 1/2; CREB, cAMP-calcium response element binding protein; Ras, GTP binding protein; Raf, Ras associated factor; MEK, MAP/Erk kinase; mTOR, mammalian target of rapamycin; (Picture taken from Cunha et al., 2010)

1.4.3 Energy metabolism and BDNF

BDNF is intimately connected with energy metabolism. Mice that either lack one copy of the BDNF gene or have a conditioned deletion of BDNF in the postnatal brain are hyperphagic and develop obesity (Rios et al., 2001). Moreover, mice with reduced BDNF levels are both obese and hyperglycemic (Lyons et al., 1999; Kernie et al., 2000). Peripheral or central infusion of BDNF has been found to reduce body weight, normalize glucose levels (Tonra et al., 1999), ameliorate lipid metabolism in diabetic rodents (Tsuchida et al., 2002), and increase insulin sensitivity (Pelleymounter et al., 1995; Nakagawa et al., 2000). Hypoglycemia and intermittent fasting both increase BDNF levels whereas hyperphagia and high oxidative stress (OS) levels, the harmful by-products of energy metabolism, decrease

BDNF levels (Lindvall et al., 1992; Lee et al., 2002; Wu et al., 2004a). It is also notable that, in the mature CNS, the BDNF protein is most abundant in brain areas foremost associated with cognitive and neuroendocrine regulation, the hippocampus and hypothalamus, respectively (Nawa et al., 1995). Evidence showed that during exercise, cellular energy metabolism can modulate BDNF-mediated synaptic plasticity in the hippocampus. Indeed, by infusing 1,25-dihydroxyvitamin D3 (D3), a modulator of energy metabolism that acts on mitochondria, directly into the hippocampus during 3 days of voluntary wheel running, Vaymann and colleagues found that BDNF, synapsin I, and CREB where significantly reduced (Vaynman et al., 2006b). Moreover, disrupting energy metabolism in the hippocampus reduced the expression p-CAMKII, the signal transduction cascade downstream to BDNF action. Other findings from the study showed that exercise increases the expression of the uncoupling protein 2 (UCP2), a mitochondrial protein, which uncouples substrate oxidation from ATP synthesis (Bouillaud et al., 1985; Boss et al., 1997; Vidal-Puig et al., 1997; Sanchis et al., 1998; Mao et al., 1999). In human, a study by Yeo et al. (2004) documented a case that may provide clues to evaluate the role of BDNF in metabolism and cognition in human subjects. A human patient with a de novo mutation affecting TrKB, the consort receptor of BDNF, exhibited both hyperphagia and obesity and also suffered developmental delays and other defects in higher order neurologic functions. Thus, metabolism and cognitive function seem to have common signaling pathways (Gomez-Pinilla et al., 2008).

1.4.4 Metabolic plasticity: between synaptic plasticity and energy metabolism

Metabolic plasticity is defined by processes that underlie synaptic plasticity at the energy metabolism level, resulting in correlated metabolic adaptations. The notion of metabolic plasticity has indeed found experimental validation (Magistretti 2006). Evidence has been obtained in a restricted number of experimental paradigms for activity dependent long-term metabolic adaptations (Barrett et al., 2003; Gonzalez-Lima and Garrosa, 1991; Hyden et al., 2000; Maviel et al., 2004; Welker et al., 1992; Zhang and Wong-Riley, 1999). Such adaptations in metabolic pathways are mediated by transcriptional mechanisms that modulate the expression of genes involved in energy metabolism (Allaman et al., 2000; 2003; 2004; Debernardi et al., 2003; Pierre et al., 2003; Sorg and Magistretti, 1992). As mentioned earlier, MCT2 might be one of those target proteins. A restricted set of neuroactive substances regulates neuronal MCT2 expression. It is the case of noradrenaline, insulin and IGF-1, all known to be involved in synaptic plasticity (Pierre et al., 2003; Chenal and Pellerin, 2007; Chenal et al., 2008). Furthermore, based on the peculiar MCT2 distribution (localization at the postsynaptic density, colocalization with PSD95 and GluR2 subunit AMPA receptor,…), evidences suggest that lactate metabolism and synaptic plasticity could be linked processes.

However, most of the data on metabolic plasticity collected so far relate to *in vitro* analyses at the molecular level. The mechanisms of metabolic plasticity in a well-established paradigm of learning and memory are starting to be explored. Recently, Suzuki and colleagues demonstrated for the first time that lactate transfer through monocarboxylate transporters, notably MCT2 is essential for the establishment of long-term memory (Suzuki et al., 2011). In this context, such evidences suggest that metabolic plasticity could be a new essential component for synaptic plasticity processes and memory formation.

1.4.5 Metabolic plasticity in a learning and memory paradigm

It is now widely recognized that learning and memory processes are correlated with functional and structural modifications at the synaptic level, resulting in cellular and molecular changes, named neuronal plasticity. A variety of behavioural paradigms are available to explore the mechanisms of learning and memory in laboratory animals. Spatial learning is one of the best established of such behavioural paradigms; in addition the role of a particular brain area, the

hippocampus in such spatial learning has been extensively characterized. Indeed, the hippocampus is involved in coding, consolidation as well as retrieval of spatial memory in rodents (O'Keefe and Nadel, 1979; Riedel et al., 1999). However, it is still unclear how the hippocampus is working. Electrophysiological studies, partial hippocampal lesion studies and, more recently human brain imaging, have suggested the existence of functional specialization within the hippocampus (Jung et al., 1994; Lepage et al., 1998; Moser and Moser, 1998; Poucet and Buhot, 1994). In addition, studies in rodents have shown that markers of activity (in particular metabolic markers such as 2-deoxyglucose 2-DG), evolve spatially and temporally over the different phases of the learning paradigm, including during recall (Bontempi et al., 1999). Glucose utilization was mapped in a spatial learning paradigm, in the eight-arm radial maze (Ros et al., 2006). Results obtained indicate that the metabolic demand during the various learning phases evolved spatially and temporally in the areas engaged by the task, in particular in the hippocampus. In keeping with the initial hypothesis, distinct patterns of metabolic activity were observed during the learning and recall phases. Analysis of metabolic activity in the hippocampus revealed different patterns over the rostro-caudal axis in its three major subregions, the CA1, CA3 and dentate gyrus (DG). Thus, as learning proceeded, more portions of the CA1 and CA3 became engaged metabolically, moving from the posterior and intermediate parts toward the anterior level. In addition, during recall, increased metabolic activity could be observed only in the anterior parts of the dentate gyrus (Ros et al., 2006). This set of data is in keeping with the notion that metabolic adaptations (plasticity) are occurring as a correlate of learning and recall.

1.5 Questions and aim of the present study

Few data are available concerning MCT regulation in the brain. Previously, it was demonstrated that the predominant neuronal monocarboxylate transporter MCT2 was enhanced by three different factors: noradrenaline, a neurotransmitter (Pierre et al., 2003; Chenal and Pellerin, 2007), insulin, a hormone (Chenal et al., 2008) and insulin-like growth factor (IGF-1), a neurotrophic factor (Chenal et al., 2008). Common points among these neuroactive substances were that the MCT2 regulation occurred by a translational mechanism and they were known to induce long-term changes in synaptic transmission (Kobayashi and Yasoshima, 2001; Trejo et al, 2007; van der Heide et al, 2006).

Synaptic plasticity and behaviour are likely dependent on the capacity of neurons to meet the energy demands imposed by neuronal activity. As described in the introduction, lactate seems to be the preferred energetic substrate for neurons during glutamatergic activity (Pellerin and Magistretti, 1994). The concept that energy metabolism might be coupled to synaptic plasticity has first been proposed together with the implication of BDNF in such interactions (Vaynman et al., 2006 ; Gomez-Pinilla et al., 2008). Considering the critical role of MCT2 in lactate uptake and its utilization by neurons, we studied first the intriguing possibility of a coupling between neuronal MCT2 regulation to adapt the supply of lactate in synaptic plasticity context, and then if these mechanisms of regulation are essential to learning and memory processes.

In the present study we addressed three main questions:

1- Does MCT2 expression regulated by BDNF *in vitro*? If yes, by which pathways and by which mechanism?
2- Does intrahippocampal BDNF injection affect the expression of MCTs?
3- Does an *in vivo* downregulation of hippocampal MCT2 affect mice cognition?

In the first part of this thesis, it became of interest to investigate whether brain derived neurotrophic factor (BDNF), a key molecule involved in synaptic plasticity, could regulate MCT2 expression in cultured neurons and to characterize signal transduction pathways involved in this effect. We were also interested to investigate if MCT2 could be regulated by a miRNA, known to be constitutively expressed in cells and to exert a repressor effect on protein translation. Some of them are known to regulate proteins involved in synaptic plasticity and neuronal growth (Fiore et al., 2008). Then, we investigated *in vivo* the effect of BDNF in the hippocampus on MCT expression in parallel with several pre- and postsynaptic proteins and with two immediate early genes (*Arc* and *Zif268*), both transiently and rapidly activated during LTP as well as along the process of learning and memory formation (Davis et al., 2003; Plath et al., 2006; Lonergan et al., 2010). As hippocampal activation is known to be required for spatial learning tasks, we investigated in the last part, the impact of reducing hippocampal MCT2 expression on spatial performance in mice. Overall, our findings suggest the intriguing possibility that energy supply, via monocarboxylate transporters, could be an important determinant of learning and memory processes.

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Chapter 2

Chapter 2

Brain-derived neurotrophic factor enhances the expression of the monocarboxylate transporter 2 through translational activation in mouse cultured cortical neurons (Robinet C and Pellerin L, 2010).

2.1 Abstract

MCT2 is the predominant neuronal monocarboxylate transporter allowing lactate use as an alternative energy substrate. It is suggested that MCT2 is upregulated to meet enhanced energy demands after modifications in synaptic transmission. Brain-derived neurotrophic factor (BDNF), a promoter of synaptic plasticity, significantly increased MCT2 protein expression in cultured cortical neurons (as shown by immunocytochemistry and western blot) through a translational regulation at the synaptic level. Brain-derived neurotrophic factor can cause translational activation through different signaling pathways. Western blot analyses showed that p44/p42 mitogen-activated protein kinase (MAPK), Akt, and S6 were strongly phosphorylated on BDNF treatment. To determine by which signal transduction pathway(s) BDNF mediates its upregulation of MCT2 protein expression, the effect of specific inhibitors for p38 MAPK, phosphoinositide 3-kinase (PI3K), mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK), p44/p42 MAPK (ERK), and Janus kinase 2 (JAK2) was evaluated. It could be observed that the BDNF-induced increase in MCT2 protein expression was almost completely blocked by all inhibitors, except for JAK2. These data indicate that BDNF induces an increase in neuronal MCT2 protein expression by a mechanism involving a concomitant stimulation of PI3K/Akt/mTOR/S6, p38 MAPK, and p44/p42 MAPK. Moreover, our observations suggest that changes in MCT2 expression could participate in the process of synaptic plasticity induced by BDNF.

2.2 Introduction

Brain-derived neurotrophic factor (BDNF) is a widely expressed neurotrophin in the central nervous system (Skup, 1994). Acting through specific tyrosine kinase receptors, BDNF affects neuronal survival, differentiation, and synaptic plasticity after
the activation of multiple intracellular signal transduction mechanisms, such as phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) signaling pathways (Bramham and Messaoudi, 2005). Presynaptically, BDNF potentiates depolarization-evoked Ca2+-dependent glutamate release while causing direct glutamate release through Ca2+ mobilization from Ins(1,4,5)P3-sensitive stores, whereas the postsynaptic actions of BDNF include changes in glutamate receptor phosphorylation, subcellular localization and synthesis, and local alterations in protein synthesis, as well as long-term changes in gene expression (Carvalho et al, 2008). These effects of BDNF contribute not only to synaptic plasticity but also to modifications in spine density and morphology (Carvalho et al, 2008). However, one aspect that has not been explored in the context of synaptic plasticity concerns putative changes in neuroenergetics. Indeed, it is likely that as a consequence of alterations in synaptic efficacy, the supply of energy substrates must be adapted to meet the energy needs imposed by new levels of synaptic response. In recent years, the role of monocarboxylates such as lactate as additional energy substrates for neurons has attracted increasing attention (Pellerin, 2003), raising interest for the identification of specific transporters in the central nervous system. MCT2 was shown to be the predominant monocarboxylate transporter expressed by neurons (Pierre et al, 2002). It belongs to a family of proton-linked carriers involved in the transport of lactate, pyruvate, and ketone bodies (Garcia et al, 1994, 1995). MCT2 immunoreactivity was found to be abundant in the neuronal processes of various brain regions, including the cortex, hippocampus, and cerebellum (Bergersen et al, 2002; Pierre et al, 2002). At the subcellular level, MCT2 is expressed on axons and dendrites (Pierre et al, 2002, 2009). Moreover, MCT2 is present at glutamatergic synapses and exclusively on postsynaptic elements (Bergersen et al, 2002, 2005; Pierre et al, 2009). It is particularly enriched in the postsynaptic density as well as in an intracellular pool within the spines (Bergersen et al, 2005). Recent observations have shown that MCT2 expression can be regulated in cultured neurons. Thus, it was found that noradrenaline, insulin, and insulin-like growth factor-1 (IGF-1) increase MCT2 protein expression in neurons through a translational mechanism (Chenal and Pellerin, 2007; Chenal et al, 2008). Interestingly, each of these neuroactive substances is known to induce long-term changes in

synaptic transmission (Kobayashi and Yasoshima, 2001; Trejo et al, 2007; van der Heide et al, 2006). Therefore, it became of interest to investigate whether BDNF could regulate MCT2 expression in cultured neurons and to characterize the signal transduction pathways involved in this effect. In addition, the nature of the mechanism (transcriptional or translational) by which MCT2 expression is regulated by BDNF has been investigated.

2.3 Material and methods

Neuronal Cultures and Pharmacological Treatments

Primary cultures of mouse cortical neurons were prepared from embryonic day 17 OF1 mice (Charles River, Lyon, France). As described previously (Debernardi et al, 2003), after decapitation and brain dissection, cortices were mechanically dissociated in phosphate-buffered saline (PBS) supplemented with glucose (NaCl, 150 mmol/L; KCl, 3 mmol/L; KH_2PO_4, 1.5 mmol/L; Na_2HPO_4, 7.9 mmol/L; glucose, 33 mmol/L; penicillin, 0.006 g/L; streptomycin, 0.1 g/L; pH 7.4). Cells were plated on poly L-ornithine (15 mg/L)-precoated dishes and cultured in neurobasal-B27 medium (Brewer et al, 1993) supplemented with 0.5 mmol/L L-glutamine. All experiments were carried out on day 7 *in vitro*. At this stage, cultures contained <5% of glial cells (Debernardi et al, 2003). Neuronal treatments with pharmacological agents were carried out without changing the medium before or during the incubation time. Brain-derived neurotrophic factor (CYT-207; Brunschwig, Basel, Switzerland) was added directly into the culture medium at various concentrations and cells were incubated for the indicated times. Rapamycin, 20 ng/mL (mammalian target of rapamycin (mTOR) inhibitor), SB202190 HCl, 10 μmol/L and SB203580 HCl, 10 μmol/L (p38 MAPK inhibitors), LY294002, 10 μmol/L (PI3K inhibitor), PD98059, 50 μmol/L (MAPK/ERK kinase (MEK inhibitor), UO126, 10 μmol/L (p44/p42 MAPK (ERK, extracellular signal-regulated kinase) inhibitor), and AG490 25 μmol/L (JAK2 (Janus kinase 2) inhibitor) were added directly to the medium 30 mins before BDNF. All these inhibitors were purchased from Alexis Biochemicals (Lausen, Switzerland), except LY294002 (L9908; Sigma, Buchs, Switzerland) and SB203580 (S8307; Sigma). Transcription and translation inhibitors (5 μmol/L actinomycin D (ActD) and 10 μmol/L cycloheximide, respectively) were added 30 mins before pharmacological agents. All other chemicals were purchased from Sigma. Data represent mean±s.e.m. of '*n*' determinations, which are independent

measurements (from different culture plates) obtained from at least three separate neuronal cultures.

Immunocytochemistry and Related Quantification

After removal of the culture medium, cells were carefully rinsed in PBS at 37°C and directly postfixed in an ice-cold paraformaldehyde fixative (4% in PBS for 30 mins at 20°C). Fixed cells were treated with casein (0.5% in PBS) for 1 h at room temperature to block nonspecific sites. For immunostaining, cultures were incubated overnight at 4°C in 50 μL of freshly prepared MCT2 antibody solution (anti-MCT2 diluted 1:500 in PBS containing 0.25% bovine serum albumin) (Pierre et al, 2000). After carefully rinsing in PBS, cultures were incubated in a solution containing Cy3-conjugated anti-rabbit Igs (diluted 1:500 for 2 h at room temperature; Jackson Immunoresearch, Baltimore, MD, USA). After rinsing in PBS twice and a final rinsing in water, coverslips were mounted with Vectashield (Reactolab SA, Burlingame, CA, USA). Coverslips were examined and photographed with an Axioplan2 microscope (Zeiss, Hallbergmoos, Germany) using epifluorescence with an appropriate filter.

To quantitatively assess the influence of different treatments on MCT2 protein expression, a quantitative analysis of images obtained by epifluorescence with a × 20 objective and acquired using a cooled CCD camera (Axiocam, Zeiss), together with the 4.6 Axiovision software (Zeiss) was carried out. Three fields were chosen randomly on each coverslip; they contained at least 20 MCT2-labeled neurons per field. All pictures were acquired and presented as different levels of gray with identical acquisition time for all. Pictures were then analyzed using NIH software (National Institutes of Health Image program, version 1.62, Rockville Pike, MD, USA). The fluorescence intensity of eight isolated cells taken randomly in each of the three captured areas was assessed. The average fluorescence intensity representing neuronal MCT2 expression was obtained by calculating the average of 24 measurements per coverslip. Measurements were obtained in a blinded manner with the investigator unaware of the culture treatments. Mean and s.e.m. for a particular condition were calculated from average fluorescence intensity values of distinct coverslips representing independent determinations (numbers indicated on each bar of the graph). Data were statistically analyzed with an ANOVA (analysis of variance), followed by Dunnett's or Bonferroni's test.

Western Blot and Related Quantification

Neurons in each culture dish were homogenized in 50 μL of buffer containing the following: Tris-HCl, pH 6.8, 20 mmol/L; sucrose, 0.27 mol/L; EGTA (ethylene glycol tetraacetic acid), 1 mmol/L; EDTA (ethylene diamine tetraacetic acid), 1 mmol/L; NaF, 50 mmol/L; Triton X-100, 1%; β-glycerophosphate, 10 mmol/L; DTT (dithiothreitol), 10 mmol/L; 4-nitrophenylphosphate, 10 mmol/L; and a mixture of protease inhibitors (Complete, Roche Molecular Biochemicals, Mannheim, Germany). Each stimulated condition was examined in duplicate and the contents of the two Petri dishes were pooled. Protein samples were sonicated and heated at 95°C for 5 mins in half the final volume of SDS-PAGE sample buffer (Tris-HCl, 62.5 mmol/L; DTT, 50 mmol/L; SDS, 2%; glycerol, 10%; and bromophenol blue, 0.1%). Samples were loaded onto polyacrylamide gels composed of a 10% or 6% acrylamide-bisacrylamide running gel and a 4.5% acrylamide-bisacrylamide stacking gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes (Trans-Blot Transfer Medium 162-0115; Bio-Rad, Reinach, Switzerland) using a Transblot semi-dry transfer cell (Bio-Rad). For protein detection, membranes were incubated in a blocking solution of Tris-buffered saline supplemented with Tween-20 (TBST; Tris-HCl, pH 7.5, 50 mmol/L; NaCl, 150 mmol/L; and Tween-20, 0.1%) containing 5% nonfat milk for 1 h at room temperature. Membranes were incubated overnight at 4°C with the antiphospho-serine/threonine protein kinase from AKT virus (Akt)-Ser473 (1:700), antiphospho-p44/p42 MAPK-Thr202/Tyr204 (both 1:1,000), antiphospho-mTOR-Ser1448 (1:1,000), anti-mTOR (1:1,000), antiphospho-S6-Ser235/236 ribosomal protein (1:1,000), and anti-β-actin (A5441; Sigma). All primary antibodies were purchased from Cell Signalling (BioConcept, Allschwil, Switzerland), except anti-β-actin (A5441; Sigma). After three washes in TBST, membranes were incubated with the secondary antibodies Alexa Fluor 680 goat anti-IgG (Juro, Lucerne, Switzerland) and IRDye 800 goat anti-mouse IgG (BioConcept), diluted at 1:5,000 in TBST containing 1% nonfat milk, for 2 h at room temperature, and protected from light. After three washes in TBST, membranes were scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA), which permits detection and quantification of proteins of interest. β-Actin, revealed in green, was used for normalization and the proteins of interest were revealed in red. As phospho-mTOR has a very high molecular weight (289 kDa), actin was not visible on the same gel. To normalize western blots for phospho-mTOR, samples were loaded in duplicate onto a 6% running gel and proteins were transferred onto a nitrocellulose membrane using a 10% methanol transfer buffer. The membrane was then cut

into two identical pieces and probed either with the phospho-mTOR antibody (1:1,000) or with the mTOR antibody (1:1,000). Thus, quantifications were performed on samples resolved on the same gel and transferred onto the same membrane, and normalization was conducted against mTOR (instead of β-actin). The remaining of the procedure was unchanged.

Quantitative Real-Time Reverse Transcriptase-PCR

Quantitative determination of MCT2 mRNA expression levels was performed by quantitative reverse transcriptase-PCR according to Heid *et al* (1996) using an ABI Prism 7000 sequence detection system from Applied Biosystems (Rotkreuz, Switzerland). The following sets of oligonucleotides were used: 5′ → 3′; ActinFo, GCTTCTTTGCAGCTCCTTCGT; ActinRe, ATATCGTCATCCATGGCGAAC (Embl: X03672); MCT2Fo, CAGCAACAGCGTGATAGAGCTT; MCT2Re, TGGTTGCAGGTTGAATGCTAAT (Embl: NM_011391); NPYFo, ACCAGACAGAGATATGGCAAGAGA; NPYRe, GGCGTTTTCTGTGC (produced by Microsynth, Balgach, Switzerland).

Preparation and Stimulation of Synaptoneurosomes

Synaptoneurosomes were prepared from the forebrains of 8- to 14-day-old mice pups (10 to 15 pups per preparation) according to a previously published protocol (Rao and Steward (1991), as modified by Schratt *et al* (2004)). Briefly, the total forebrains were dissected in 20 mL of homogenization buffer (0.32 mol/L sucrose, 0.1 mmol/L EDTA, 0.25 mmol/L DTT, and 3 mmol/L HEPES, pH 7.4) and disrupted using a Teflon-coated Dounce-Potter homogenizer (B. Braun, Crissier, Switzerland) by eight up-and-down strokes. Nuclei and cell debris were pelleted by 2 mins of centrifugation at 2,000 g. The supernatant was collected and centrifuged for an additional 10 mins at 14,000 g to pellet a crude synaptoneurosome-containing fraction (P2). The pellet was then brought up to 8 mL total volume (with a solution of 0.32 mol/L sucrose and 1 mmol/L $NaHCO_3$). This suspension (4 × 2 mL) was layered onto three different discontinuous sucrose gradients (0.85, 1, 1.2 mmol/L) that had been equilibrated at 4°C for 1 h. The gradient was centrifuged at 45,000 g for 45 mins in a Centrikon T-1075 ultracentrifuge using a SW 41.14Ti swinging bucket rotor. Synaptoneurosomes were collected from the 1/1.2 mol/L interface (500 μL for 2 mL of P2), washed twice in 1 × PBS (in a 2-mL tube), with a centrifugation at 7,000 g for 2 mins. Synaptoneurosomes were resuspended in 500 μL of synaptoneurosome incubation buffer (10 mmol/L Tris, pH 7.5,

2.2 mmol/L $CaCl_2$, 0.5 mmol/L Na_2HPO_4, 0.4 mmol/L KH_2PO_4, 4 mmol/L $NaHCO_3$, and 80 mmol/L NaCl). Synaptoneurosomes were centrifuged at 7,000 g for 2 mins, the supernatant was discarded, and synaptoneurosomes in the lower fraction were either used immediately or stored at −80°C. No significant differences were observed between results from freshly prepared or frozen synaptosomes such that results were pooled. Enrichment of the synaptosomal fraction was controlled by performing western blotting for the postsynaptic protein PSD-95 (data not shown). The synaptoneurosome pellet was thawed on ice for 30 mins and diluted in a prewarmed (37°C) synaptoneurosome incubation buffer containing a mixture of antiproteases (Complete 11257000; Roche Molecular Biochemicals) to yield a protein concentration between 2 and 9 mg/mL. Synaptoneurosome samples (100 μL) were exposed to either ActD (5 μmol/L) or cycloheximide (10 μmol/L) for 30 mins before application of 100 ng/mL BDNF for 6 h. The reaction was stopped by adding 20 μL of SDS buffer (5 ×), and samples were boiled for 5 mins and served as starting material for western blotting.

2.3 Results

2.3.1 Effect of BDNF on MCT2 protein expression in cultured neurons

To assess the effect of BDNF on neuronal MCT2 protein expression, cultured cortical neurons were treated with BDNF for 6 h at various concentrations up to 100 ng/mL. Experiments conducted with BDNF concentrations of 10, 50, and 100 ng/mL followed by immunocytochemistry led to a striking enhancement of fluorescence intensity corresponding to higher levels of MCT2 immunoreactivity (IR) at 100 ng/mL (Figure 1A). Western blot analysis showed that the maximal increase of MCT2 expression was found with 100 ng/mL. At lower concentrations, MCT2 expression had a tendency to be increased, but the effect was not significant statistically (Figure 1B).

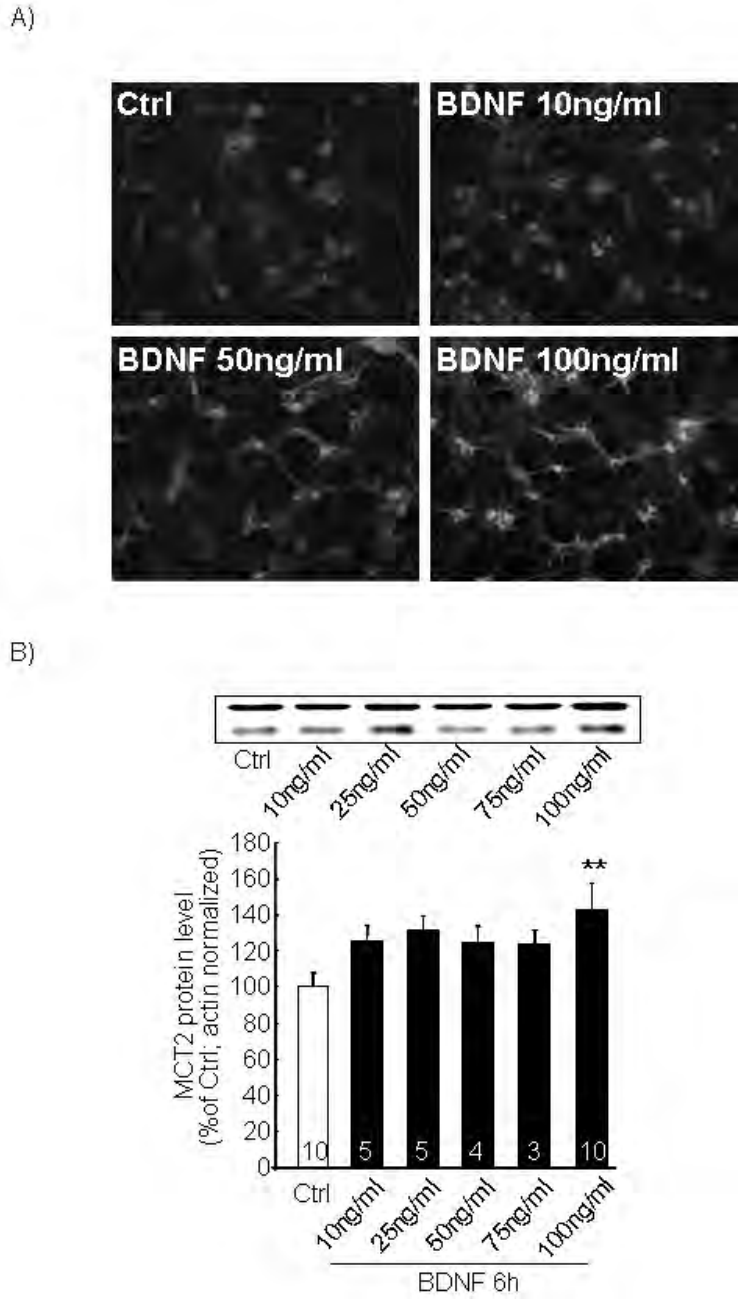

Fig.1. Concentration-dependent effect of BDNF on MCT2 expression in primary cultures of mouse cortical neurons. A) Immunocytochemical stainings for MCT2 in untreated cultures (Ctrl) and cultures treated with BDNF for 6h at different concentrations. B) Western blot analysis of MCT2 expression in primary cultures of mouse cortical neurons treated with BDNF for 6h at various concentrations up to 100ng/ml. Western-blot were quantified using Odyssey software (LI-COR). Results are expressed as percent of control after the values had been normalized using β-actin signal as reference. Statistical analysis was performed using ANOVA followed by Dunnett's test. ** indicates MCT2 protein levels significantly different from control with $p<0.01$. Numbers in the graph bars represent the number of independent experiments for each condition.

Changes in the levels of MCT2 IR induced by BDNF were also studied as a function of time (1, 6, 12, and 24 h). It was observed that 100 ng/mL BDNF caused a 40% increase in MCT2 IR 6 h after the beginning of the treatment that remained elevated at 12 and 24 h (Figure 2A). Western blot analysis showed that the increase of MCT2 expression was already found significant as early as after 1 h of BDNF stimulation (100 ng/mL) and that this significant

increase was still present up to 24 h (Figure 2B). The effect of BDNF was found to be as robust as the previously described effect of noradrenaline (100 mmol/L) on MCT2 expression after 6 h of treatment (Figure 2C).

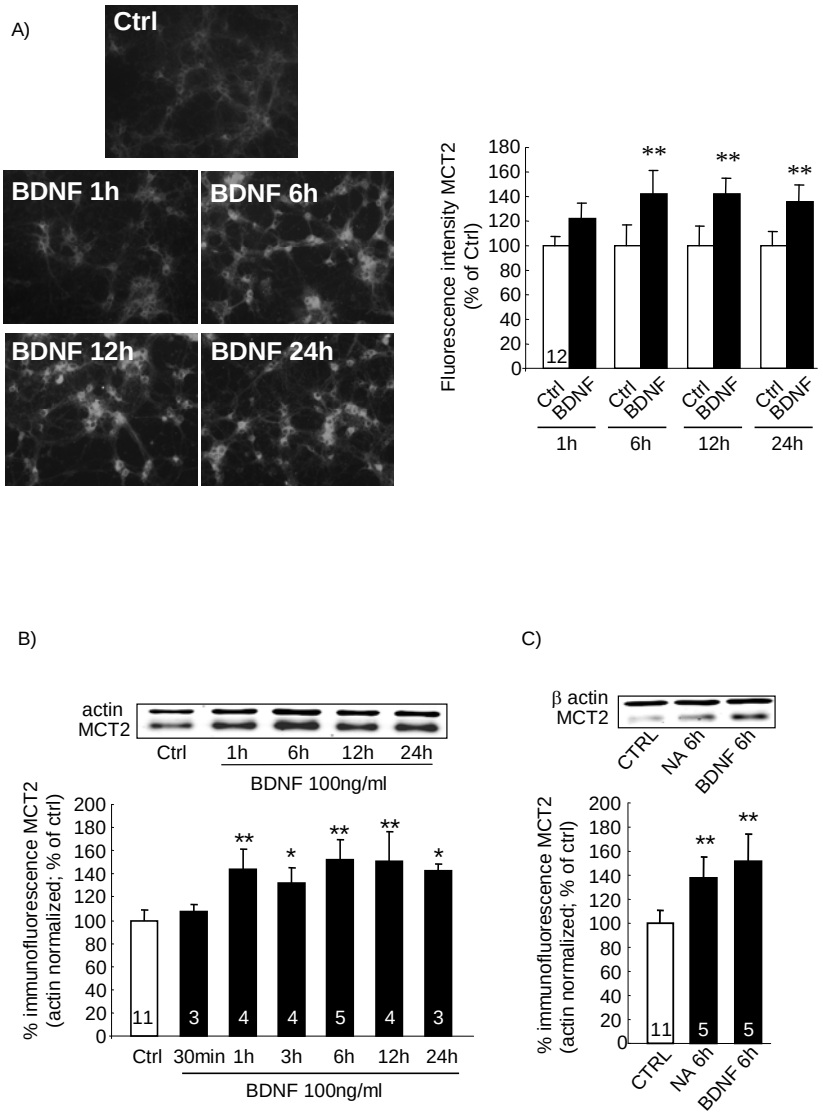

Fig.2. Time course of the effect of BDNF on MCT2 protein expression in primary cultures of mouse cortical neurons. A) MCT2 IR and related quantification of primary cultures of mouse cortical neurons treated with BDNF at a final concentration of 100ng/ml for various periods of time up to 24h. Left panels represent immunocytochemical stainings for MCT2 in untreated cultures (Ctrl) or cultures treated with BDNF for 6h. The bar graph represents the quantitative determination of fluorescence intensity corresponding to MCT2 IR in cultured neurons treated with BDNF for various periods of time up to 24h. Results are expressed as percentage of control fluorescence intensity and represent the mean±SEM of independent determinations (numbers indicated on bars) from four distinct experiments. The value of fluorescence intensity for each determination represents the average level from 24 cells on the same coverslip. B) and C) Western blot analysis of MCT2 protein expression in cultures of mouse cortical neurons treated with BDNF 100ng/ml or Noradrenaline (NA 6h) for the indicated times as compared with untreated cells (Ctrl). Western blots were quantified using Odyssey sofware (LI-COR). Results are expressed as percentage of control (mean±SEM) after the values had been normalized using β-actin signal as reference. Statistical analysis was performed using ANOVA followed by Dunnett's test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control (Ctrl) for MCT2 protein levels. Numbers in the graph bars represent the number of independent experiments for each condition.

2.3.2 Putative involvement of distinct signal transduction pathways in BDNF-induced MCT2 expression

Involvement of specific signal transduction mechanisms involved in the effect of BDNF on MCT2 expression was investigated with a series of inhibitors. Cultured cortical neurons were pretreated with five different inhibitors (LY294002, a specific PI3K inhibitor; rapamycin, a specific mTOR inhibitor; PD98058, a specific MEK inhibitor; SB202190, a specific p38 MAPK inhibitor; and AG490, a specific JAK2 inhibitor) 30 mins before the addition of BDNF (100 ng/mL) for 6 h. The six panels on the left of Figure 3A show that a 30-min pretreatment of cultured mouse cortical neurons with LY294002 (10 mmol/L), rapamycin (20 ng/mL), PD98058 (50 mmol/L), or SB202190 (10 mmol/L) blocked the BDNF-induced increase in MCT2 IR after 6 h of treatment. In contrast, AG490 (25 mmol/L) had no effect (not shown). The quantification of MCT2 IR shows that all inhibitors (with the exception of AG490) prevented the induction of MCT2 protein expression by BDNF, reducing the overall expression by ~40% (Figure 3A). Similar results were obtained by western blot, confirming the MCT2 IR data (Figure 3B). The effect of all five inhibitors alone (without posttreatment with BDNF) was tested previously on cultured cortical neurons and no change in MCT2 IR was observed compared with the control condition (data not shown). To further validate our observations, two more inhibitors were tested for their effect on BDNF-induced MCT2 expression (UO126, a specific p44/p42 MAPK inhibitor, and SB203580, another specific p38 MAPK inhibitor). Cultured cortical neurons were pretreated with UO126 (10 mmol/L) and SB203580 (10 mmol/L) 30 mins before the addition of BDNF (100 ng/mL) for 6 h. Western blot analysis shows that UO126 and SB203580 significantly reduced MCT2 protein expression induced by BDNF (Figure 3C).

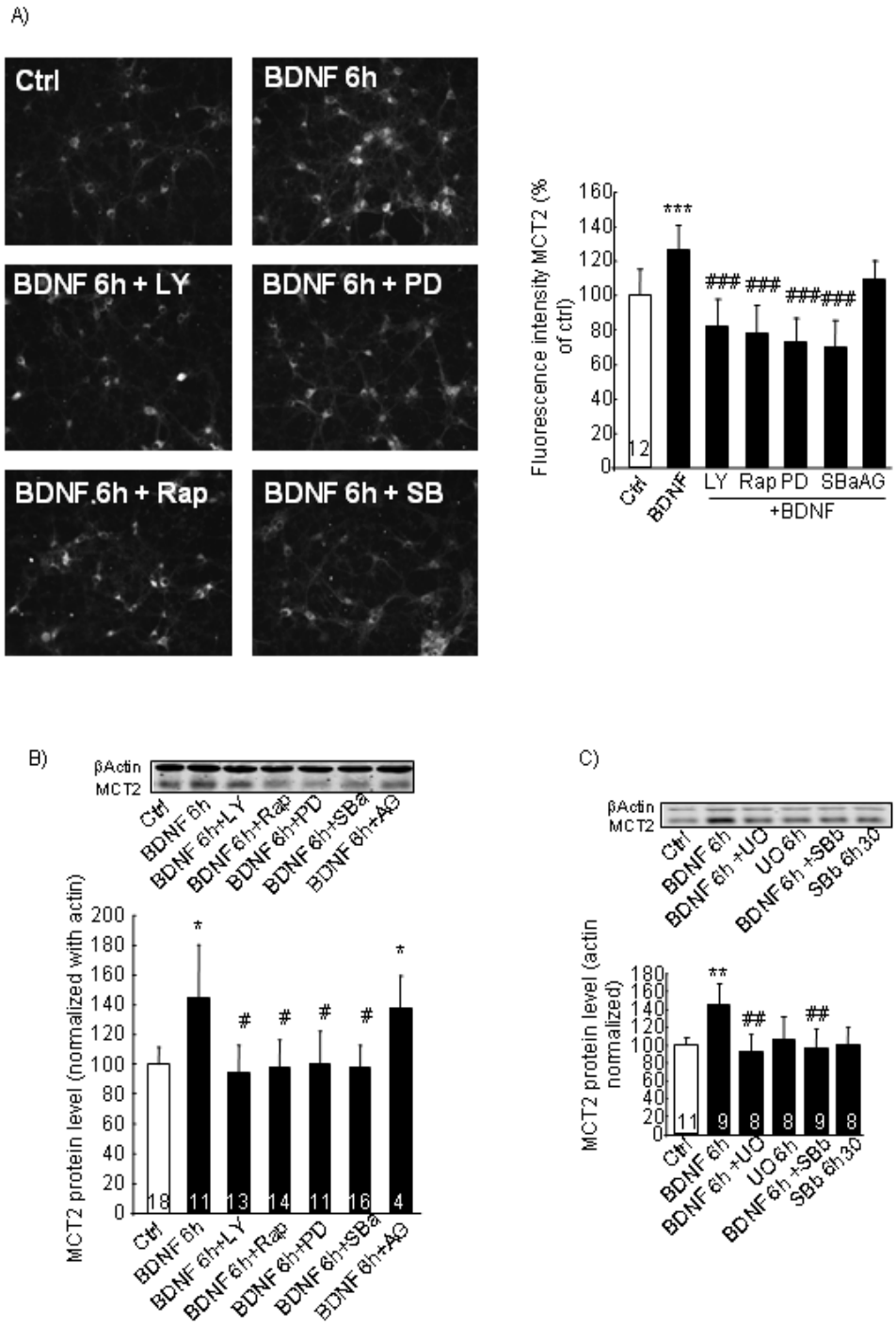

Fig.3. Effect of different signaling pathway inhibitors on BDNF-stimulated MCT2 expression in primary cultures of mouse cortical neurons. Primary cultures of mouse cortical neurons were pretreated for 30 mins with specific signaling pathway inhibitors (PD98095, 50 μmol/L (PD); SB202190, 10 μmol/L (SB_a); LY294002, 10 μmol/L (LY); rapamycin, 20 ng/mL (Rap); AG490 25 μmol/L (AG); UO126, 10 μmol/L (UO); SB203580, 10 μmol/L (SB_b)) before stimulation with BDNF 100 ng/mL for 6 h (BDNF 6 h). (**A**) Left panels represent immunocytochemical stainings for MCT2 in untreated cells (Ctrl), cultures treated with BDNF alone for 6 h (BDNF), or pretreated for 30 mins with each inhibitor (LY, Rap, PD, SB_a) before the addition of BDNF. The bar graph represents the quantitative determination of fluorescence intensity corresponding to MCT2 IR in cultured neurons treated with the specific signaling pathway inhibitors followed by exposure to BDNF for 6 h. Results are expressed as percentage of control fluorescence intensity and represent the mean±s.e.m. of independent determinations (numbers indicated on bars) from four distinct experiments. The value of fluorescence intensity for each determination represents the average level from 24 cells on the same coverslip. (**B** and **C**) Western blot analysis of MCT2 expression in cultures of mouse cortical neurons treated with specific signaling pathway inhibitors (+LY, Rap, PD, SB_a, AG, UO, SB_b) before stimulation with BDNF 100 ng/mL for 6 h (BDNF 6 h). Western blots were quantified using Odyssey software (LI-COR Biosciences, Lincoln, NE, USA). Results are expressed as percentage of control (mean±s.e.m.) after the values had been normalized using β-actin signal as the reference. Statistical analysis was performed using ANOVA followed by Bonferroni's test. $^{*}P<0.05$, $^{**}P<0.01$ versus control (Ctrl). $^{\#}P<0.05$ versus 6 h BDNF treatment. Numbers in the graph bars represent the number of independent experiments for each condition.

2.3.3 Activation by BDNF of the different signal transduction pathways implicated in MCT2 upregulation

It became necessary to assess the effect of BDNF on each signal transduction pathway putatively implicated in MCT2 upregulation and to verify the efficacy of each inhibitor used. Cultured cortical neurons were treated with BDNF (100 ng/mL) for three time periods (5 mins, 30 mins, and 1 h). First, to evaluate the activation of the PI3K/Akt/mTOR/S6 pathway, the phosphorylation levels of Akt on Ser473, mTOR on Ser2448, and S6 ribosomal protein on Ser235/236 were determined by western blot. Brain-derived neurotrophic factor induced the phosphorylation of Akt after 5 mins of treatment (Figure 4A). The level of phosphorylation was increased by ~300% above control (set at 100%) after 5 mins of treatment. Maximal activation was reached after 30 mins. Phosphorylation of Akt was sustained for 1 h of BDNF treatment. To obtain the confirmation that Akt signaling can be inhibited in a manner similar to MCT2 upregulation, cultured cortical neurons were pretreated with 10 mmol/L LY294002 for 30 mins before the addition of BDNF (100 ng/mL) for 5 mins, 30 mins, and 1 h. Indeed, LY294002 pretreatment prevented the phosphorylation of Akt induced by BDNF in cultured cortical neurons (Figure 4A). Activation of mTOR by BDNF treatment was investigated by monitoring its level of phosphorylation. Brain-derived neurotrophic factor induced the phosphorylation of mTOR within 5 mins after the beginning of the treatment (Figure 4B). Phosphorylation of mTOR was further increased by more than 50% above the control level after 1 h of BDNF treatment. To determine whether mTOR activation can be prevented in the same manner as MCT2 upregulation, cultured cortical neurons were pretreated with 20 ng/mL rapamycin for 30 mins before adding BDNF (100 ng/mL) for 5 mins, 30 mins, and 1 h. Rapamycin pretreatment completely prevented the phosphorylation of mTOR induced by BDNF in cultured cortical neurons at 30 mins (Figure 4B). S6 phosphorylation represents one of the late steps in the activation of the PI3K/Akt/mTOR/S6 pathway. Phosphorylation levels of S6 were assessed after BDNF treatment. Brain-derived neurotrophic factor induced the phosphorylation of S6 within 5 mins after the beginning of the treatment (80% above control; Figure 4C). Maximal activation was reached after 1 h with an increase of 350% above control. As the S6 protein is known to be a downstream effector of the Akt/mTOR signaling pathway, cultured cortical neurons were pretreated with 20 ng/mL rapamycin (inhibitor of mTOR) for 30 mins before the addition of BDNF for 5 mins, 30 mins, and 1 h. It was observed that rapamycin pretreatment completely prevented the phosphorylation of S6 ribosomal protein induced by BDNF in cultured cortical neurons (Figure 4C).

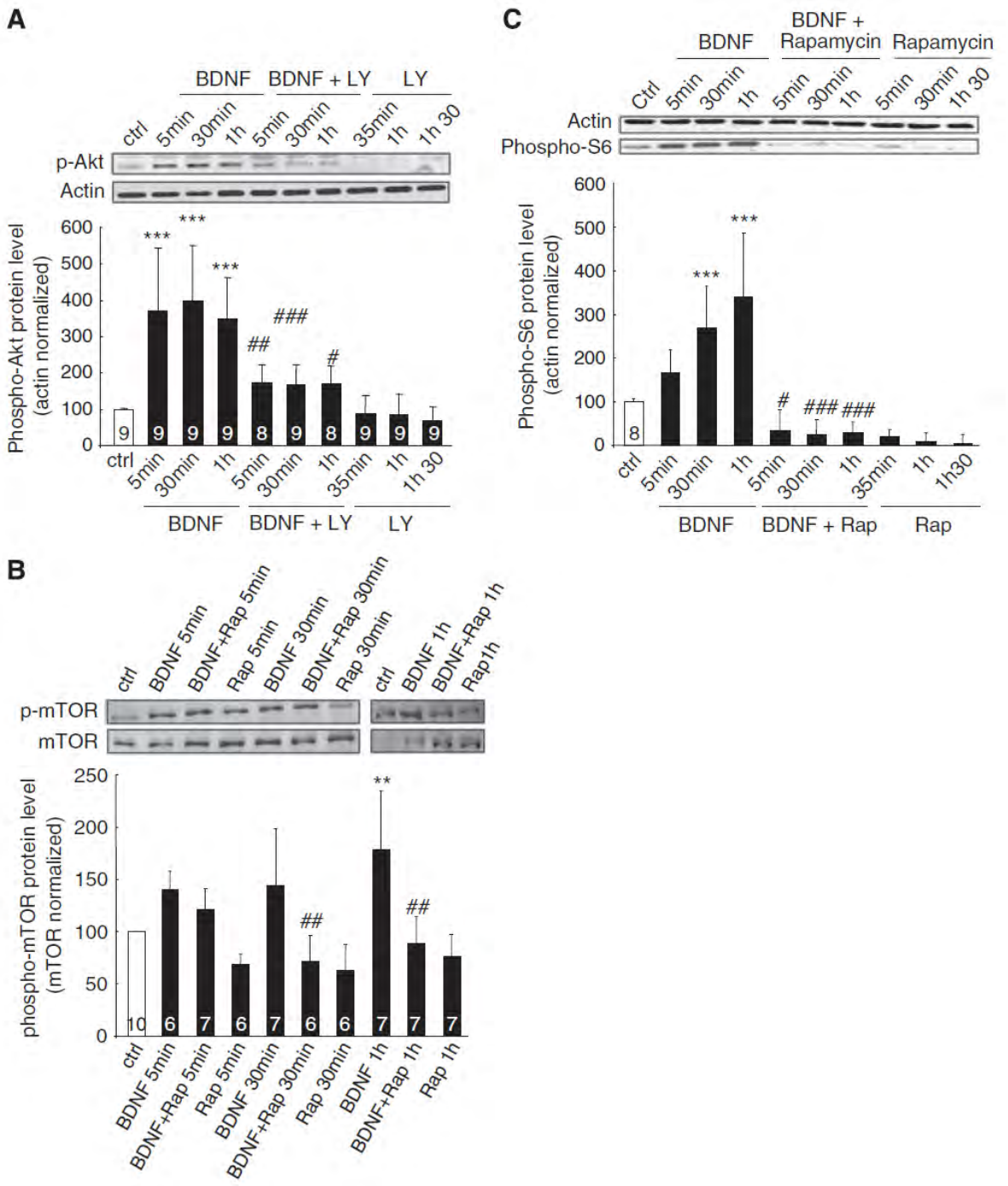

Fig.4. Effect of BDNF on the phosphorylation levels of Akt, mTOR and S6 in cultured mouse cortical neurons. Western blot analysis of phospho-Akt (A), phospho-mTOR (B), phospho-S6 (C) levels in cultures of mouse cortical neurons treated with BDNF 100ng/ml for the indicated times as compared with untreated cells (ctrl). A) LY294002, a specific PI3K inhibitor, was added to the culture medium at the concentration of 10μM, 30min before to incubation with BDNF 100ng/ml during 5min, 30min and 1h. Western blots were quantified using Odyssey software (LI-COR). Results are expressed as percent of control after the values had been normalized using β-actin signal as reference. B) and C) Rapamycin, a specific mTOR inhibitor, was added to the culture medium at the concentration of 20ng/ml, 30min prior to incubation with BDNF 100ng/ml. Results are expressed as percent of control after the values had been normalized using either β-actin signal (A,C) or mTOR signal (B) as reference. Statistical analysis was performed using ANOVA followed by Bonferroni's test. **, *** indicates phospho-Akt, phospho-mTOR or phospho-S6 protein levels significantly different from control with $p< 0.01$, $p<0.001$ respectively. #, ##, ### indicates phospho-Akt, phospho-mTOR or phospho-S6 protein levels significantly different from BDNF-treated condition with $p<0.05$, $p<0.01$, $p<0.001$ respectively. Numbers in the graph bars represent the number of independent experiments for each condition.

Mitogen-activated protein kinase activation is purported to be involved in the effect of BDNF on MCT2 expression. Phosphorylation levels of p44 and p42 MAPK (phospho-ERK) on Thr202/Tyr204 were determined by western blot after treatment of cultured cortical neurons with BDNF (100 ng/mL) for three different time periods (5 mins, 30 mins, and 1 h). Brain-derived neurotrophic factor induced the phosphorylation of p44 and p42 MAPK after 5 mins of treatment (Figure 5A). The level of phosphorylation was increased by more than 500% (for both p44 and p42) above control (set at 100%) at that time. Phosphorylation of p44 and p42 MAPK was sustained for 1 h of BDNF treatment. To ascertain whether MAPK activation can be prevented similar to MCT2 upregulation, cultured cortical neurons were pretreated with 50 mmol/L PD98059 for 30 mins before the addition of BDNF (100 ng/mL) for 5 mins, 30 mins, and 1 h. As expected, PD98059 pretreatment attenuated the phosphorylation of p44 and p42 MAPK induced by BDNF in cultured cortical neurons (Figure 5A).

Activation of p38 MAPK is another possible signaling mechanism involved in BDNF-induced MCT2 upregulation. Phosphorylation levels of p38 MAPK on Thr180/Tyr182 were determined by western blot. Brain-derived neurotrophic factor induced the phosphorylation of p38 MAPK that reached a maximum after 1 h with an increase of B150% above control (set at 100%; Figure 5B). To investigate whether p38 MAPK activation can be prevented using the inhibitor previously used, cultured cortical neurons were pretreated with 10 mmol/L SB202190 for 30 mins before the addition of BDNF (100 ng/mL) for 5 mins, 30 mins, and 1 h. SB202190 pretreatment was found to prevent phosphorylation of p38 MAPK induced by BDNF in cultured cortical neurons (Figure 5B).

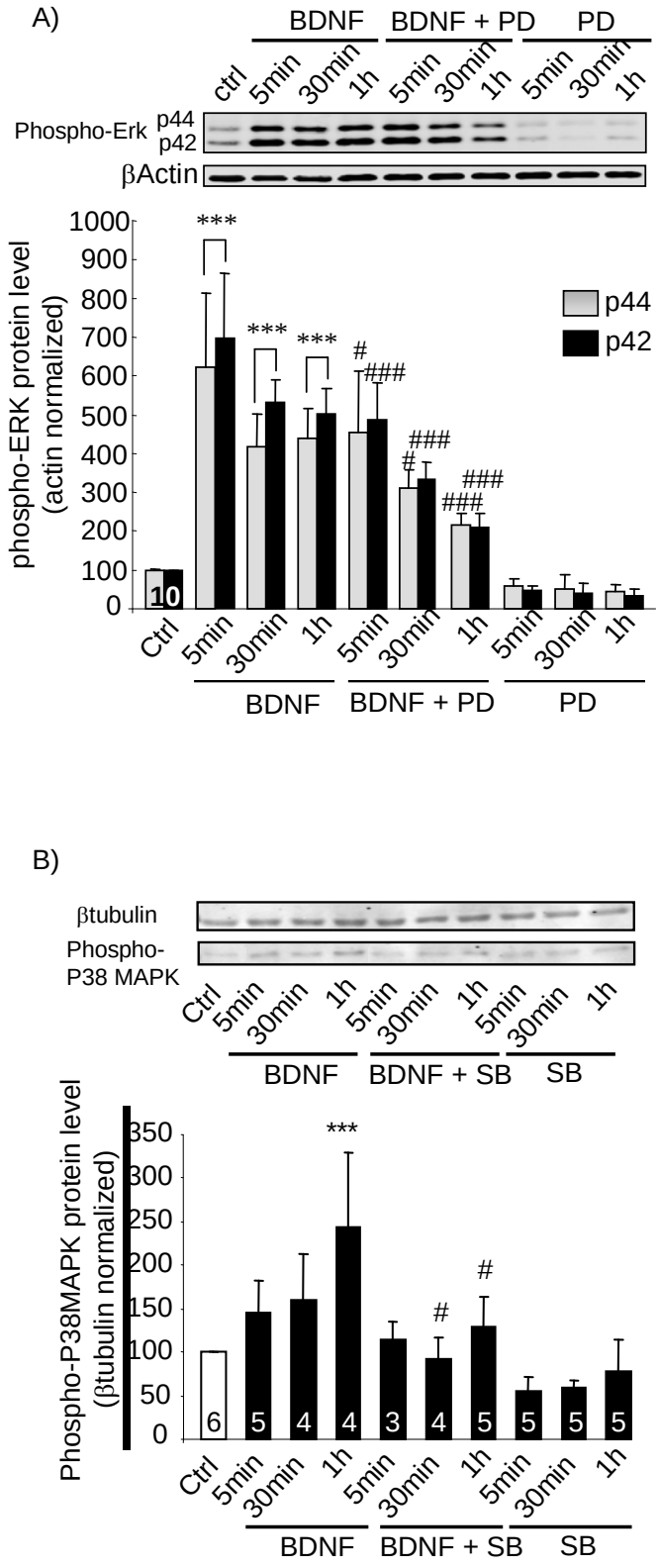

Fig.5. Effect of BDNF on the phosphorylation levels of ERK and p38 MAPK in cultured mouse cortical neurons. Western blot analysis of phospho-ERK (A) and phospho-p38 MAPK (B) levels in cultures of mouse cortical neurons treated with BDNF 100ng/ml for the indicated times as compared with untreated cells (ctrl). A) PD98058, a specific MEK inhibitor, was added to the culture medium at the concentration of 50µM, 30min before to incubation with BDNF 100ng/ml during 5min, 30min and 1h. B) SB202190, a specific p38 MAPK inhibitor, was added to the culture medium at the concentration of 10µM, 30min prior to incubation with BDNF 100ng/ml. Western blots were quantified using Odyssey software (LI-COR). Results are expressed as percent of control after the values had been normalized using β-actin signal as reference. Statistical analysis was performed using ANOVA followed by Bonferroni's test. *** indicates phospho-ERK or phospho-p38 MAPK levels significantly different from control with $p<0.001$. ##, ### indicates phospho-ERK or phospho-p38 MAPK levels significantly different from BDNF-treated condition with $p<0.01$, $p<0.001$ respectively. Numbers in the graph bars represent the number of independent experiments for each condition.

2.3.4 BDNF enhances MCT2 expression by a translational mechanism

The effect of BDNF on MCT2 mRNA expression was investigated by quantitative reverse transcriptase-PCR on total RNA from mouse cortical neurons. Neurons were treated with BDNF (100 ng/mL) for various time points up to 24 h. Figure 6A shows that BDNF exerted no enhancing effect on MCT2 mRNA levels at any time point, whereas NPY mRNA levels (a peptide known to be transcriptionally regulated) showed a significant increase after 12 and 24 h. A small but significant decrease in MCT2 mRNA levels was observed at 3 h. To ascertain whether the effect of BDNF on MCT2 expression requires the activation of translation but not of transcription, cultured neurons were treated with inhibitors for these two processes. Application of cycloheximide (10 mmol/L), a protein synthesis inhibitor, 30 mins before BDNF stimulation (100 ng/mL), prevented the enhancement of MCT2 protein expression (Figure 6B). Quite unexpectedly, the mRNA synthesis inhibitor ActD (5 mmol/L) also blocked the effect of BDNF on MCT2 protein expression. To determine whether the enhancement in MCT2 protein synthesis occurs at the synaptic level and requires transcriptional activation, an experiment was conducted on synaptoneurosomes (Figure 6C). Brain-derived neurotrophic factor (100 ng/mL) induced an increase in MCT2 protein expression after 6h in this preparation. The effect of BDNF was prevented by cycloheximide (10 mmol/L) but not by ActD (5mmol/L).

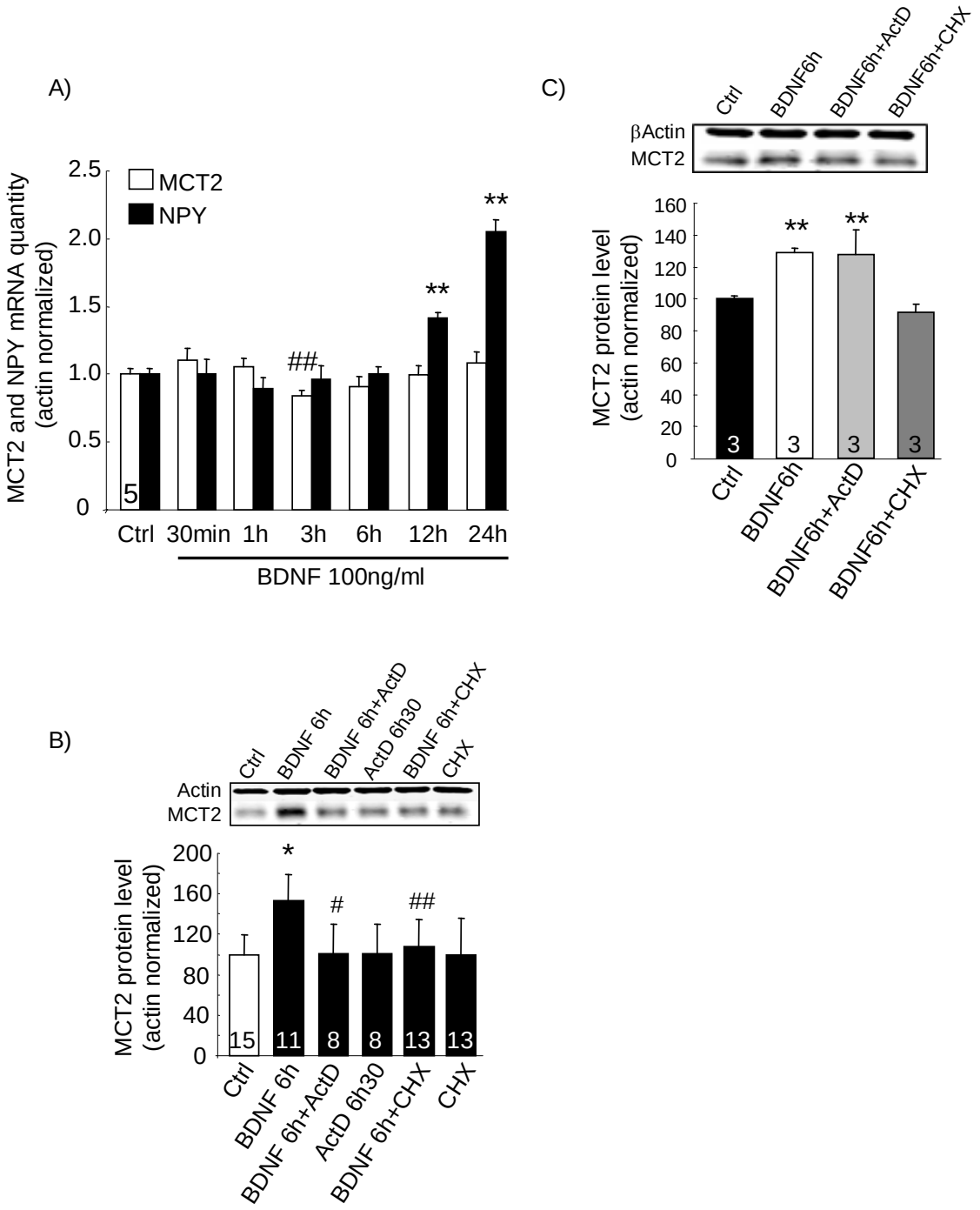

Fig.6. Effect of transcription and translation inhibitors on BDNF-induced enhancement of MCT2 protein expression in primary cultures of mouse cortical neurons. Western blot analysis of MCT2 expression in primary cultures of mouse cortical neurons exposed to either actinomycin D (ActD) or cycloheximide (CHX) for 30min prior to application of 100ng/ml BDNF for 6h. Western blots were quantified using Odyssey software (LI-COR). Results are expressed as percent of control after the values had been normalized using β-actin signal as reference. Statistical analysis was performed using ANOVA followed by Bonferroni's test. * indicates MCT2 protein levels significantly different from control with $p<0.05$. #, ## indicates MCT2 protein levels significantly different from BDNF-treated condition with $p<0.05$, $p<0.01$ respectively. Numbers in the graph bars represent the number of independent experiments for each condition

2.4 Discussion

In recent years, BDNF has emerged as a major regulator of synaptic transmission and plasticity at adult synapses in many regions of the central nervous system (Bramham and Messaoudi, 2005). Among the mechanisms subserving synaptic plasticity, enhancement of localized protein synthesis constitutes a critical process for long-term adaptation of synaptic efficacy. Brain-derived neurotrophic factor is one of the major activity-dependent modulators of dendritic protein synthesis and it is known to activate specific components of the translational machinery in neurons (Bramham and Messaoudi, 2005; Steward and Schuman, 2003). Previously, it has been shown that the rapid induction of protein synthesis by BDNF is mediated both through the PI3K pathway, as it involves the activation of PI3K and mTOR (Bramham and Messaoudi, 2005; Schratt et al, 2004; Yoshii and Constantine-Paton, 2007), as well as through the MAPK signaling pathway (Kelleher et al, 2004). Interestingly, BDNF was shown in this study to enhance MCT2 protein expression in cultured cortical neurons through a stimulation of translation. This is supported by the observations that no changes in MCT2 mRNA levels were detected, whereas MCT2 protein expression was enhanced by BDNF in synaptoneurosomes, a preparation that can sustain translation but not transcriptional activation. The observation that the mRNA synthesis inhibitor ActD can prevent BDNFinduced enhancement of MCT2 protein expression in intact cells but not in synaptoneurosomes is intriguing. It cannot be excluded that as a general transcription inhibitor, ActD indirectly interferes with the translation process in intact cells by reducing the expression of some essential components. The effect of BDNF on MCT2 protein expression was shown to involve the activation of three distinct signaling pathways, all classically implicated in the regulation of translation initiation (Hay and Sonenberg, 2004). Thus, a concomitant activation of PI3K/Akt/mTOR, p38 MAPK as well as MEK/ ERK kinases was found to be necessary for the enhancement of MCT2 protein expression by BDNF (Figure 7).

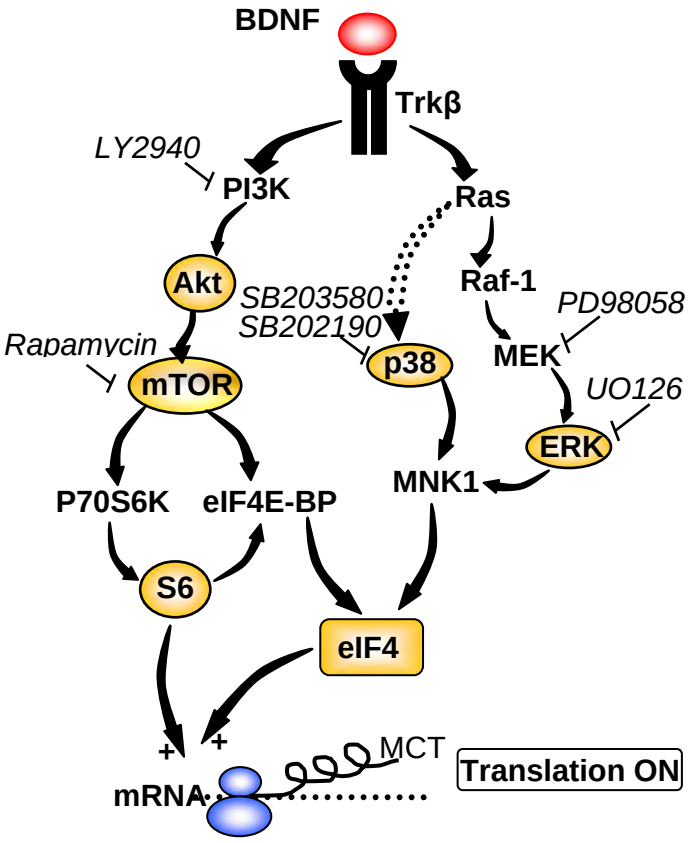

Fig.7. Putative signalling pathways leading to translational activation and enhanced MCT2 protein synthesis following BDNF treatment in cultured cortical neurons. BDNF, acting via TrkB receptors, can activate distinct signal transduction pathways involving specific kinases leading to translation initiation. Two pathways investigated in this study are illustrated here (PI3K/Akt/mTOR/S6 and MAPK signalling pathways). Proteins highlighted were directly investigated for their level of phosphorylation. First, phosphorylation of PI3K can cause the phosphorylation and activation of Akt. Akt then directly phosphorylates mTOR which in turn phosphorylates p70S6K. The target of p70S6K, the ribosomal S6 protein, once phosphorylated, participates in the tranlational machinery as part of the 40S complex. Second, the MAPK cascade is also activated by BDNF requiring activation of MEK. MEK phosphorylates the p44- and p42- MAPK which can activate, among others, MNK1. Once activated, MNK1 phosphorylates eIF4E on Ser209 and it correlates with enhanced rates of translation of capped mRNA. Specific inhibitors for some kinases have been used to distinguish the implication of each pathway in the effect of BDNF: LY294002, PI3K inhibitor; Rapamycin, mTOR inhibitor; PD98058, MEK inhibitor; SB202190 and SB203580, p38 MAPK inhibitors; AG490, JAK2 inhibitor.

Such an observation suggests a putative cross talk between the different signaling pathways activated by BDNF to regulate translational activation, a possibility previously proposed by others (Almeida et al, 2005; Bramham and Messaoudi, 2005). It is noted that noradrenaline (a neurotransmitter), insulin (a hormone), and IGF-1 (a neurotrophic factor) were also previously shown to regulate MCT2 protein expression at the translational level, notably by activating the PI3K/Akt/mTOR/S6 kinase pathway in cultured cortical neurons (Chenal and Pellerin, 2007; Chenal et al, 2008). As each of these neuroactive substances, similar to BDNF, has been implicated in different forms of synaptic plasticity, it suggests that MCT2 represents a

common target for such signals, pointing to a putatively important role of MCT2 in relation to synaptic modifications.

In view of the current results, it appears that MCT2 belongs to a group of neuronal proteins specifically regulated at the translational level. Until now, more than 100 different mRNAs coding for proteins involved in neurotransmission and in the modulation of synaptic activity have been identified in dendrites. Local protein synthesis from these mRNAs is postulated to provide the basic mechanism of long-term changes in the strength of neuronal connections (Skup, 2008). Postsynaptic proteins that undergo enhancement of their synthesis locally include calmodulin kinase II (Ouyang et al, 1999; Wu and Cline, 1998), MAP2 (Blichenberg et al, 1999), Arc/Arg 3.1 (Steward and Schuman, 2001), and GluR1, as well as GluR2 AMPA receptor subunits (Ju et al, 2004). Although the presence of MCT2 mRNA has not been described yet in dendrites, it seems likely that the translational regulation of MCT2 expression would occur locally. Our results obtained with synaptoneurosomes support this conclusion. The MCT2 protein is not only present in dendrites but it is also specifically associated with spines, where it was found to be expressed in the postsynaptic density as well as on vesicle-like structures forming an intracellular pool (Bergersen et al, 2005). MCT2 was recently found to interact with a specific subset of postsynaptic proteins (Pierre et al, 2009). This is particularly the case with GluR2, a subunit of the glutamatergic AMPA receptor subtype. It was shown that MCT2 seems not only to determine the subcellular localization of GluR2 in neural cells but also to regulate its expression levels (Maekawa et al, 2009). In addition, it was observed that MCT2 together with GluR2 undergoes a trafficking process between the plasma membrane and an intracellular pool under conditions inducing synaptic plasticity (Pierre et al, 2009). Translocation of GluR2 to and from the plasma membrane has been implicated in synaptic plasticity mechanisms, such as long-term potentiation and long-term depression (Kessels and Malinow, 2009; Malenka, 2003; Sheng and Kim, 2002). Thus, MCT2 localization and interaction with specific synaptic proteins involved in plasticity mechanisms strengthen the view that its expression may be regulated in a manner similar to its partners. In this regard, increased MCT2 protein expression by BDNF may be part of a coordinated mechanism of local synthesis for various postsynaptic proteins involved in the long-term regulation of glutamatergic transmission. Although a major effort has been devoted to decipher the molecular events involved in synaptic plasticity, including those induced by BDNF, few studies have explored the concomitant changes in neuroenergetics that could take place with alterations in synaptic transmission. Recently, the concept that energy metabolism

might be coupled to synaptic plasticity has been proposed together with the implication of BDNF in such interactions (Vaynman et al, 2006). Indeed, BDNF was shown to enhance mitochondrial activity (El Idrissi and Trenkner, 1999). Regarding the energy substrates that might be concerned, lactate has attracted much attention recently. Lactate has been shown to be a preferential oxidative substrate for neurons both in vitro (McKenna et al, 1993, 1994) and in vivo (Hyder et al, 2006; Serres et al, 2005). Lactate is not only able to sustain synaptic vesicle turnover and synaptic transmission (Morgenthaler et al, 2006; Rouach et al, 2008; Schurr et al, 1988) but it is also shown to allow, at least in part, the establishment of long-term potentiation (Izumi et al, 1997; Yang et al, 2003). In addition, glutamatergic activity increases the production and release of lactate by astrocytes (Pellerin and Magistretti, 1994), and it has been purported that such an effect participates in a mechanism to provide lactate as an additional energy substrate to neurons to sustain their activity (Pellerin et al, 2007). As the primary role of MCT2 is to supply neurons with nonglucose energy substrates, e.g., lactate, changes in MCT2 expression might facilitate the utilization of alternative substrates. Indeed, overexpression of MCT2 in neurons was shown to enhance lactate consumption by neurons stimulated with kainite (Bliss et al, 2004). Our observation that MCT2 can be upregulated by BDNF is consistent with the possibility of a coupling between lactate utilization and synaptic plasticity. It is hypothesized that to meet higher energy demands caused by enhanced synaptic transmission after synaptic plasticity, MCT2 expression is increased to facilitate lactate supply at potentiated synapses.

In conclusion, we have shown that BDNF enhances the expression of the monocarboxylate transporter MCT2 in cultured cortical neurons through a translational regulation. A possible role for such an effect could be to enlarge the local lactate transporter pool to allow potentiated synapses to meet higher energy demands on activation.

2.5 References

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Chapter 3

Chapter 3

Possible regulation of MCT2 protein synthesis via miRNA

3.1 Introduction

3.1.1 Protein synthesis, from mRNA to protein: an overview

Following transcription, processing and nucleocytoplasmic export, mRNAs are competent for translation. The complex process of translating mRNA into protein can be divided into four stages: initiation, elongation, termination and recycling (Figure 3.1). During initiation, the ribosome is assembled at the initiation codon in the mRNA with a methionyl initiator tRNA bound in its peptidyl (P) site. During elongation, aminoacyl tRNAs enter the acceptor (A) site where decoding takes place. If the correct tRNA is present, the ribosome catalyzes the formation of a peptide bond. After, tRNAs and the mRNA are translocated such that the next codon is moved into the A site, and the process is repeated. Termination takes place when a stop codon is encountered and the finished peptide is released from the ribosome. In the final stage, recycling, the ribosomal subunits are dissociated, releasing the mRNA and deacylated tRNA and setting the stage for another round of initiation (Kapp and Lorsch 2004).

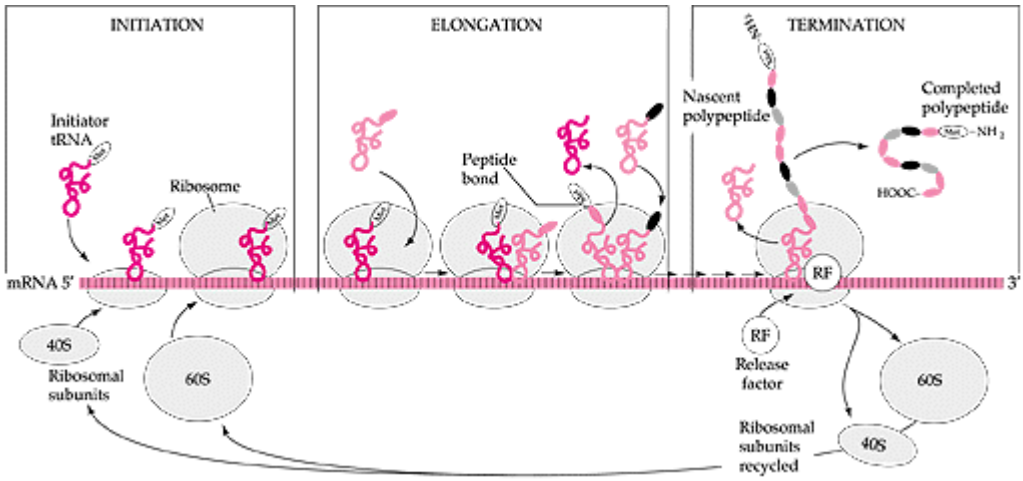

Figure 3.1 Schematic representation of the events of eukaryotic translation. The initiation steps bring together the 40S and 60S ribosomal subunits, mRNA, and the initiator tRNA, which is complexed to the amino acid methionine (Met). During elongation, amino acids are brought to the polysome, and peptide bonds are formed between the amino acids. The sequence of amino acids in the growing protein is directed by the sequence of nucleic acid codons in the mRNA. After the last peptide bond of the protein has been made, one of the codons UAG, UGA, or UAA signals the termination of translation. The ribosomal subunits and message can be reutilized (Picture taken from Scott F. Gilbert, 7th edition, "A companion to developmental biology").

One mechanism used by eukaryotes to regulate gene expression is the control of translation rate. Translational regulation plays critical roles in cell growth, proliferation, and development. Advantages of translational control include rapid response and a mean to affect the level of gene product in the absence of transcription (Mathews 1996). Translation rates can be controlled at each of the three steps of translation: initiation, elongation and termination. However, regulation occurs predominantly during the initiation step. The rate-limiting step in translation initiation under most circumstances is binding of the 43S preinitiation complex to mRNA. This step is the primary target for translational control (Gingras et al., 1999). Two general modes of control exist. There is a global control in which the translation of most mRNAs in the cell is regulated and mRNA-specific control whereby the translation of a defined group of mRNAs is modulated without affecting general protein biosynthesis (Gebauer and Hentze 2004). Global regulation mainly occurs via the modification of eIFs (translation initiation factors), whereas mRNA-specific regulation is driven by regulatory protein complexes that recognize specific elements usually present in 5' and /or 3'-UTRs of target elements (Dever 2002; Wilkie et al., 2003; Gebauer and Hentze 2004)

3.1.2 Dendrites as separate translational compartments - local protein synthesis

Although direct behavioral evidence for the contribution of local translation in memory storage is limited, there is clear evidence that local translation plays a key role in synaptic plasticity (Sutton and Schuman, 2006). Given that transcriptional activation at the cell soma is required for late-phase LTP in normal hippocampal slices (Nguyen et al., 1994), initial ideas regarding the sites of translational control naturally focused on the cell soma, and early studies provided some support for this idea (Frey et al., 1989). More recent studies have suggested, however, that in some circumstances, translation in the dendrites themselves is critical, and even that somatic translation may be dispensable. For example, the neurotrophin BDNF induces potentiation of CA3-CA1 synaptic transmission in hippocampal slices from which CA1 dendrites have been surgically isolated from their cell bodies, and this effect requires new protein synthesis (Kang and Schuman, 1996). In a similar slice preparation, activation of group 1 metabotropic glutamate receptors (mGluRs) or paired-pulse low-frequency stimulation can induce a form of LTD that requires dendritic, but not somatic, protein synthesis (Huber et al., 2000). Moreover, several recent studies have also demonstrated that isolated hippocampal dendrites of pyramidal cells can support protein-synthesis-dependent forms of LTP (Cracco et al., 2005; Vickers et al., 2005; Huang and Kandel, 2005), and that focal dendritic application of protein-synthesis inhibitors in intact slices inhibits late LTP (Bradshaw et al., 2003).

A study has demonstrated a rapid form of synaptic plasticity, elicited by dopamine agonists, that requires local protein synthesis (Smith et al., 2005). Restricted application of a dopamine D1/D5 receptor agonist to a dendritic segment led to an increase in endogenous protein synthesis in cultured hippocampal neurons. In addition, D1/D5 receptor activation led to a rapid, protein-synthesis inhibitor-sensitive increase in the frequency of spontaneous miniature excitatory postsynaptic currents (mEPSCs or “minis”), likely mediated by an increase in the number and size of synaptic GluR1 particles. These data suggest that local protein synthesis may be important for the conversion of synapses from a silent to an active state. Taken together, these studies indicate that the site where plasticity is initiated can determine the cellular location where protein synthesis is required.

3.1.3 MicroRNA: role in regulation of protein synthesis

The discovery of microRNAs (miRNAs) has introduced an important new layer of regulatory control of gene expression. miRNAs are short non-coding RNAs, about 21 nucleotides long, that modulate gene expression at the post-transcriptional level by guiding cellular machinery to the 3′-untranslated region (3′-UTR) of specific messenger RNAs (mRNAs) to control their expression. Most miRNAs are transcribed by polymerase II, although a few human miRNAs have been shown to be transcribed by polymerase III (Borchert et al., 2006). The primary transcript (pri-miRNA) can be up to several thousands nucleotides long and contains internal hairpin structures. Within the nucleus, the pri-miRNA is processed by the RNase III enzyme Drosha, resulting in a ~70 nt long hairpin precursor miRNA (pre-miRNA) containing a 2-nt 3′overhang. This overhang is recognized by Exportin-5, which transports the pre-miRNA into the cytoplasm (Figure 3.2). In the cytoplasm, the pre-miRNA is further cleaved by the RNase III enzyme Dicer. This results in the formation of an intermediary miRNA: miRNA* duplex consisting of the ~21 nt mature miRNA and its star sequence, miRNA*. Following unwinding of the miRNA duplex by a helicase, the mature miRNA is incorporated into the RNA-induced silencing complex (RISC), whereas the miRNA* is usually degraded. Binding of a miRNA to its target mRNA requires both RISC and the presence of Argonaute (Ago) proteins. Depending on the degree of complementarity between the miRNA and its target, the target mRNA can either be cleaved and degraded or translationally repressed (Figure 3.2). Perfect complementarity induces degradation of the mRNA, whereas non-perfect complementarity results in translational inhibition. In animals, miRNA silencing of gene expression is predominantly mediated by translational blockade. The miRNA induced translational inhibition appears to be reversible in a few instances (Bhattacharyya et al., 2006; Schratt et al., 2006), rendering the miRNA mediated regulation dynamic and responsive to specific cellular needs.

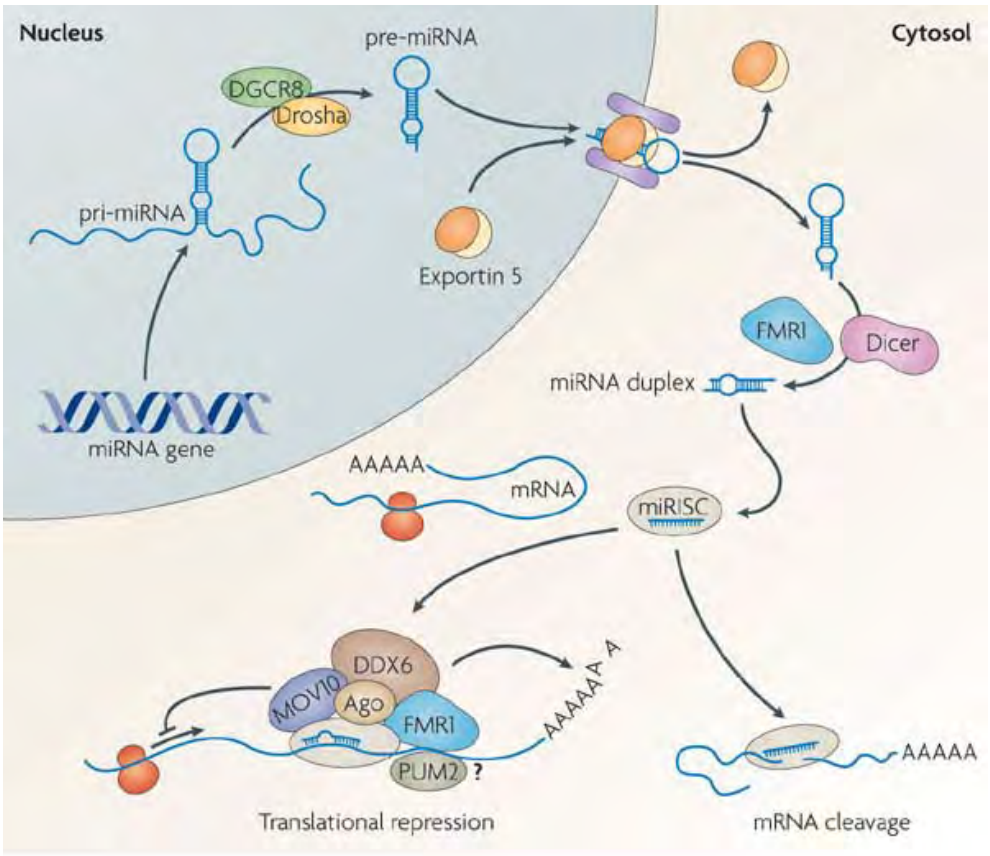

Figure 3.2. RNA biogenesis and mode of action (Picture taken from Schratt 2009).

3.1.4 Synaptic protein synthesis associated with synaptic plasticity

New protein synthesis is required for certain forms of long-term memory establishment and in some cases newly synthesized proteins derive from the local translation of mRNAs within neuronal processes. A subset of these mRNAs is located in the dendritic shaft and in spines (Sutton and Schuman, 2006; Siegel et al., 2011), small actin-rich protrusions from dendrites and the primary sites of excitatory synaptic contact. Several recent findings suggest that miRNA function in the translational control of dendritically localized mRNAs. Both miRNAs and pre-miRNAs have been detected in synaptoneurosomes (Lugli et al., 2008; Siegel et al., 2009). The involvement of miRNAs in the regulatory control of local protein synthesis at the mammalian synapse was provided by a study of brain-specific miRNA, miR-134, in cultured hippocampal neurons (Figure 3.3). miR-134 is located in the synaptodendritic compartment where it co-localizes and inhibits translation of Lim-domain containing protein kinase 1 (Limk1), a regulator of actin filament dynamics involved in dendritic spine morphology. The downregulation of Limk1 protein synthesis restricts the growth of dendritic spines, thereby limiting excitatory synapse development. Interestingly, the interaction between Limk1 mRNA and miR-134 is relieved by exposure of neurons to brain-derived neurotrophic factor (BDNF), a neurotrophin secreted in response to synaptic stimulation (Figure 3.3) (Schratt et al., 2006; Fiore et al., 2008). The overexpression of miR-134 produced a dendritic spine volume phenotype similar to Limk1 loss of function. Schratt and colleagues have found that membrane depolarization elevated the expression of the miR-134 precursor. The induction of miR-134 expression could form part of a negative feedback loop that restores RISC silencing after an initial burst of synaptic protein synthesis. But the mechanism underlying the switch between translational inhibition and activation of Limk1 or other proteins by miR-134 is currently unkown.

In an interestingly related study, Vo et al. found the expression of a microRNA (miR-132) to be a direct target of BDNF regulation, which was mediated through the transcription factor CREB (Vo et al., 2005). A computational search predicted that P250GAP (a possible regulator of Rho or Rac) would be a target of miRNA132. Both miRNA-132 overexpression and Knockdown of P250GAP expression induced neurite outgrowth in transfected rat primary cortical neurons. These observations indicate that CREB acts through the miR-132-mediated silencing of P250GAP to promote neurite growth (Vo et al., 2005).

miR-138, another brain-specific miRNA, is located in dendrites and regulates negatively spine size. miR-138 inhibits the expression of Acyl protein thioesterase1 (APT1), a depalmitoylating enzyme controlling the palmitoylation status of proteins at the synapse and hence their membrane association. Several signalling molecules are substrates for APT1, notably G protein alpha subunits (Gα). All together, evidences show that synaptic protein synthesis under miRNA control is associated with synaptic plasticity.

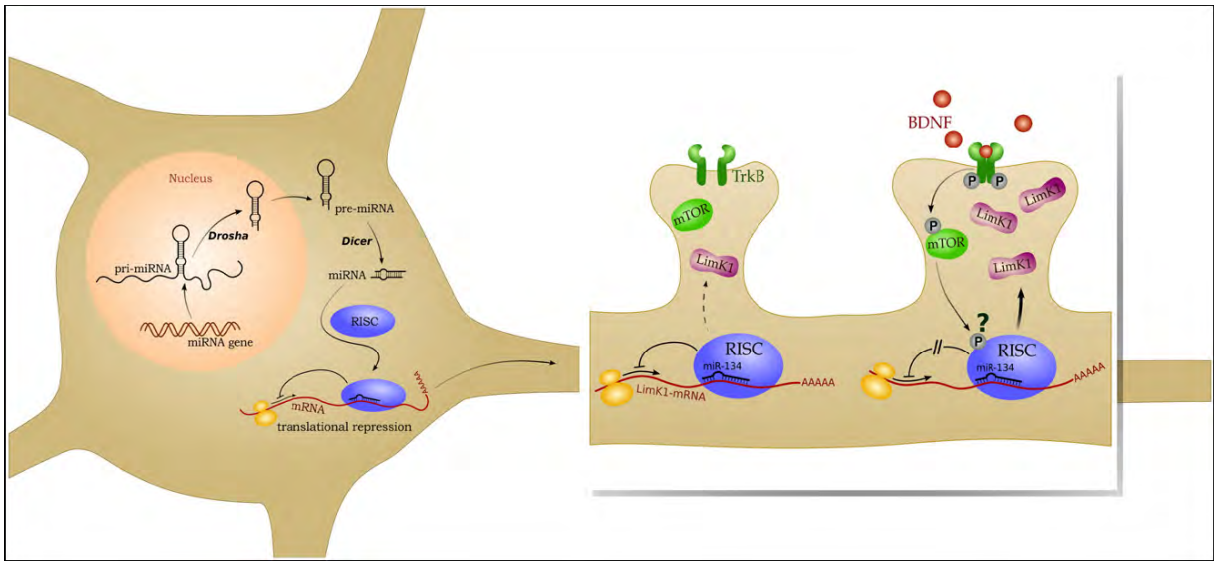

Figure 3.3. MicroRNA function in dendritic spine morphogenesis and synaptic plasticity. Dendritic microRNAs, such as miR-134, are synthesized and processed in the neuronal cell soma, where they are presumably assembled together with their target mRNAs (e.g. Limk1) into transport granules that mediate localization to the synaptodendritic compartment. Inset: within dendrites, the miR-134/Limk1 complex is stored close to dendritic spines in a translationally inactive state due to the inhibitory function of miR-134. Upon synaptic stimulation, the release of BDNF inactivates miR-134 by an unknown mechanism, giving rise to Limk1 mRNA translation and spine remodeling. Note that the mechanism of dendritic transport of miR-134 and other miRNAs has not yet been experimentally addressed and could involve transport of miRNA precursors into dendrites and local processing. (Taken from Fiore et al., 2008)

Mammalian hippocampal neurons are enriched in miRNA-134 that inhibits translation of Limk1 (a protein involved in spine development), thus blocking excitatory synaptic transmission and size of dendritic spines. But, the molecular mechanism by which miRNAs silence the expression of their target mRNAs remains unclear. As previously described, neuronal activity via BDNF release derepressed the miR-134–mediated translation inhibition of Limk1 mRNA (Schratt et al., 2006). In chapter 2, we observed that MCT2 seems to be regulated by a translational mechanism following BDNF stimulation both in primary neuronal cultures and in synaptoneurosome preparations (Robinet and Pellerin 2010). Because

miRNAs regulate the expression of target genes affecting protein translation and since a single miRNA is able to target up to a few hundreds of different mRNAs (Lewis et al., 2005), we decided to investigate the putative regulation of MCT2 translation by miRNA, and more specifically as a new target of miR-134 following BDNF stimulation.

3.2 Experimental procedure

Neuronal Cultures and Pharmacological Treatments

Primary cultures of mouse cortical neurons were prepared from embryonic day 17 OF1 mice (Charles River, Lyon, France). As described previously, after decapitation and brain dissection, cortices were mechanically dissociated in phosphate-buffered saline (PBS) supplemented with glucose (NaCl, 150 mmol/L; KCl, 3 mmol/L; KH_2PO_4, 1.5 mmol/L; Na_2HPO_4, 7.9 mmol/L; glucose, 33 mmol/L; penicillin, 0.006 g/L; streptomycin, 0.1 g/L; pH 7.4). Cells were plated on poly L-ornithine (15 mg/L)-precoated dishes and cultured in neurobasal-B27 medium supplemented with 0.5 mmol/L L-glutamine. All experiments were carried out on day 7 *in vitro*. At this stage, cultures contained <5% of glial cells. Neuronal treatments with pharmacological agents were carried out without changing the medium before or during the incubation time. Brain-derived neurotrophic factor (CYT-207; Brunschwig, Basel, Switzerland) was added directly into the culture medium at various concentrations and cells were incubated for the indicated times. All other chemicals were purchased from Sigma.

Neuronal transfection

After 6 days, primary neuronal cultures were transfected either with the siRNA Alexafluor 488 or with the anti-miRNA (anti-miR134 or anti-miR132) using a lipid-based transfection reagent (HiPerFect, Qiagen). The appropriate amount of siRNA or anti-miRNA (5µl from initial concentration of 20µM, for a final concentration of 50nM) was diluted in Neurobasal medium, and then, 5 μL of transfecting agent (lyophilized samples were diluted in H_2O at a concentration of 1 mg/mL, storage at 4 °C) were added and diluted in Neurobasal medium to a final volume of 1ml. The medium was mixed and incubated at room temperature for 10 min to allow transfection complex formation. The transfection complexes were added dropwise onto the cells. The cells were incubated at 37°C until silencing analysis. The transfection was visualized by immunocytochemistry.

Immunocytochemistry and Related Quantification

After removal of the culture medium, cells were carefully rinsed in PBS at 37°C and directly postfixed in an ice-cold paraformaldehyde fixative (4% in PBS for 30 mins at 20°C). Fixed cells were treated with casein (0.5% in PBS) for 1 h at room temperature to block nonspecific sites. For immunostaining, cultures were incubated overnight at 4°C in 50 μL of freshly prepared MAP2 antibody solution (anti-MAP2 diluted 1:200 in PBS containing 0.25% bovine serum albumin). After carefully rinsing in PBS, cultures were incubated in a solution containing Cy3-conjugated anti-rabbit Igs (diluted 1:500 for 2 h at room temperature; Jackson Immunoresearch, Baltimore, MD, USA). After rinsing in PBS twice and a final rinsing in water, coverslips were mounted with Vectashield (Reactolab SA, Burlingame, CA, USA). Coverslips were examined and photographed with an Axioplan2 microscope (Zeiss, Hallbergmoos, Germany) using epifluorescence with an appropriate filter.

Western Blot and Related Quantification

Neurons in each culture dish were homogenized in 50 μL of buffer containing the following: Tris-HCl, pH 6.8, 20 mmol/L; sucrose, 0.27 mol/L; EGTA (ethylene glycol tetraacetic acid), 1 mmol/L; EDTA (ethylene diamine tetraacetic acid), 1 mmol/L; NaF, 50 mmol/L; Triton X-100, 1%; β-glycerophosphate, 10 mmol/L; DTT (dithiothreitol), 10 mmol/L; 4-nitrophenylphosphate, 10 mmol/L; and a mixture of protease inhibitors (Complete, Roche Molecular Biochemicals, Mannheim, Germany). Each stimulated condition was examined in duplicate and the contents of the two Petri dishes were pooled. Protein samples were sonicated and heated at 95°C for 5 mins in half the final volume of SDS-PAGE sample buffer (Tris-HCl, 62.5 mmol/L; DTT, 50 mmol/L; SDS, 2%; glycerol, 10%; and bromophenol blue, 0.1%). Samples were loaded onto polyacrylamide gels composed of a 10% or 6% acrylamide-bisacrylamide running gel and a 4.5% acrylamide-bisacrylamide stacking gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes (Trans-Blot Transfer Medium 162-0115; Bio-Rad, Reinach, Switzerland) using a Transblot semi-dry transfer cell (Bio-Rad). For protein detection, membranes were incubated in a blocking solution of Tris-buffered saline supplemented with Tween-20 (TBST; Tris-HCl, pH 7.5, 50 mmol/L; NaCl, 150 mmol/L; and Tween-20, 0.1%) containing 5% nonfat milk for 1 h at room temperature. Membranes were incubated overnight at 4°C with anti-MCT2 (Pierre et al., 2000), anti-Limk1 (Cell signaling) and anti-β-actin (Sigma). After three washes in TBST, membranes were

incubated with the secondary antibodies Alexa Fluor 680 goat anti-IgG (Juro, Lucerne, Switzerland) and IRDye 800 goat anti-mouse IgG (BioConcept), diluted at 1:5,000 in TBST containing 1% nonfat milk, for 2 h at room temperature, and protected from light. After three washes in TBST, membranes were scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA), which permits detection and quantification of proteins of interest. β-Actin, revealed in green, was used for normalization and MCT2 was revealed in red.

3.3 Preliminary results

In order to study the putative control of miR-134 on MCT2 expression following BDNF stimulation, the transfection of an inhibitor of miR-134 (Anti-miR134) was performed on primary cultures of cortical neurons. If miR-134 regulates MCT2 expression, we would expect to obtain an upregulation of MCT2 after transfection of the Anti-miR-134. First, we controlled the transfection quality and transfection rate of a fluorescent siRNA (siRNA Alexafluor 488) using the Hiperfect transfection reagent on primary neuronal cultures (Figure 3.4, a,b,c). We performed a MAP2 immunostaining (in red) of neuronal cultures to localize siRNA Alexafluor 488 (in green) in neurons. Results showed that siRNA Alexafluor 488 seems to be associated with neuronal processes, suggesting that some siRNA Alexafluor 488 has entered into the cell (Figure 3.4, a). However, we also observed that these siRNAs were bound together, often forming fluorescent clusters outside cells, that probably partially prevented their internalization into cells (Figure 3.4, b,c).

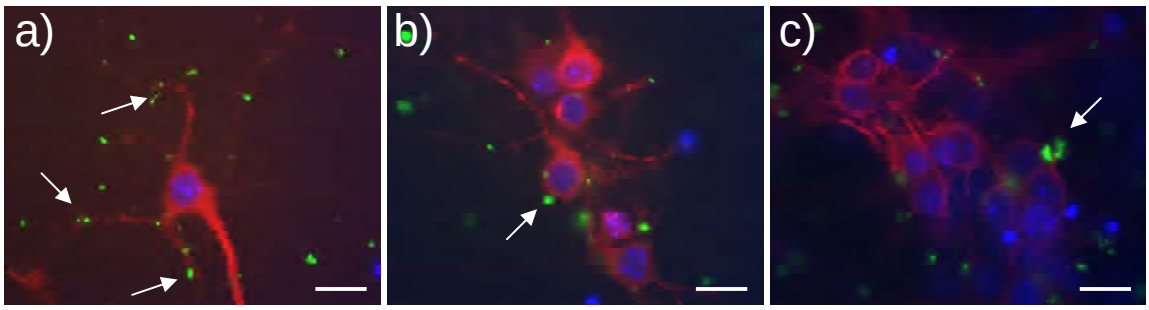

Figure 3.4. Transfection of primary cultures of mouse cortical neurons treated with siRNA Alexafluor 488 (in green) using HiPerFect transfection Reagent (Qiagen) at a final concentration 2.75µl/ml. Panels represent immunocytochemical stainings for MAP2 (in red). The nuclei are labelled with DAPI (in blue). Magnification, 63X ; calibration bar, 15µm. As pointed out by white arrows, fluorescent siRNAs were associated with neuronal processes (a) but often formed clusters outside cells (b,c).

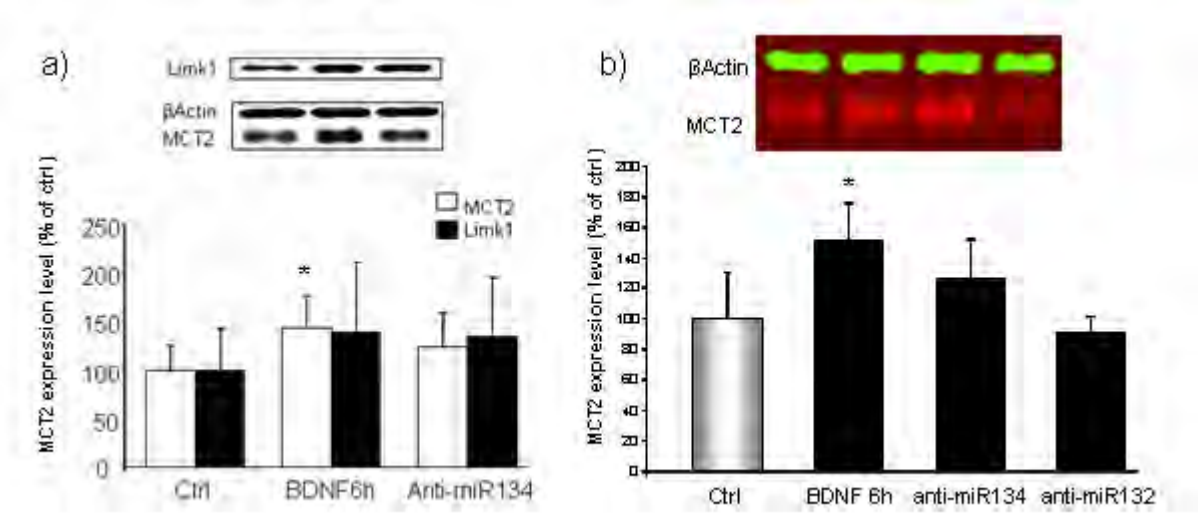

Figure 3.5. Effect of miRNA inhibitors (anti-miR134 and anti-miR132) on MCT2 expression following BDNF stimulation in primary neuronal cultures. Primary cultures of mouse cortical neurons are pretreared for 10 mins with anti-miR (2.75µl/ml). Neurons treated with BDNF 100ng/ml for 6h were used as positive control. The bar graph represents the quantitative determination of fluorescent intensity corresponding to MCT2 IR in cultured neurons treated or not with anti-miR followed by exposure to BDNF for 6h. Results are expressed as percentage of control fluorescence intensity and reprensent the mean +/- s.e.m. from ten distinct experiments. One-way ANOVA followed by Dunnet's post hoc test were used for data analysis. P-values < 0.05 were considered as statistically significant.

To assess the effect of Anti-miR134 on neuronal MCT2 protein and Limk1 protein expression (used as positive control), cultured cortical neurons were treated with the Anti-miR134 for 24h prior to add BDNF (100 ng/mL) for 6 h. We used the miR-132 repressor as negative control. Indeed, it is known that miR-132 is a direct target of BDNF regulation, which seems to act through the transcription factor CREB. Western blot analysis showed that BDNF significantly increased MCT2 expression ($p<0.05$). However, BDNF did not significantly increase Limk1 expression due to the high variability obtained (Figure 3.5a). The transfection of the Anti-miR134 had a tendency to increase the level of MCT2 and Limk1 but these effects were not significant statistically ($p=0.061$, $n=10$) (Figure 3.5a). Concerning the effect of anti-miR132, we did not observe any effect on MCT2 expression (Figure 3.5b).

Despite the fact that transfection levels were very low, we could still observed a tendency toward an increase in the level of MCT2 protein by inhibiting the miR-134. These results suggest to us that if we could improve the transfection rate, we would obtain a significant effect of the Anti-miR134 on MCT2 expression. Here, the use of viral vectors might be a useful alternative approach that was used successfully by Christensen and co-workers for this

purpose (Christensen et al., 2010). Indeed, they obtained very good transfection rate and low cell death.

3.4 Discussion and perspectives

Brain-specific miRNAs play a role in synaptic function, and in CNS neurons, BDNF is also a key player in synaptic plasticity. The interaction between BDNF and brain-specific miRNAs is an area of recent focus. Remarkably, the loss of brain-specific miRNAs enhances learning and memory in mice (Konopka et al., 2010). The authors show that in Dicer mutant mice, the efficacy at hippocampal synapses and translation of BDNF was higher than in control (Konopka et al., 2010). As mentioned above, Schratt et al. reported involvement of miR134 in BDNF-regulated dendritic spine size in hippocampal neurons (Schratt et al., 2006). The miR-134 is localized in dendrites, and negatively regulates spine size by repressing the translation of Limk1 mRNA, which is known to regulate dendritic structures (Meng, 2004). They demonstrated that BDNF relieves suppression of Limk1 translation caused by miR-134 (Schratt et al., 2006). In the same manner, our results tend to show that BDNF seems to inhibit the suppression of MCT2 translation caused by miR-134 in primary neuronal culture. A recent study shows that miR-134 regulates memory and neuronal plasticity (Gao et al., 2010). The mammalian Sir2 homolog SIRT1 seems to promote synaptic plasticity by an upregulation of CREB expression via a repression of miR-134, as mutant mice lacking SIRT1 catalytic activity showed reduction in both CREB and BDNF proteins and upregulation of miR134. A knockdown of miR134 rescues the hippocampal long-term potentiation and memory impairments caused by SIRT1 deficiency in mutant mice lacking SIRT1 catalytic activity in a brain-specific manner (Gao et al., 2010). However, until now no study was performed on a putative regulation of miR134 on some synaptic proteins involved in synaptic plasticity, such as AMPA receptor and NMDA receptors. Thus, if miR-134 is involved in synaptic plasticity, and that BDNF increases the level of MCT2 by inhibiting the repressor effect of miR-134, it would further suggest that MCT2 is also implicated in synaptic plasticity processes (as previously suggested in the discussion of chapter 2). However, to confirm these findings, it would be interesting to demonstrate a similar localization of miR-134 and MCT2 mRNA in dendrites by using a technique such as fluorescent in situ hybridization (Fish) (as already performed for MCT2 by Chenal J., thesis report, 2007). Indeed, miR134 was found in the dendritic compartment of hippocampal neurons (Schratt et al., 2006).

Therefore it is tempting to speculate that miRNAs (for exemple miR-134) might also control local protein synthesis, synaptic plasticity (reviewed by Konopka et al., 2011) and energy metabolism in neurons (lactate uptake through MCT2, figure 3.6). But further studies are necessary to test this hypothesis. It would be first important to test the complementarity between the 5' end of the miRNA, called the seed region, and the 3' UTR of the target mRNA, as it appears to be critical for binding. More variability is tolerated in the base pairing at the 3'end of the miRNA (reviewed by Bushati N and Cohen SM, 2007). Then, it would be interesting to study the putative regulation of MCT2 and other postsynaptic proteins such as the AMPA receptor GluR2 subunit via miR-134 in the context of synaptic plasticity. Indeed, we recently demonstrated in our lab that MCT2 and GluR2 are colocalized, follow the same trafficking process and influence their respective levels of expression (Bergersen et al., 2001; Pierre et al., 2009, Maekawa et al., 2009).

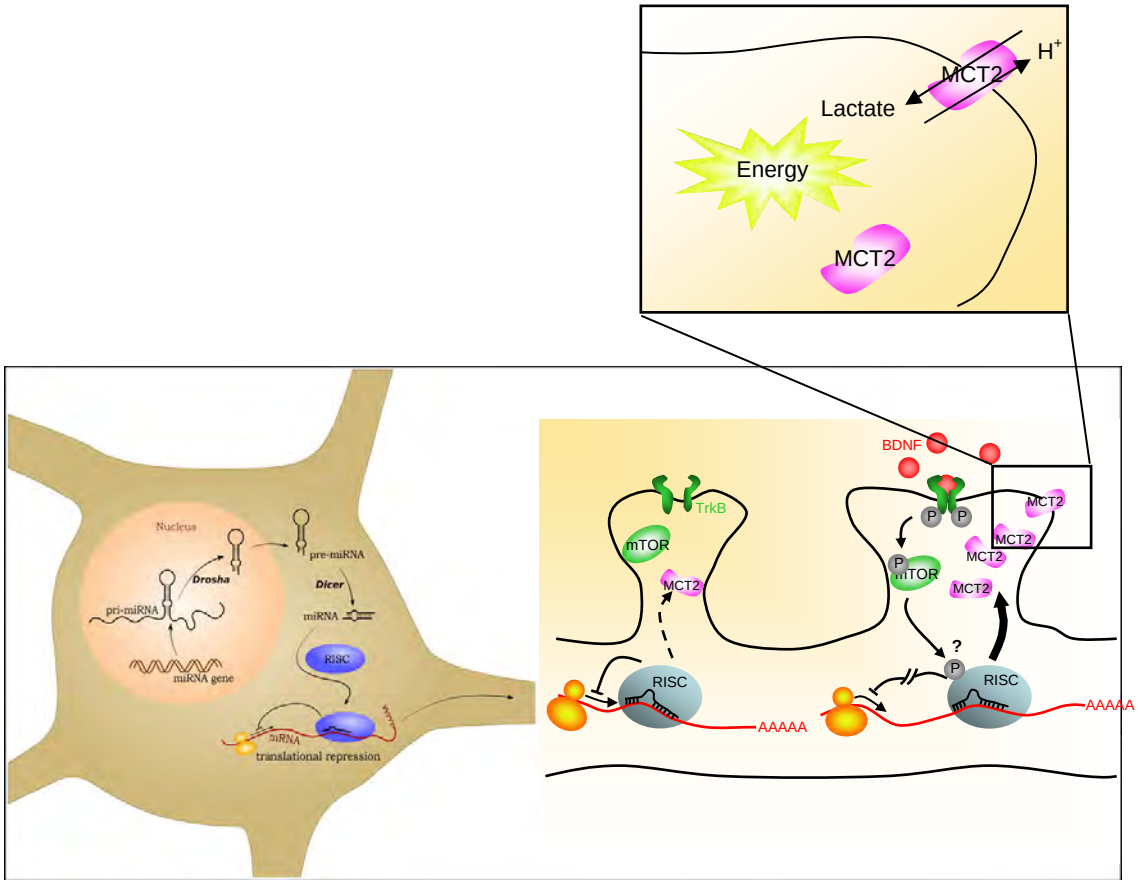

Figure 3.6. MicroRNA function in dendritic synaptic plasticity and energy metabolism. Dendritic microRNAs, such as miR-134, are synthesized and processed in the neuronal cell soma, where they are presumably assembled together with their target mRNAs (e.g. MCT2) into transport granules that mediate localization to the synaptodendritic compartment. Inset: within dendrites, the miR-134/MCT2 complex is stored close to dendritic spines in a translationally inactive state due to the inhibitory function of miR-134. Upon synaptic stimulation, the release of BDNF inactivates miR-134 by an unknown mechanism, giving rise to MCT2 mRNA translation and lactate uptake. (Modified from Fiore et al., 2008)

In contrast to miR-134, miR-132 contributes to the action of BDNF. miR-132 has been identified as a direct target of CREB (cAMP response element binding protein). miR-132 exerts its positive effect by inhibiting the GTPase activating protein p250GAP, a negative regulator of neurite outgrowth. In cultured cortical neurons, BDNF induces upregulation of miR-132 (Vo et al., 2005). Interestingly, overexpression of miR-132 increases the outgrowth of primary neurites, and transfection of an antisense RNA for miR-132 results in a substantial decrease in neurite outgrowth (Vo et al., 2005). Our results showed that miR-132 inhibitor (Anti-miR-132) had no effect on MCT2 expression (Figure 3.5b). An explanation could be that MCT2 expression is independent of the transcription factor CREB. However, we previously observed that the transcription inhibitor Actinomycin D (ActD) prevented BDNF effect on MCT2 expression, suggesting that BDNF needs to activate a transcription factor to have a positive effect on MCT2 translation following BDNF stimulation (Robinet and Pellerin, 2010). In this case, miR-132 was not an appropriate miRNA control for this experiment. Recently it was also reported that BDNF induced a marked increase in miR-132 levels in cultured cortical neurons (Kawashima et al., 2010). While BDNF increased the expression of synaptic proteins including NR2A, NR2B, and GluR1, it was demonstrated that transfection of double strand-miR-132 also up-regulates these glutamate receptors (Kawashima et al., 2010). Similar experiments could be performed in primary neuronal cultures by overexpression of miR-132 to quantify the level of MCT2 expression.

Finally, another miRNA that might regulate MCT2 could be miR-29 (miR-29 a,b,c). miR-29 is abundantly expressed in many tissues including brain, pancreas, liver, heart, in both murine and human models (Smirnova et al., 2005 ; Pullen et al., 2011 ; Roderburg C et al., 2011; Soci UP et al., 2011). It has been studied particularly in pancreatic beta cells. Its overexpression in beta cells is notably associated with type 2 diabetes, leading to insulin resistance (He A et al., 2007). Recently, it has been shown that miR-29 isoforms (miR-29a and miR-29b) contribute to the beta-cell-specific silencing of the MCT1 transporter. Indeed, inhibition of miR-29 isoforms in primary mouse islets increases MCT1 mRNA levels (Pullen et al., 2011). In brain, miR-29 expression is minimal throughout the embryonic and perinatal periods. Expression of miR-29 is enhanced at P14 (Smirnova L et al., 2005). Since MCT1 shares features with MCT2, we would suspect that miR-29 could also bind MCT2 and could regulate its expression. However, miR-29 is more strongly expressed in astrocytes than in neurons (Smirnova L et al., 2005) and there are differences in the 3'-UTR sequences between MCT1

and MCT2 (Halestrap P and Price 1999). Nevertheless, as it constitutes the first example of a MCT regulated by a miRNA, it might indicate that expression of other MCTs in other tissues might be also under control of different miRNAs.

We believed it is likely that MCT2 synthesis can be also regulated by a miRNAs (e.g. miR-134 or miR-132). Until now, very few data are available on the negative control of synaptic protein synthesis during synaptic plasticity. Furthermore, the fascinating possibility of a miRNA-dependent synthesis regulation in neuronal metabolism has never been studied.

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Chapter 4

Chapter 4

Brain-Derived Neurotrophic Factor enhances the hippocampal expression of key postsynaptic proteins *in vivo* including the monocarboxylate transporter MCT2 (Robinet and Pellerin, 2011).

4.1 Abstract

Brain-derived neurotrophic factor (BDNF) promotes synaptic plasticity via an enhancement in expression of specific synaptic proteins. Recent results suggest that the neuronal monocarboxylate transporter MCT2 is a postsynaptic protein critically involved in synaptic plasticity and long-term memory. To investigate *in vivo* whether BDNF can modulate the expression of MCT2 as well as other proteins involved in synaptic plasticity, acute injection of BDNF was performed in mouse dorsal hippocampal CA1 area. Using immunohistochemistry, it was found that MCT2 expression was enhanced in part of the CA1 area and in the dentate gyrus 6 hours after a single intrahippocampal injection of BDNF. Similarly, expression of the immediate early genes Arc and Zif268 was enhanced in the same hippocampal areas, in accordance with their role in synaptic plasticity. Immunoblot analysis confirmed the significant enhancement in MCT2 protein expression. In contrast, no changes were observed for the glial monocarboxylate transporters MCT1 and MCT4. When other synaptic proteins were investigated, it was found that PSD95 and GluR2 protein levels were significantly enhanced while no effect could be detected for synaptophysin, SNAP25, αCaMKII and GluR1. These results demonstrate that MCT2 expression can be upregulated together with other key postsynaptic proteins *in vivo* under conditions related to synaptic plasticity, further suggesting the importance of energetics for memory formation.

4.2 Introduction

The neurotrophin BDNF plays an important role in activity-dependent synaptic plasticity mechanisms such as long-term potentiation (LTP) (Bramham and Messaoudi, 2005). Moreover, it has been proposed to participate in processes leading to memory formation (Yamada and Nabeshima, 2003). Indeed, several studies have shown an increase in BDNF mRNA expression and TrkB activation during memory acquisition and consolidation (reviewed in Yamada and Nabeshima, 2003). Application of BDNF to hippocampal slices facilitates the induction of synaptic potentiation and enhances basal synaptic transmission in CA1 pyramidal neurons (Ji et al., 2010). Similarly, acute intrahippocampal infusion of BDNF leads to a long-lasting enhancement of synaptic transmission in the rat dentate gyrus (Messaoudi et al., 1998) and induces LTP in intact adult hippocampus (Ying et al., 2002). The BDNF-induced long-lasting potentiation requires ERK pathway activation and the upregulation of Arc and Zif268 synthesis (Rosenblum et al., 2002; Ying et al.,2002). *Arc* and *Zif268* are two immediate early genes (IEGs) transiently and rapidly activated during LTP as well as along the process of learning and memory formation (Davis et al., 2003; Plath et al., 2006; Lonergan et al., 2010). Ultimately, it is purported that BDNF exerts its effect on synaptic function by regulating local translation of a specific subset of synaptic proteins (Santos, 2010). While a few of these proteins have been described, more remain to be identified. Moreover, BDNF-induced enhancement in expression of several key synaptic proteins still awaits confirmation *in vivo*.

Apart from classical proteins involved in synaptic transmission, BDNF possibly regulates the expression of proteins involved in other cellular functions. In this regard, putative changes in energetics occurring upon synaptic plasticity and possible regulation of associated proteins have not been explored. Recently, the role of monocarboxylates, and particularly lactate, as additional energy substrates for neurons has attracted attention (Pellerin, 2003). Release and uptake of these substrates are dependent on the presence of specific carriers known as monocarboxylate transporters (MCTs) that are expressed by both neurons and glial cells (Pierre and Pellerin, 2005). Possible BDNF-mediated alterations in expression of these transporters *in vivo* has never been investigated. In the present study, the level of expression of six synaptic proteins, two IEGs, as well as three monocarboxylate transporters were determined in mouse hippocampus 6 hours after a single intrahippocampal infusion of BDNF.

Results clearly indicate that the neuronal monocarboxylate transporter MCT2 is one of the key postsynaptic proteins upregulated as part of the synaptic changes induced by BDNF.

4.3 Experimental procedures

Animals

Seven-week-old C57Bl/6Rj male mice (~20g) were obtained from the Janvier Breeding Center (Le Genest Saint-Isle, France) and received 1 week prior to the experiments to acclimatize them to the animal facility. They were housed in a temperature-controlled environment with 12:12h light-dark cycle and given free access to food and water. Animal experiments were performed in accordance with the Animal Care and Use Committee guidelines (Service vétérinaire du Canton de Vaud, Switzerland).

Surgery and intrahippocampal injection of BDNF

Mice were weighed and anesthetized by intraperitoneal injection with a mixture of Ketamine (24µl) and Xylazine (10µl) diluted in 100µl NaCl 0.9%, and positioned in a stereotaxic apparatus (Stoelting, WI, USA). Rectal temperature was maintained at 36°C with a thermostatically controlled electric heating pad. Stereotaxic coordinates relative to Bregma were: anteroposterior (AP) : -1.85 ; mediolateral (ML) : -1.25 ; dorsoventral (DV): -1.4. A single intrahippocampal injection was performed using a 5 µL Hamilton syringe with a 33-gauge needle. The needle was inserted into the dorsal hippocampal CA1 area and 2 µl of phosphate buffered saline (PBS) or 2 µg of Brain-derived neurotrophic factor (BDNF) in 2 µl PBS were injected slowly during 20 min (Brain-Derived neurotrophic factor human, recombinant expressed in E.coli, Sigma-Aldrich). Mice completely recovered 1 h after the surgery. Mice were sacrificed 6 h after the end of surgery.

Western blotting and related quantification

Once mice had been sacrificed, the brain was rapidly removed and put into a cold solution of phosphate buffered saline (PBS 1X). Hippocampi were rapidly dissected on ice, directly freezed in liquid nitrogen and stored at -80°C.

For western blotting, hippocampal tissue was homogenized and sonicated in a 0.32 M sucrose solution containing HEPES 1 mM, $MgCl_2$ 1 mM, $NaHCO_3$ 1 mM, phenyl-methyl-sulphonyl fluoride 0.1 mM, pH 7.4, in presence of a complete set of protease inhibitors (Complete, Roche, Switzerland). Protein concentrations were determined by the BCA method. Ten

micrograms of protein were heated at 95°C in SDS-PAGE sample buffer (62.5 mM Tris-HCl, 50 mM DTT, 2% SDS, 10% glycerol and 0.1% bromophenol blue) and loaded onto 10% polyacrylamide gels. After electrophoresis, samples were transferred on nitrocellulose membranes (Biorad Laboratories). For protein detection, membranes were incubated in a blocking solution of Tris-buffered saline supplemented with Tween-20 (TBST; Tris-HCl 50 mM pH 7.5, NaCl 150 mM, and Tween-20, 0.1%) containing 5% nonfat milk for 1 h at room temperature. Membranes were incubated overnight at 4°C with a mixture containing the primary antibodies: anti-MCT4 (Santa Cruz, CA, USA), anti-GluR1 and anti-GluR2/3 (Chemicon, CA, USA) diluted 1/500 ; anti-MCT1, anti-MCT2 antibodies, anti-synaptophysin (Sigma, Buchs, Switzerland), anti-SNAP25 (Alomone labs, Jerusalem, Israel), anti-PSD95 and anti-CamKIIα (Cell Signaling, MA, USA) diluted 1/1000 ; anti-βactin (Sigma, Buchs, Switzerland), diluted 1/10000; anti-βtubulin (Sigma, Switzerland), diluted 1/1000. These antibodies were diluted in TBS, 0.1% Tween, 5% bovine serum albumin (BSA). After three washes in TBST, membranes were incubated with the secondary antibodies Alexa Fluor 680 goat anti IgG (Juro, Lucerne, Switzerland) and IRDye_800 antimouse IgG (BioConcept, Allschwil, Switzerland), diluted at 1:5000 in TBST containing 1% nonfat milk, for 2 h at room temperature, and protected from light. For anti-MCT1, peroxydase-conjugated donkey anti-rabbit IgG was used as secondary antibodies (Amersham, Piscataway, NJ, USA) diluted 1:10000. After three washes in TBST, membranes were scanned and quantified with the Odyssey®Infrared Imaging System (LI-COR® Biosciences, Lincoln, NE, USA). For MCT1 detection, Enhanced Chemiluminescence (ECL) was required for detection and ImageJ software (http://rsbweb.nih.gov/ij/) was used for the quantification. β-Actin or β-Tubulin was used for normalization of proteins of interest.

Immunohistochemistry

Under urethane anaesthesia (1.5g/kg i.p.), animals were perfused intracardially with a heparinised solution of saline (25 IU/ml in 0.9% NaCl, during 2 min) followed by a freshly prepared solution of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4, for 15 min). Brains were removed and fixed in 4% paraformaldehyde for 2h, then cut on a vibratome (Leica VT1000M) to obtain coronal sections (40 µm thick), which were collected in phosphate buffer saline (PBS). Sections were rinsed several times in PBS and incubated with casein (0.5% in PBS) for 1 h to block non-specific sites. They were incubated overnight at 4°C in freshly prepared primary antibody solution diluted in PBS containing 0.25% BSA:

anti-MCT2 (1:500), anti-MAP2 (1:200; sigma), anti-Arc (1:200; Santa Cruz Biotechnology, Heidelberg, Germany), anti-EGR1 (1:500; Cell Signaling, Allschwil, Switzerland).
After careful rinsing in PBS, sections were incubated in a solution containing Cy3-conjugated anti-rabbit Igs (diluted 1/500, 2 h, room temperature; Jackson Immunoresearch, MD, USA) and/or anti-mouse FITC-conjugated Igs (diluted 1:200, 2h, room temperature; Jackson Immnoresearch). After rinsing in PBS twice and a final rinsing in water, slices were mounted with Vectashield-DAPI (Reactolab SA, CA, USA).

Microscopy

Brain slices were examined and photographed with an Eclipse 80i microscope (Nikon, Kingston, England) using epifluorescence with an appropriate filter together with the NIS-Elements Microscope Imaging 5.1 software (Nikon). They were also examined under a Zeiss LSM 710 Quasar confocal microscope using the DPSS 561-10, 561nm laser illumination. Stacks were made of images taken with 0.5 to 0.8µm step size. Pictures were treated with Imaris software, version 7.0.0.

Statistical analysis

All values are presented as mean ± SEM. Mann Whitney's test, or one-way ANOVA followed by Dunnett's post-hoc test were used for data analysis with GraphPad Prism® software, version 5.0 (La Jolla,CA). P values < 0.05 were considered as statistically significant.

4.4 Results

Intrahippocampal injections of BDNF (or PBS) were made in the right (or left) hippocampus of 7 week-old C57/Bl6 mice. Six hours later, hippocampal expression of MCT2 was examined by immunohistochemistry on coronal sections taken at different levels along the anteroposterior axis. Results showed that MCT2 immunofluorescence was stronger in a portion of the pyramidal cell layer of the CA1 area as well as in the granule cell layer of the dentate gyrus for the BDNF-injected hippocampus compared to the contralateral, PBS-injected structure, as indicated by arrows (Fig.1).

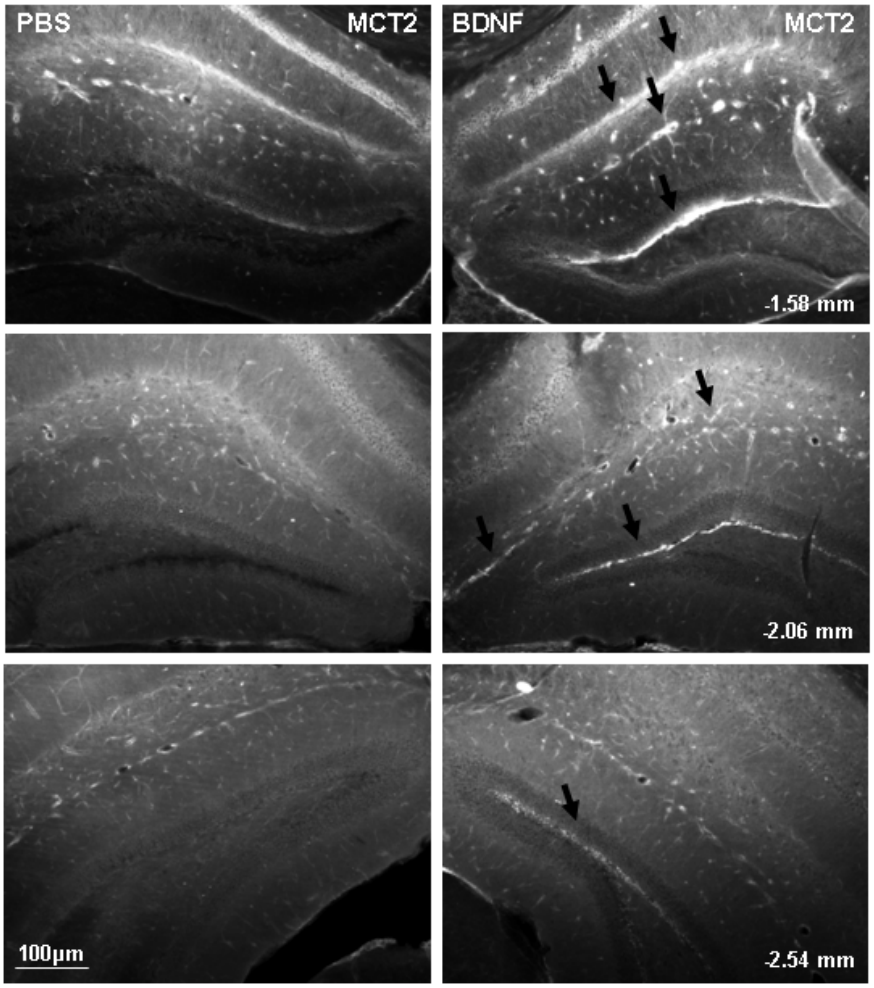

Figure 1. Effect of acute intrahippocampal injection of BDNF *in vivo* on MCT2 protein expression. MCT2 immunolabelling in coronal brain sections at three different hippocampal levels 6 hours after injection of either PBS (left column) or 2 µg BDNF (right column). As pointed out by arrows, stronger immunolabelling is visible in portions of the hippocampus injected with BDNF compared to PBS, particularly in the CA1 area and in the dentate gyrus. Magnification, 10x; Calibration bar, 100µm. Numbers at the bottom right on right column images refer to position from bregma.

No sign of lesion was detected in the surroundings of the needle tract. Confocal microscopy showed at high magnification a visible increase of MCT2 staining in neurons after BDNF treatment compared to PBS control (Fig. 2).

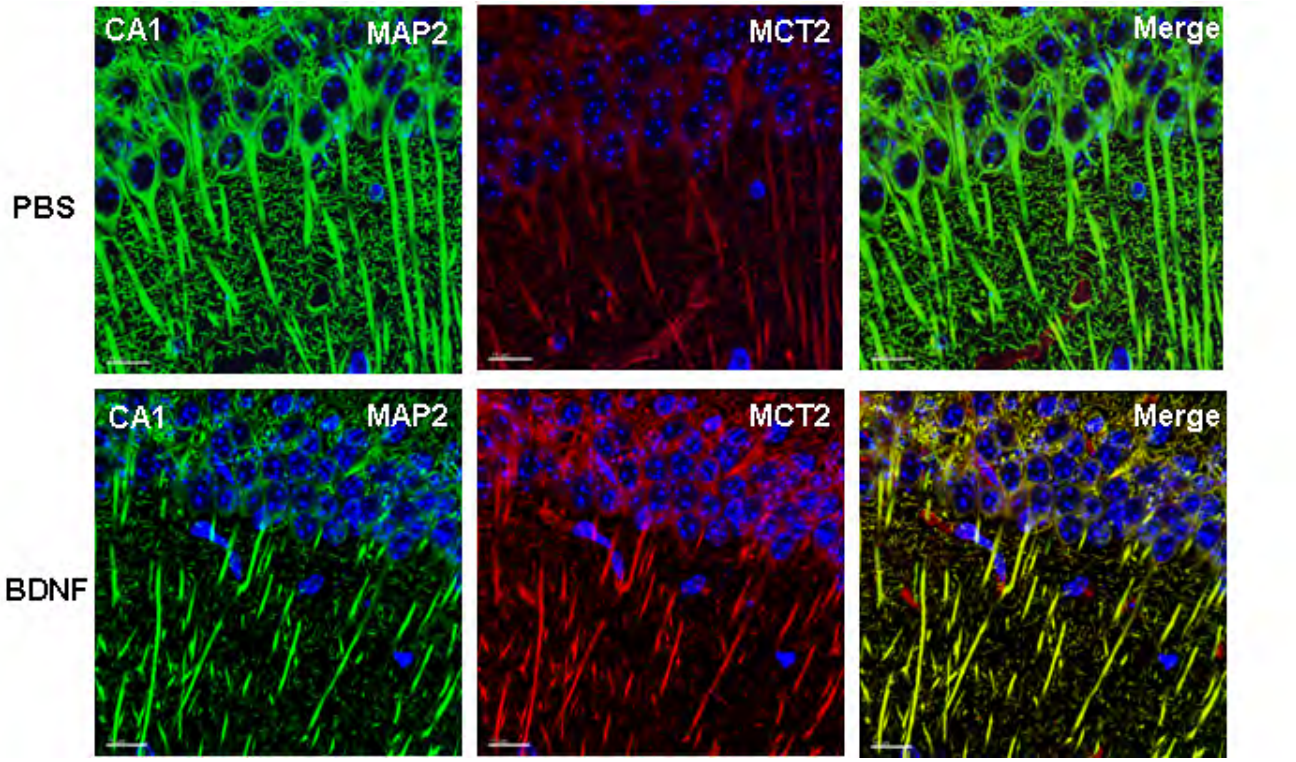

Figure 2. Effect of acute intrahippocampal injection of BDNF *in vivo* on neuronal MCT2 expression in the hippocampal CA1 area. Double immunofluorescent labellings for the neuronal marker MAP2 (green) and MCT2 (red) in coronal hippocampal sections (-2.06 mm from bregma) obtained after 6 hours from animals injected with either PBS (top row) or 2 µg BDNF (bottom row). A stronger MCT2 immunolabelling is clearly visible in CA1 pyramidal neurons after BDNF injection compared to control (PBS). Pictures were obtained with a confocal microscope and images correspond to a projection of 18 sections of 0.5µm. Magnification, 63x ; Calibration bar, 15µm.

Under the same conditions, the expression of two immediate early genes, *Arc* and *Zif268*, known to be implicated in synaptic plasticity, was evaluated by immunohistochemistry. Double immunostainings for Arc or Zif268 proteins with the neuronal marker MAP2 revealed an enhancement in neuronal expression of these two immediate early gene products after BDNF injection compared to PBS control in the same hippocampal area as for MCT2 (Fig. 3).

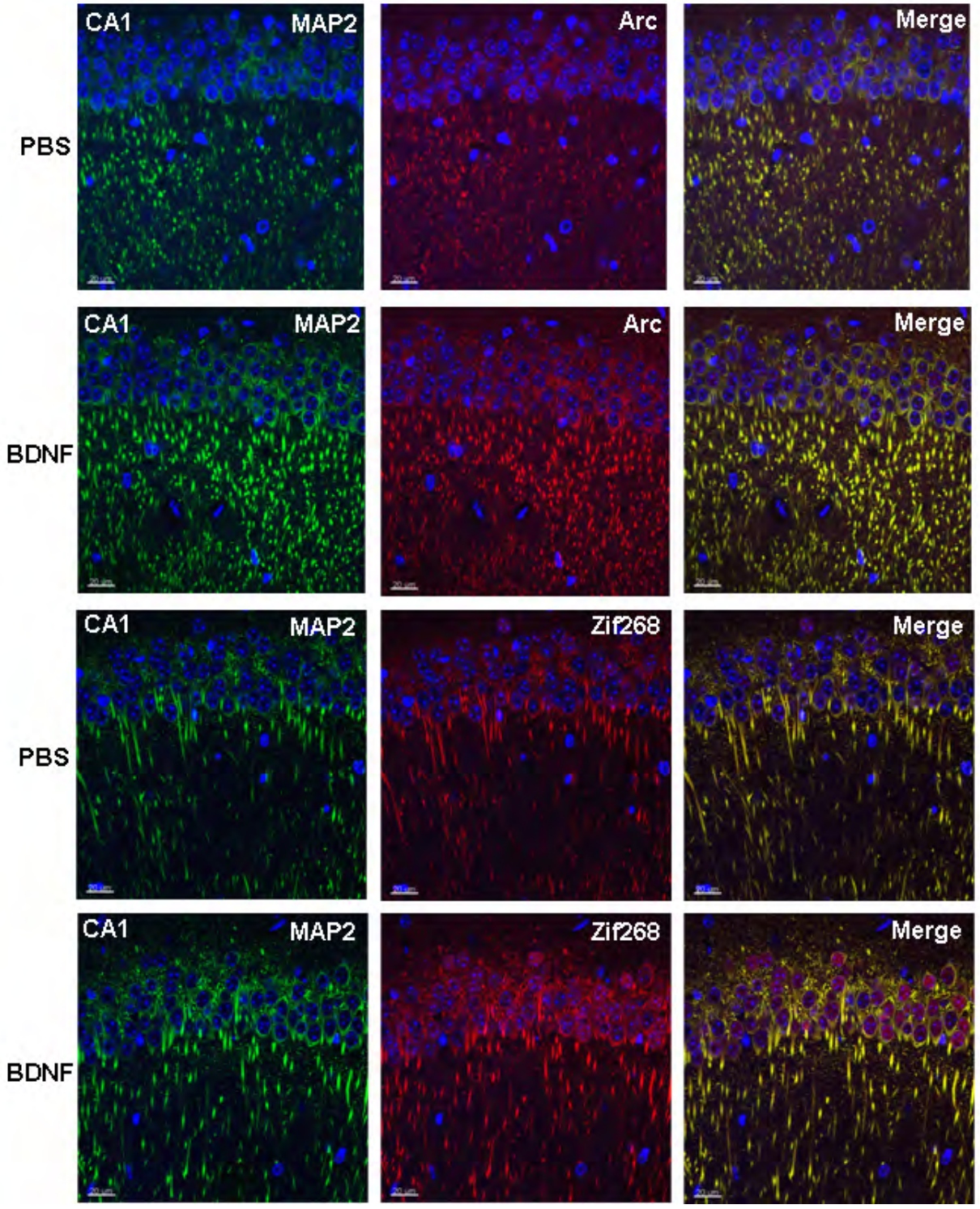

Figure 3. Effect of acute intrahippocampal injection of BDNF *in vivo* on neuronal Arc and Zif268 expression in the hippocampal CA1 area. Double immunofluorescent labellings for the neuronal marker MAP2 (green) and either Arc or Zif268 (red) in coronal hippocampal sections (-2.06 mm from bregma) obtained after 6 hours from animals injected with either PBS (top rows) or 2 µg BDNF (bottom rows). A strong immunolabelling for both Arc and Zif268 is observed in CA1 hippocampal neurons after BDNF injection compared to control (PBS). Pictures were obtained with a confocal microscope and images correspond to a projection of 18 sections of 0.5µm. Magnification, 40x; Calibration bar, 20µm.

In order to further characterize the extent and nature of the changes induced by BDNF, expression levels of several synaptic and extra-synaptic proteins, including MCT2, were determined by immunoblot on hippocampal extracts. Western blot analysis of hippocampal extracts from BDNF-injected tissue showed a significant increase (125 ± 12% vs. control) of MCT2 expression compared to control (no injection, contralateral side) or PBS-injected animals (Fig. 4A). Basal hippocampal MCT2 expression levels were not different between anesthetized (Keta/Xyla+) and unanesthetized (Keta/Xyla-) mice, excluding a confounding effect of anesthesia (Fig. 4B). Expression levels of MCT1 and MCT4, two other monocarboxylate transporters present in the brain but predominantly on glial cells, were not altered (Fig. 4C and D, respectively).

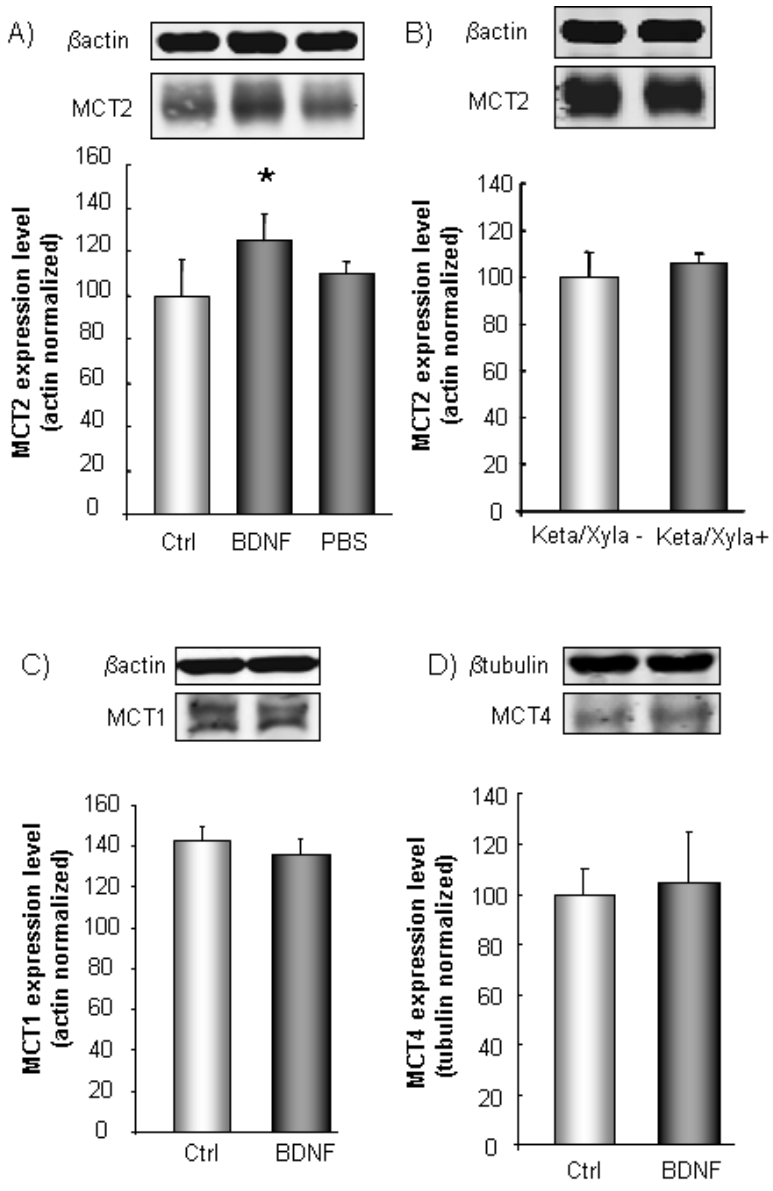

Figure 4. Effect of intrahippocampal injection of BDNF *in vivo* on MCT1, MCT2 and MCT4 protein expression levels. Western blot analysis of MCT2 (A and B), MCT1 (C) and MCT4 (D) expression in hippocampal protein extracts. Right hippocampi were injected with 2µg BDNF and collected after 6h. Left hippocampi were used as control. Effect of anesthesia (Ketamine/xylazine) on hippocampal MCT2 protein expression was also performed as control (B). Western blots were quantified using Odyssey software (LI-COR Biosciences, Lincoln, NE, USA) or ImageJ software (http://rsbweb.nih.gov/ij/). Results are expressed as percentage of control (mean ± s.e.m.) after the values were normalized using β-actin or β-tubulin signal as reference. Statistical analysis was performed using one-way ANOVA followed by Dunnett's test (A) or performed using a Mann Whitney's test (B,C,D). Asterisk indicates MCT2 protein levels significantly different from control (Ctrl) with $p < 0.05$. n = 6 mice for each condition.

In parallel to monocarboxylate transporters, the effect of BNDF on the hippocampal expression of several pre- and postsynaptic proteins was determined by Western blot (Fig. 5A). Quantitative analysis of immunoblots for synaptophysin and SNAP25, two presynaptic proteins, showed no expression change 6 hours after BDNF injection (Fig. 5B and C, respectively). A similar analysis on some postsynaptic proteins gave differential results. No expression change was observed for the protein kinase aCamKII and the glutamate receptor subunit GluR1 (Fig. 5D and F). In contrast, a significant increase of the postsynaptic density scaffold protein PSD95 (118 ± 9%) and of the glutamate receptor subunit GluR2/3 (124 ± 7%) compared to control (no injection) was observed 6 hours after BDNF injection (Fig. 5E and G).

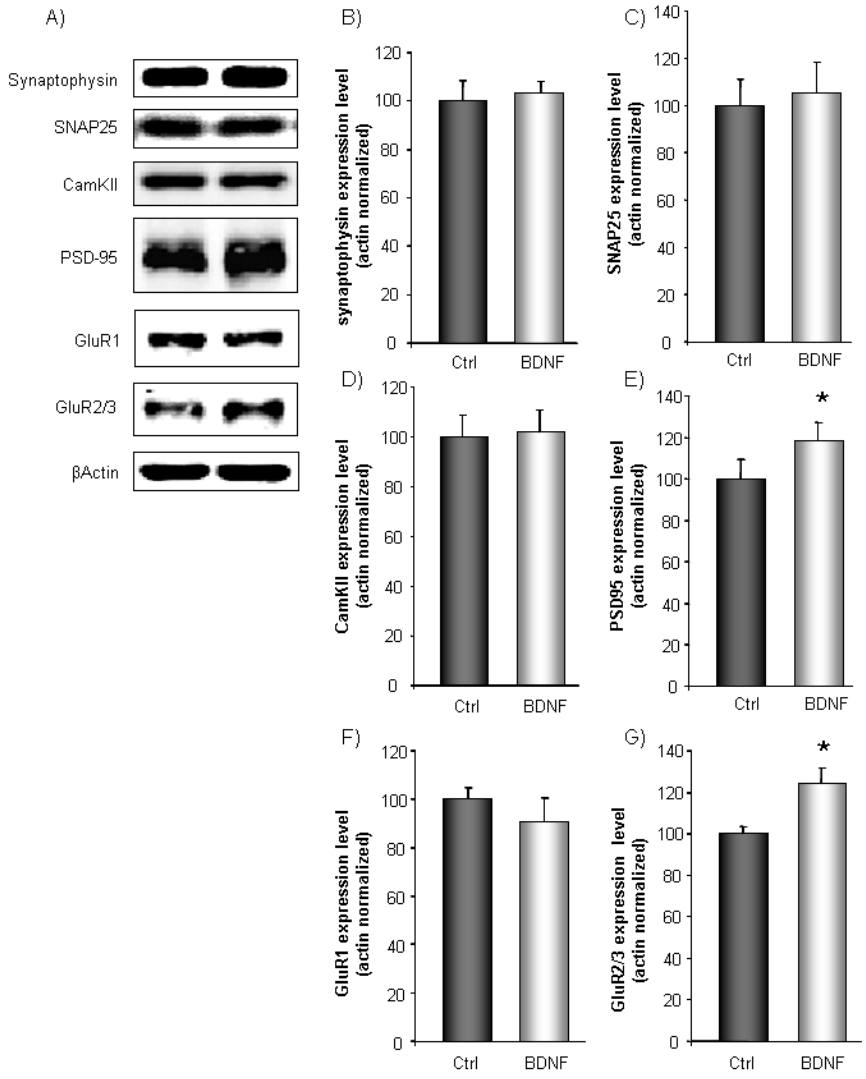

Figure 5. Effect of Intra-hippocampal injection of BDNF *in vivo* on expression levels of several presynaptic and postsynaptic proteins. Western blot analysis of synaptophysin, SNAP25, aCamKII, PSD95, GluR1 and GluR2/3 expression on hippocampal protein extracts (A). Right hippocampi were injected with 2µg BDNF and collected after 6h. Left hippocampi were used as control. Western blot quantification for synaptophysin (B), SNAP25 (C), CamKII (D), PSD95 (E), GluR1 (F) and GluR2/3 (G), performed with Odyssey sofware (LI-COR Biosciences, Lincoln, NE, USA). Results are expressed as percentage of control (mean ± s.e.m.) after the values were normalized using β-actin signal as reference. Statistical analysis was performed using a Mann Whitney's test. Asterisk indicates PSD95 and GluR2/3 protein levels different from control (Ctrl) with $p < 0.05$. $n = 6$ mice for each condition.

4.5 Discussion

As it is proposed to provide the underlying mechanism subserving learning and memory processes, synaptic plasticity is the subject of intense attention. Brain-derived neurotrophic factor represents an essential signal released under learning-associated conditions that contributes to the modifications of synaptic transmission taking place in this context (Gottmann et al., 2009; Yoshii and Constantine-Paton, 2010). Then, activation of TrkB receptors by BDNF leads to a cascade of events including transcriptional and translational steps that will result in the synthesis of a specific subset of synaptic proteins (Santos et al., 2010). However, relatively few information exist *in vivo* about the specific proteins that are subject to regulation by BDNF. Moreover, evidence for a possible modulation of proteins essential in energy metabolism are scarce and limited to *in vitro* (Burkhalter et al. 2007; Robinet and Pellerin 2010). This question was addressed here by investigating the putative changes in expression of several key synaptic proteins including monocarboxylate transporters, a group of proteins involved in energy substrate transfer and supply to neurons, following an intrahippocampal BDNF injection.

MCT2 is the predominant monocarboxylate transporter expressed by neurons whereas MCT1 and MCT4 are expressed by glial cells (Pellerin et al., 2005). At the subcellular level, MCT2 is expressed on axons and dendrites (Pierre et al., 2002; 2009). Moreover, MCT2 is present at glutamatergic synapses and exclusively on postsynaptic elements (Bergersen et al., 2002 ; Pierre et al., 2009). It is particularly enriched in the postsynaptic density of asymmetric synapses as well as in an intracellular pool within spines (Bergersen et al., 2005). Recently, it was demonstrated in cultured neocortical neurons that BDNF induces the expression of MCT2 via a regulation of local translation (Robinet and Pellerin, 2010). Data obtained in the present study showing increased MCT2 protein levels after BDNF injection further extend this observation to the hippocampus *in vivo*.

Interestingly, this increased neuronal expression of MCT2 in the hippocampus following BDNF injection is matched in terms of distribution by a similar enhancement in neuronal expression of both Arc and Zif268 proteins. Induction of the immediate early genes (IEGs) *Arc* (also known as *Arg3.1*) and *Zif268* (also known as *EGR1*) is required for late long-term potentiation (l-LTP) and long-term memory formation (Guzowski et al., 2000; Jones et al., 2001). In parallel, BDNF-induced long-lasting potentiation leads to an upregulation of Arc

and Zif268 synthesis (Rosenblum et al., 2002; Ying et al., 2002). *Arc* mRNA is rapidly transported to dendrites and translated locally where it plays a critical role in local translation as well as in trafficking of AMPA receptors (Bramham et al., 2008). In parallel, Zif268 regulates the transcription of some late response genes and is involved in the degradation of synaptic proteins as well as in receptor trafficking (McDade et al., 2009). Considering their spatially- and timely-related appearance, it is not excluded that Arc and Zif268 could be involved in the regulation of MCT2 expression and subcellular distribution induced by BDNF. Indeed, it was previously shown that BDNF-induced upregulation of MCT2 protein expression in cultured neurons requires a transcriptional step although MCT2 mRNA levels were not affected (Robinet and Pellerin, 2010). Thus, transcriptional activation of *Arc* and *Zif268* might be critical to allow a translationally-regulated enhancement of MCT2 protein levels. Such a possibility will require to be further investigated both *in vitro* and *in vivo*.

BDNF was shown previously *in vitro* to enhance the expression of a few synaptic proteins. PSD95 is a scaffold protein of the postsynaptic density that plays an important role in synaptic plasticity (Gardoni et al., 2009). BDNF can induce the transport of PSD95 to dendrites and it was suggested that activation of the PI3K-Akt pathway by BDNF could lead to enhanced PSD95 synthesis (Yoshii and Constantine-Paton, 2007). Indeed, it was reported that activation of the PI3K-Akt pathway causes an increase of PSD95 expression in hippocampal slices (Lee et al., 2005). In cultured cortical neurons, BDNF was shown to enhance PSD95 protein expression (Matsumoto et al., 2006). Our data provide a confirmation that BDNF can enhance PSD95 protein levels *in vivo*, supporting the idea that more PSD95 proteins are necessary to sustain BDNF-induced synaptic modifications. PSD95 colocalizes with MCT2 both *in vitro* and *in vivo* (Pierre et al. 2009). As PSD95 is a necessary component of the postsynaptic density, it might be required to ensure proper insertion of newly formed MCT2 in the postsynaptic membrane.

AMPA-type glutamate receptors are composed of distinct subunits known as GluR1 to GluR4. The synaptic distribution and levels of GluR1 and GluR2 subunits have been shown to be modulated as part of the mechanism of synaptic plasticity (Sprengel 2006; Keifer and Zheng, 2010). It was demonstrated in both neocortical and hippocampal neurons in culture that BDNF treatment causes an enhancement of GluR1 and GluR2 protein expression levels (Narisawa-Saito et al., 1999; Matsumoto et al., 2006). Our observations *in vivo* that BDNF raises GluR2/3 protein levels partly confirm these data. It also emphasizes the important role

that the GluR2 subunit appears to play in synaptic plasticity (Isaac et al., 2007; Bassani et al., 2009). MCT2 was shown previously to not only colocalize but also directly interact with GluR2 (Pierre et al. 2009). Moreover, changes in the expression levels of MCT2 have been shown to modify both the expression levels and the subcellular distribution of GluR2 subunits (Maekawa et al. 2009). Thus, it appears that MCT2 and GluR2 must be concomitantly regulated in order to fulfill their respective roles in the adaptation to changes in synaptic transmission.

In contrast to PSD95 and GluR2, no change could be detected in the expression of several other proteins. This is the case for the other monocarboxylate transporters MCT1 and MCT4. Since these transporters are rather expressed by glial cells, regulation of their expression might be under the control of other signals that remain to be identified. The protein kinase αCamKII is a postsynaptic protein that has been involved in the process of synaptic plasticity (Miyamoto, 2006). BDNF has been reported to enhance αCamKII protein levels, notably in synaptosomes (Schratt et al., 2004). Surprisingly, no change in αCamKII protein levels could be evidenced *in vivo* under our experimental conditions. Similarly, the expression of the GluR1 subunit was not modified despite being enhanced by BDNF *in vitro* (Matsumoto et al., 2006). A possible explanation for these differences could be related to our experimental protocol. Since a single dose of BDNF was used and observation was made at only one time-point, possible changes in GluR1 and αCamKII protein expression might have been overlooked. Moreover, since hippocampal extracts were prepared from the entire hippocampus while the effect of injected BDNF was restricted to only portions of the tissue, putative differences might have been diluted in Western blot analyses. Further experiments investigating changes in expression at different times after BNDF injection (and at different doses), as well as using more restricted portions of the hippocampus, will be needed to clarify this point.

The presynaptic proteins synaptophysin and SNAP25 are involved in the mechanism of vesicle-dependent neurotransmitter release (Valtorta et al., 2004). BDNF was reported to enhance the expression of synaptophysin but not SNAP25 in neocortical neuronal cultures (Matsumoto et al., 2006). In our study, protein levels of synaptophysin and SNAP25 were not altered by intrahippocampal infusion of BDNF. The difference between *in vitro* and *in vivo* results might come from the developmental stage of each preparation. Since BDNF is known to promote synaptic maturation (Gottmann et al., 2009), involving synthesis of pre- and

postsynaptic proteins, such an effect might be more prominent in cultured cells prepared from embryonic animals than in adult mice.

Our data emphasize the fact that neuronal expression of MCT2, in parallel with other proteins known or highly suspected to participate in the mechanism of synaptic plasticity such as the immediate early genes *Arc* and *Zif268*, the postsynaptic scaffold protein PSD95 as well as the AMPA receptor GluR2 subunit, is upregulated by BDNF in the hippocampus *in vivo*. Considering the critical role of MCT2 in lactate uptake and utilization by neurons, our results point to the intriguing possibility of a coupling between neuronal lactate utilization and synaptic plasticity. Indeed, the concept that energy metabolism might be coupled to synaptic plasticity has been proposed together with the implication of BDNF in such interactions (Vaynman et al., 2006 ; Gomez-Pinilla et al., 2008). In addition, a recent study has shown that lactate import into neurons through MCT2 is necessary for long-term memory formation (Suzuki et al., 2011). In this context, it is tempting to hypothesize that to meet higher energy demands caused by enhanced synaptic transmission after synaptic plasticity, increased MCT2 expression induced by BDNF would facilitate lactate supply at potentiated synapses.

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Chapter 5

Chapter 5

The neuronal lactate transporter MCT2 is required for hippocampal-dependent short-term memory (Robinet C et al., in progress).

5.1 Abstract

Lactate has been proposed as an essential energy substrate to sustain neuronal functions. Neuronal lactate supply and utilization is critically dependent on expression of the neuronal monocarboxylate transporter MCT2. Here, we present evidence that reducing hippocampal MCT2 expression interferes with the acquisition of some forms of memory in mice. MCT2 knockdown primarily affecting the hippocampal dentate gyrus area produced working memory deficits in two spatial tasks while reference memory appeared unimpaired. In parallel, it reduced performance for short but not long latencies in a passive avoidance task. These results suggest that neuronal lactate supply via MCT2 is critical for the establishment of short-term memory and support the notion that a clear dissociation can be made between short-term and long-term memory on the basis of selective, region-specific protein downregulation.

5.2 Introduction

Monocarboxylate transporters (MCTs) are a small group of proton-linked membrane carriers that allow the release, passage and uptake of energy substrates such as lactate, pyruvate and ketone bodies (Pierre and Pellerin, 2005). Three MCT isoforms are expressed in the central nervous system and MCT2 represents the predominant neuronal monocarboxylate transporter. MCT2 is expressed not only on axons and dendrites, but it is particularly enriched in dendritic spines, being associated with the postsynaptic density of glutamatergic synapses (PSD) (Bergersen et al., 2005). It was also shown to interact with the AMPA receptor subunit GluR2/3 and to undergo a similar trafficking between the plasma membrane and an intracellular pool under conditions related to synaptic plasticity (Pierre et al., 2009). Such findings suggest the intriguing possibility that energy supply, via monocarboxylate transporters, could be an important determinant of learning and memory processes. Indeed, it has been recently demonstrated that interfering with the hippocampal expression of monocarboxylate transporters, including MCT2, and lactate supply to neurons prevented the formation of long-term memory in the context of an inhibitory avoidance learning paradigm (Suzuki et al. 2011). As hippocampal activation is known to be required for spatial learning tasks, we investigated the impact of reducing hippocampal MCT2 expression on spatial performance in mice.

5.3 Material and Methods

Animals

Twenty-four male C57BL/6 Rj mice were purchased at seven weeks of age (~20g), from Janvier Breeding Center (Le-Genest-Saint-Isle, France) and group housed in a temperature-controlled environment with 12:12h light-dark cycle with free access to food and water. Surgery took place at 8 weeks, and behavioural testing started 2 weeks thereafter.

Construction of shRNA plasmids

Oligonucleotides containing 17 nucleotides from the H1 promoter, the sense-strand, a loop, the anti-sense strand for Rat and mouse siMCT2 and a universal control were synthesized.
siMCT2 :
CTAGTTTCCAAAAATAGGATTAATAGCCAACACTATGACAGGAAGTAGTGTTGGC
TATTAATCCTAGGGGATCTGTGGTCTCATACAGAAC,
siUNIV:
CTAGTTTCCAAAAAGTATCGATCACGAGACTAGTGACAGGAAGCTAGTCTCGTGA
TCGATACGGGGATCTGTGGTCTCATACAGAAC.
These oligonucleotides and the primer H1-3F: CACCGAACGCTGACGTCATCAACCCG were used to perform a PCR reaction on the pBC-H1 plasmid (Drouet et al. 2009). The PCR product was cloned in the pENTR/D-TOPO plasmid (Invitrogen, Cergy Pontoise, France). The H1-shRNA cassette was then transferred with the LR clonase recombination system (Invitrogen, Villebon sur Yvette, France) in the SIN-cPPT-PGK-GFP-WPRE-LTR-TRE-gateway vector (SIN-CWP-GFP-TRE-gateway) (Drouet et al. 2009).

Lentiviral vector production

Lentiviral vectors encoding the various siRNA were produced in 293T cells with a four-plasmid system as previously described (Hottinger et al. 2000). The lentiviral particles were produced and purified with the vivapure LentiSELECT 500 kit (Sartorius stedim, Aubagne, France). The Sartobind® ion exchange membrane adsorber technology used in LentiSELECT efficiently and rapidly capture and recover large virus particles (3000 nm pores). Briefly, 500ml of supernatant are harvested and filtrated on the provided filter. The surpernatant is pumped through the filter at 10-20ml/min. After the assembly of the lentiselect column and connection with the peristaltic pump, 150ml of loading buffer is pumped through the column at 10ml/min. The flow through is treated as biohazard waste. Once bound, virus particles are

purified by washing away nonspecifically bound protein with 120ml of washing buffer at 15ml/min. The viral particles are then eluted using a buffered 30 ml solution containing a high concentration of sodium chloride. The elution is performed with a 20ml syringe at a 1ml/min debit. Finally, the eluate is collected in PBS buffer and ultracentrifuged at 19000rpm/min, 4°C for 90min to concentrate it. The virus pellet is resuspended in PBS buffer and stored at -80° until use. The particle content of each batch of virus is determined by p24 antigen ELISA (RETROtek, Gentaur, Paris, France).

Surgery and intrahippocampal injection of lentivirus

Mice were weighed and anesthetized with isoflurane setup and positioned in a stereotaxic apparatus (Stoelting, WI, USA). Rectal temperature was maintained at 36°C with a thermostatically controlled electric heating pad. Stereotaxic coordinates relative to Bregma were based on the brain atlas of Paxinos and Watson (1986): anteroposterior (AP): -1.85 ; mediolateral (ML) : -1.25 and +1.25 ; dorsoventral (DV): -1.4. A single intrahippocampal injection was performed using a 5 µL Hamilton syringe with a 30 ½-gauge needle. The needle was inserted into the hippocampal CA1 area and 2µl of 545'000 ng P24/ ml were injected slowly during 15 min. Mice were injected with a lentiviral vector containing a siMCT2-GFP sequence (Lenti-siMCT2) into the right dorsal hippocampus (CA1 area) while the left, LentiUniv-injected hippocampus was used as control. Mice completely recovered 15 min after the surgery. Behavioral testing started 14 days after surgery.

Morris watermaze task

A circular tank (150 cm ∅) was filled with water at 25±1 °C, made opaque by addition of milk. Mice learn to localize and step on an invisible, circular platform (14 cm ∅) located at the centre of the NW quadrant of the tank. Training schedule consisted of six daily trials during four consecutive days. Release points were chosen pseudorandomly, with the exception of the first release point of each day, which was always opposite to the escape platform (SW), in order to better assess the episodic component of the task. At day 5, the platform was removed for the first, 60 s trial (probe trial) to assess spatial reference learning proficiency. At the end of the 60 s trial, the platform was placed in the SW quadrant and mice further trained during 5 trials to find the platform on this novel location (reversal learning). Swim paths were videorecorded and analyzed by a videotracking system (EthoVision,

Noldus, The Netherlands). Variables assessed were escape latencies and, for the probe trial, times in quadrants and annulus.

Radial maze task (spatial working memory)

The apparatus was an eight-arm radial maze (RAM), consisting of an open central platform of 16.5 cm diameter, from which radiate eight transparent tunnels made of Plexiglas (6 x 6 x 50 cm). Access to the tunnels was regulated by remotely operated plexiglas sliding doors. In addition, two wooden barriers (1.5 x 1 x 6 cm), placed on each arm floor at 0 and 25 cm from the entry, helped in discouraging haphazard visits of already visited arms, and in preventing sight of the bait (10 µl of condensed milk) placed at the end of the lanes. An arm visit was counted when the mouse had jumped beyond the second barrier, placed halfway in the lane.

We designed a two-trial protocol, requiring mice to discriminate recently visited vs non-visited arms within a trial (working memory), while suppressing interference of spatial information between trials (non-matching-to-place, episodic memory). Each trial was made of two steps : it started with three open, baited arms, disposed at 0, 90 and 215 degrees, to prevent chained responses. Once the baits retrieved, three more baited arms, at 45, 180 and 260 degrees were made accessible (step 2). Entries in already visited arms were counted as errors. A trial was terminated when all 6 baits had been retrieved, or after 5 minutes. Upon completion of the first trial, mice were confined in the central arena during 2 minutes under an opaque lid, baits replaced, and the second daily trial started, for which the baiting pattern had been rotated of 90 degrees (i.e. : 0, 135 and 270 degrees for the step 1, and 90, 180 and 315 degrees for step 2) (Fig. 1). This training was preceded by a two-days habituation phase during which mice where food restrained to reach 90 to 85% of their initial body weight, and familiarized to the bait taste and the arena.

Passive Avoidance Test

Memory formation and retention was also assessed in a passive avoidance paradigm. The apparatus (Med Associates, St. Alban, VT, USA) was a skinner box with a lit and a dark compartment, separated by a guillotine door, and placed in a sound-proof cubiculum. Training consisted in placing the mouse in the lit side of the box, with the guillotine door open. Upon reaching the dark side of the box, the door was closed, mice received a mild footshock (0.3 mA during 2 s), and immediately transferred to a clean cage. A second training session was administered after 5 minutes after the end of the first session. Memory for the aversive events

was tested 10 minutes after the end of the second training session, and 24 hours later. Absolute and relative latencies to enter the dark side were taken as a measure of memory proficiency.

Behavioral assessment

Working memory will be reflected by the retention of trial specific or trial unique information for short periods of time (thus rather associated with short-term memory) while reference memory will be reflected by retention of intertrial information (and involves long-term memory) (Hodges, 1996).

Immunohistochemistry

Under urethane anathesia (1.5g/kg i.p.), animals were perfused intracardially with a heparinized solution of saline (25 IU/ml in 0.9% NaCl, during 2 min) followed by a freshly prepared solution of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4, for 15 min). Brains were removed and fixed in 4% paraformaldehyde for 2h, then cut on a vibratome (Leica VT1000M) to obtain coronal sections (40 µm thick), which were collected in phosphate buffer saline (PBS). Sections were rinsed several times in PBS and incubated with casein (0.5% in PBS) for 1 h to block non-specific sites. They were incubated overnight at 4°C in freshly prepared MCT2 antibody solution (anti-MCT2 diluted 1:500 in PBS containing 0.25% BSA). After careful rinsing in PBS, sections were incubated in a solution containing Cy3-conjugated anti-rabbit Igs (diluted 1/500, 2 h, room temperature; Jackson Immunoresearch, MD, USA). After rinsing in PBS twice and a final rinsing in water, coverslips were mounted with Vectashield (Reactolab SA, CA, USA). Coverslips were examined and photographed with an Axioplan2 microscope (Zeiss, Hallbergmoos, Germany) using epifluorescence with an appropriate filter.

Western blotting and related quantification

For western blotting, hippocampal tissue was homogenized and sonicated in a buffer of 0.32 M sucrose containing HEPES 1 mM, $MgCl_2$ 1 mM, $NaHCO_3$ 1 mM, phenyl-methyl-sulphonyl fluoride 0.1 mM, pH 7.4, in presence of a complete set of protease inhibitors (Complete, Roche, Switzerland). Protein concentrations were determined by the BCA method. Ten micrograms of protein were heated at 95°C in SDS-PAGE sample buffer (62.5 mM Tris-HCl, 50 mM DTT, 2% SDS, 10% glycerol and 0.1% bromophenol blue) and loaded onto 10% polyacrylamide gels. After electrophoresis, samples were transferred to

nitrocellulose membranes (Biorad Laboratories). For protein detection, membranes were incubated in a blocking solution of Tris-buffered saline supplemented with Tween-20 (TBST; Tris-HCl, pH 7.5, 50 mM; NaCl, 150 mM; and Tween-20, 0.1%) containing 5% nonfat milk for 1 h at room temperature. Membranes were incubated overnight at 4°C with a mixture containing the primary antibodies: anti-MCT4 (Santa Cruz, CA, USA), anti-GluR1 and anti-GluR2/3 (Chemicon, CA, USA) diluted 1/500 ; anti-MCT1, anti-MCT2 antibodies (Pierre et al., 2000), anti-synaptophysin (Sigma, Buchs, Switzerland), anti-SNAP25 (Alomone labs, Jerusalem, Israel), anti-PSD95 and anti-CamKIIα (Cell Signaling, MA, USA) diluted 1/1000 ; anti-βactin (Sigma, Buchs, Switzerland), diluted 1/10000. These antibodies were diluted in TBS, 0.1% Tween, 5% bovine serum albumin (BSA). After three washes in TBST, membranes were incubated with the secondary antibodies Alexa Fluor 680 goat anti IgG (Juro, Lucerne, Switzerland) and IRDye_800 antimouse IgG (BioConcept, Allschwil, Switzerland), diluted at 1/5000 in TBST containing 1% nonfat milk, for 2 h at room temperature, and protected from light. After three washes in TBST, membranes were scanned using the Odyssey®Infrared Imaging System (LI-COR® Biosciences, Lincoln, NE, USA) which permits detection and quantification of proteins of interest. β-Actin, revealed in green, was used for normalization and proteins of interest were revealed in red.

Quantitative Real-time Reverse transcriptase-PCR

Quantitative determination of MCT2 mRNA expression levels was performed from mouse hippocampal extracts by quantitative reverse transcriptase-PCR according to Heid et al (1996) using ABI Prism 7000 sequance detection system from Applied Biosystems (Rotkreuz, Switzerland). The following sets of oligonucleotides were used : 5'→3'; ActinFo, GCTTCTTTGCAGCTCCTTCGT; ActinRe, ATATCGTCATCCATGGCGAAC (Embl: X03672); MCT2Fo, CAGCAACAGCGTGATAGAGCTT; MCT2Re, TGGTTGCAGGTTGAATGCTAAT (Embl: NM_011391); NPYFo,ACCAGACAGAGATATGGCAAGAGA; NPYRe, GGCGTTTTCTGTGC (produced by Microsynth, Balgach, Switzerland).

Microscopy

Brain slices were examined and photographed with an Eclipse 80i microscope (Nikon, Kingston, England) using epifluorescence with an appropriate filter together with the NIS-Elements Microscope Imaging 5.1 software (Nikon). They were also examined under a Zeiss LSM 710 Quasar confocal microscope using the DPSS 561-10, 561nm laser illumination.

Stacks were made of images taken with 0.5 to 0.8µm step size. Pictures were treated with Imaris software, version 7.0.0.

Statistical analysis

All values are presented as mean ± SEM. Mann Whitney's test was used for data analysis with GraphPad Prism® software, version 5.0 (La Jolla,CA). P* values < 0.05; P**<0.01; P***<0.001 were considered as statistically significant.

5.4 Results

5.4.1 MCT2 downregulation in hippocampus after injection of Lenti-siMCT2 infecting primarily the dentate gyrus area

Cellular localization of transgene encoding the MCT2 siRNA was histologically verified upon the end of behavioural testing, 15 weeks after the injection. Viral infection, as revealed by GFP expression, was at that time mostly localized in dentate gyrus granule cells, while comparatively less pyramidal neurons in CA1/CA2 showed GFP labelling (Fig.1A to 1E). Confirmation of MCT2 downregulation was provided by quantitative immunoblotting (Fig.1F). A significant reduction of MCT2 expression was detected in Lenti-siMCT2-injected hippocampal extracts compared to extracts from control hippocampi that were injected with a universal siRNA control lentivirus containing a siUniv-GFP sequence (Lenti-Univ) (- 25.3 ± 11.8% vs. control). In parallel, a significant decrease in MCT2 mRNA expression was observed by quantitative RT-PCR in Lenti-siMCT2 vs control, LentiUniv-injected hippocampal extracts (- 12.5 ± 1.6% vs. control; Fig.1G). Observation at high magnification using confocal microscopy after MCT2 immunofluorescent labeling (in red) revealed a selective downregulation of MCT2 expression in infected, GFP-positive neurons (in green) compared to uninfected cells (Fig.1H and 1I).

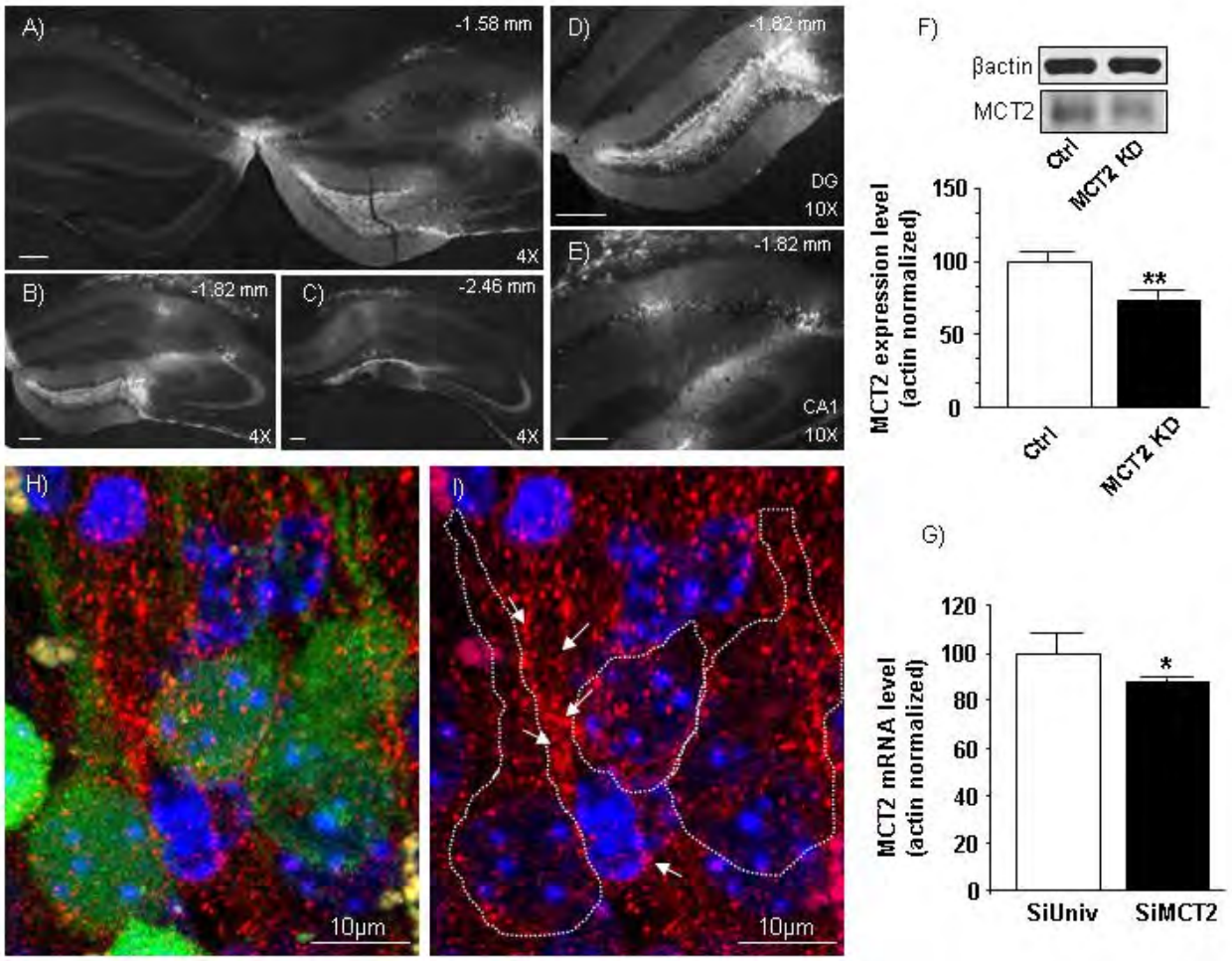

Figure1. Downregulation of MCT2 by lentiviral injection into the hippocampus . A) Right hemisphere was injected with siMCT2-GFP+ lentivirus into the CA1. Left hemisphere was used as control. B) and C) Infection pattern of hippocampal injection at different level along the anteroposterior axis. Lentiviral infection into the dentate gyrus (D) and into the CA1-CA2 (E). The expression of MCT2 (in red) was downregulated in infected siMCT2-GFP+ neurons (in green) (H) and (I). Images were taken at 4X or 10X magnification with fluorescence microscope for images A) to E), and at 63X magnification with confocal microscope for images H) and I), (Arrows point out an uninfected neuron). Quantification of MCT2 downregulation in hippocampus by immunoblot (n=6) (F). Quantification of MCT2 mRNA downregulation by quantitative RT-PCR (n=8) (G). Mann Whitney test was applied to the data. * $P<0.05$, ** $P<0.01$.

5.4.2 Spatial working memory impairment in MCT2 knockdown mice without obvious reference memory deficits.

Hippocampal-dependent learning proficiency was first assessed with a classical reference memory task in the Morris Water Maze. The experiment was conducted twice (pseudoreplication) using each time 6 MCT2-KD and 6 control mice. Testing order was such to maintain constant intertrial times of about 15 minutes, in order to evaluate short-term memory over the six daily trials. The release point of each first daily trial was kept constant (SW), while the release points of the subsequent five trials changed pseudorandomly. Therefore, while the evolution of escape latencies of the first daily trial provided a good measure of day-by-day spatial reference learning, the average escape latencies of the following trials provided a measure of intrasession, short-term memory. Escape latencies of trials 2 to 6 were significantly longer for MCT2-KD mice (Fig.2B), indicating a deficit in short-term memory; in contrast, escape latencies for the first daily trial were virtually identical for MCT2-KD and control mice (Fig. 2A), suggesting unimpaired day-to-day reference learning. This evidence is also supported by the similar preference for the target quadrant shown in the probe trial (Fig.2C). However, when considering the time spent during the probe trial within the exact perimeter of the platform position, a reduced accuracy of the MCT2-KD mice was also detected (Fig. 2D).

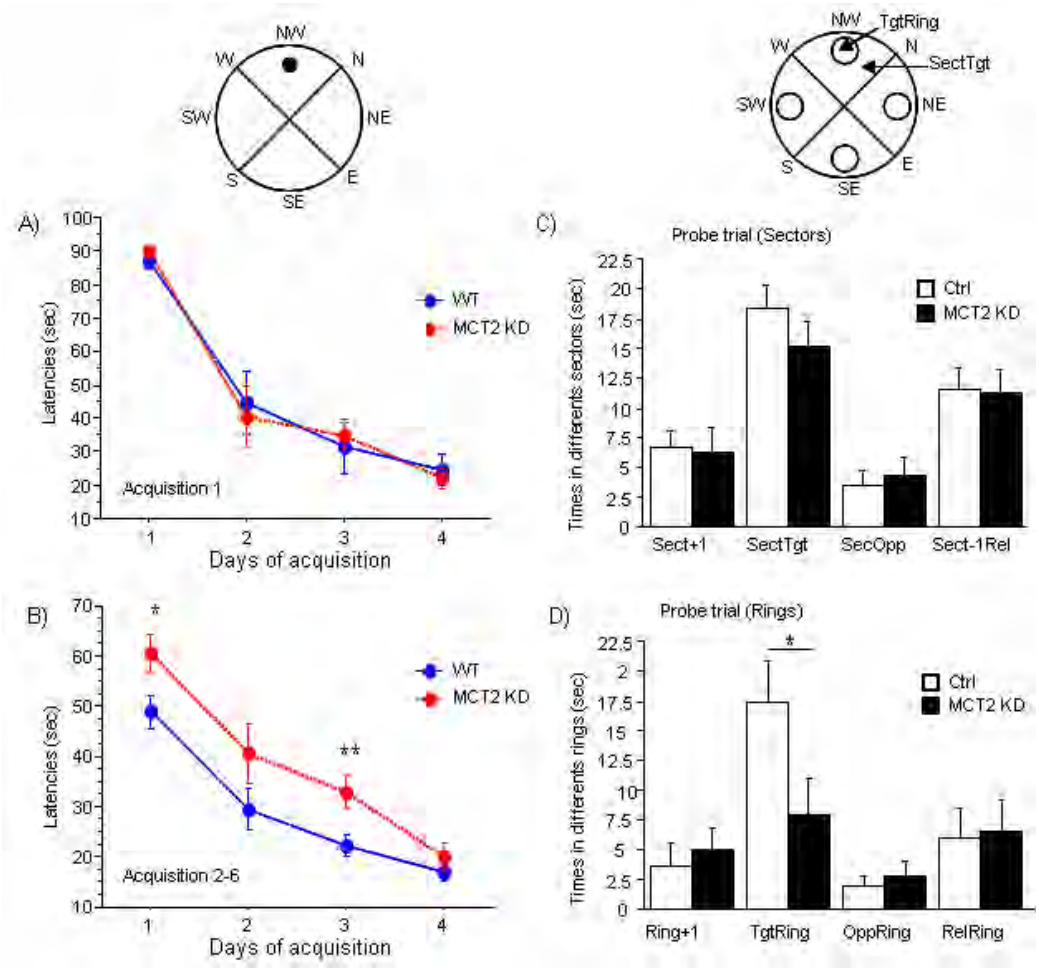

Figure. 2. Spatial reference and working memory in acquisition or probe period in the Morris watermaze. A) Spatial reference memory analysed on the first trial latency of the day. B) Spatial working memory was analysed on trial 2 to trial 6 for each day. The probe trial was evaluated the 5th day where the spent time into each sectors (Sect) (C) and each ring sectors (Ring) (D) were measured. Data are expressed as means ± SEM of 12 wildtype mice (WT) and 12 MCT2-knockdown mice (MCT2 KD). Mann Whitney test was applied to the data. *P<0.05, **P<0.01.

To further assess working memory ability of MCT2-KD mice, we designed an experiment requiring discrimination of just visited vs. non-visited arms in an 8-arm radial maze. Mice were tested in two consecutive daily trials over four days (protocol details are described in the methods section). Entries into already visited arms within each trial were counted as working memory errors. Episodic and working memory components of the task were assessed by means of repeated-measures ANOVA on the sum of errors made in the two daily trials, over the four days. While all mice reduced the number of working memory errors over time ($F_{(df\ 3,\ 30)} = 29.5$, $p < .0001$), MCT2-KD mice committed more errors at any time ($F_{(df\ 1,\ 22)} = 19$; $p < 0.005$) confirming the spatial working memory deficit observed in the watermaze (Fig. 3).

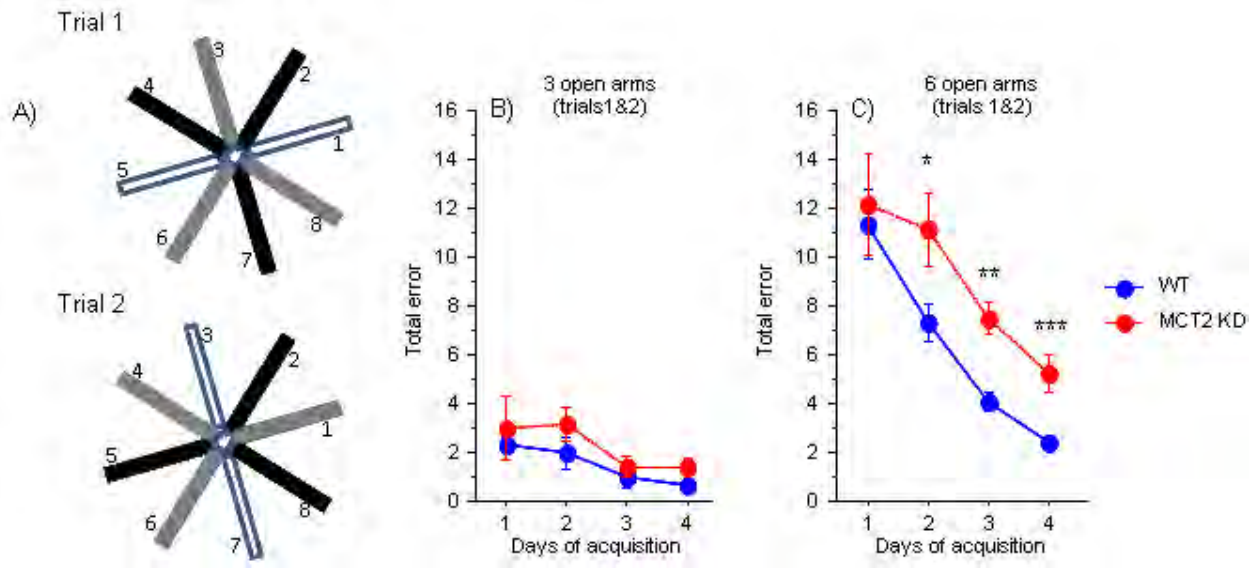

Figure 3. Spatial working memory and reference memory errors for 4 days acquisition in the RAM. A) Schematic maze protocol of trial 1 and trial 2. Black arms were open in first hand and grey arms were open once black arms were been visited/eat by mice. B) Spatial reference memory analysed from the 3-open arms of trials. C) Spatial working memory was analysed once the 6 arms were open for the both trials. Data are expressed as means ± SEM of 12 wildtype mice (WT) and 12 MCT2-knockdown mice (MCT2 KD). Mann Whitney test was applied to the data. *P<0.05, **P<0.01, ***P<0.001

Because these results also suggested a deficit in acquisition more than in retrieval of spatial information, we performed an inhibitory avoidance protocol that had been successfully used to dissociate these two steps of the learning process (Cole and Jones 1995; Decker et al. 1990). Namely, mice underwent two discrete training sessions, at 5 minutes interval, where they received a 0.3 mA footshock 3 s after having entered the dark compartment of a shuttle-box. Latency to re-enter the dark compartment, assessed 5 minutes after the first footshock event, provides a measure of learning proficiency; latencies assessed 10 minutes and 24 hours after the end of the second training session respectively provide a measure of short- and long-term memory. All mice spontaneously re-entered the shock compartment five minutes after the first training trial, yet MCT2-KD with significantly reduced latency as compared to their initial dark escape time (Fig.4A). In contrast, almost all mice presented a maximal escape inhibition at both 10 minutes and 24 hours, showing similar retention abilities (Fig.4B).

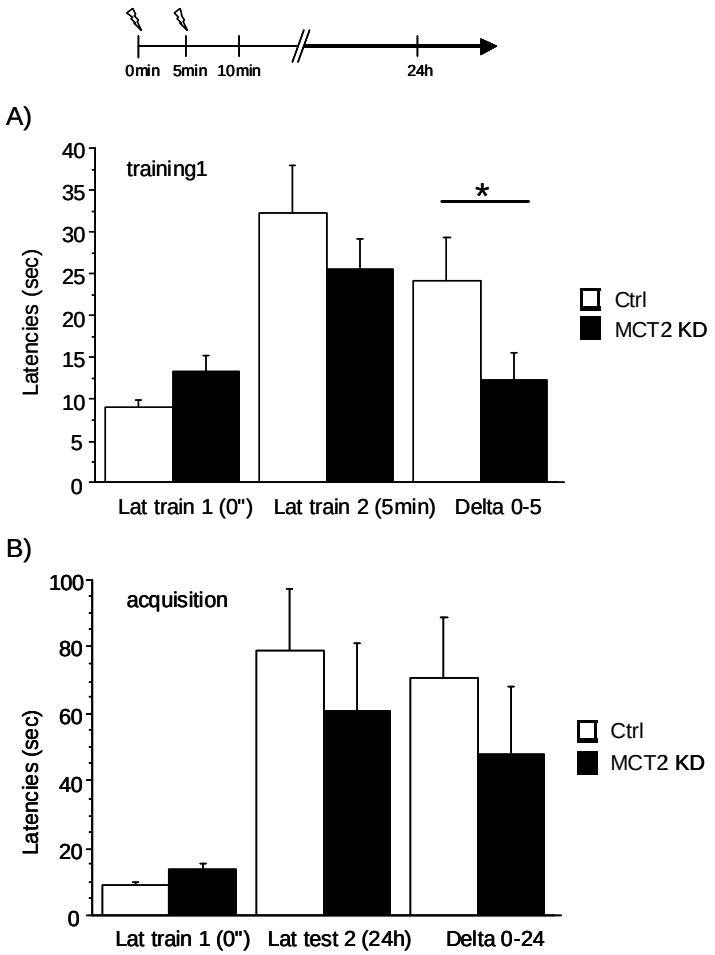

Figure 4. Dynamics of episodic memory formation evaluated with the passive avoidance task. The latencies after five minutes (5min) (A) and 24 hours (24h) (B) were evualuated after training1 (foot shock). Data are expressed as means ± SEM of 12 wildtype mice (WT) and 12 MCT2-knockdown mice (MCT2 KD). Mann Whitney test was applied to the data. *P<0.05.

5.5 Discussion

The hippocampus is a brain structure important for learning and memory processes in rodents. It is notably involved in episodic memory formation (Baddeley, 2001), especially in a spatial context (Wang and Morris, 2010). Spatial learning engages distinct processes such as working and reference memory that can be evaluated separately with appropriate tasks (Hodges, 1996). Our results show that a knockdown of MCT2 in the hippocampus (MCT2 KD) impairs spatial working memory (SWM) without effect on spatial reference memory (SRM). Since the same deficits were observed in glutamate receptor mutant mice such as GluRA $^{-/-}$ mice (Schmitt et al., 2003), NR2A$^{-/-}$ mice (Bannerman et al., 2008), as well as NR1^{deltaDG} mice (Niewoehner et al., 2007), there is a possible link between MCT2 and glutamatergic transmission in working memory. Supporting this hypothesis, some studies have previously demonstrated that MCT2 and AMPA receptor GluR2 subunits colocalize at the post-synaptic density of glutamatergic synapses and seem to be tightly bound to each other (Bergersen et al., 2005, Pierre et al., 2009). Moreover, they not only translocate to the plasma membrane together after glutamate stimulation (Pierre et al., 2009) but the level of MCT2 expression directly influences GluR2 intracellular trafficking, fate and expression levels (Maekawa et al., 2009). Thus, our results point at the possibility that energetic metabolism via MCT2 is essential for glutamate receptor-dependent working memory.

Although the role of the hippocampus in memory is well recognized, the specific role of the different sub-regions in spatial working (SWM) vs reference memory (SRM) is less clear. Studies investigating this aspect have shown that neurotoxic lesions of the dentate gyrus or the CA3, but not the CA1 sub-region induced a deficit in spatial working memory (Lee and Kesner, 2003). Furthermore, both NR1^{deltaDG} and NR1^{deltaCA3} mice revealed a spatial working memory impairment despite normal hippocampus-dependent spatial reference memory in the radial maze (Niewoehner et al., 2007; Nakazawa et al., 2002), whereas both NR1^{deltaCA1} mice and GluR2^{deltaCA1} showed a spatial reference memory deficit (Tsien et al., 1996; Wiltgen BJ et al., 2010). Therefore, evidences suggest that SWM and SRM require different specific sub-regions of the hippocampus: dentate gyrus or CA3 for SWM and CA1 for SRM. In this context, it is interesting to note that the findings of Suzuki and colleagues, showing a reference memory impairment caused by downregulating MCTs including MCT2 in the hippocampus (Suzuki et al., 2011) contrast with our results. This discrepancy could be in fact due to a difference in the degree of protein downregulation within each hippocampal sub-

region. Indeed, while in our case it seems to be the granule cells of the dentate gyrus that were primarily targeted by the lentiviral infection, it is likely that oligodeoxynucleotide injections as performed by Suzuki and collaborators might have affected more the CA1 area. Thus, it is most likely that MCT2 is important for both working and reference memory, the distinction between these two forms of memory being revealed depending of the hippocampal sub-region affected.

Little is known about the exact mechanisms implicated in SWM and SRM, although it can be suspected that working memory relies more on short-term memory processes (STM) while reference memory relies rather on long-term memory processes (LTM). These two time-dependent memory processes are based on separate molecular mechanisms and can be independently modulated (Izquierdo 1995; 1997,1999; Niewoehner et al., 2007; Bannerman et al., 2008; Sanderson et al., 2007; Wilgen et al., 2010). STM involves early-LTP (E-LTP), a synaptic plasticity process requiring NMDA receptor activation with subsequent Ca^{2+} influx (Malenka et al., 1992) as well as translocation of additional AMPA receptors into postsynaptic membrane (Kessels and Malinow 2009; Yang et al., 2008; Corera et al., 2009; Williams et al. 2007). In contrast, LTM requires late-LTP (L-LTP), which is protein synthesis-dependent (Frey and Morris 1997, Barco et al., 2002). Previously, it was demonstrated that MCT2 can be rapidly translocated to the membrane by various signals including brain-derived neurotrophic factor (BDNF) that has been implicated in long-term potentiation and synaptic plasticity (Pierre et al., 2009; Pierre K. poster; Société française des neuroscience, Bordeaux, 2010), similarly to AMPA receptor GluR subunits (Leonoudakis et al. 2004; Li and Keifer 2008; Lin et al., 2000; Lu et al., 2001; Passafaro et al., 2001). We also found that MCT2 downregulation induced STM deficits comparable to those observed in $GluR1^{-/-}$ (Sanderson et al., 2011). We can thus hypothesize that E-LTP-dependent STM involves the translocation of both AMPA receptors and MCT2. However, this hypothesis does not preclude the possibility that MCT2 is also involved in L-LTP-dependent LTM. Indeed, LTM deficits were also observed after blockade of MCT2 expression (Suzuki et al., 2011), suggesting that MCT2 synthesis could also play an important role in LTM formation. As BDNF, a key factor not only for E-LTP (via its effect on AMPA receptor subunit translocation) but also for L-LTP (via its effect on protein synthesis), was shown to enhance MCT2 protein synthesis in neurons both *in vitro* (Robinet and Pellerin, 2010) and *in vivo* (Robinet and Pellerin, 2011), this might explain the involvement of MCT2 in both mechanisms.

In conclusion, MCT2 seems to play a critical role during spatial working memory processes. As a corollary, it is possible that neuronal lactate utilization could be necessary to sustain the energy demand during high neuronal activity related to memory formation. Thus, our work highlights the putative importance of monocarboxylate transport in neurons for spatial memory formation, opening new interesting perspectives concerning the coupling between spatial memory and energy metabolism.

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Chapter 6

Chapter 6

General discussion and perspectives

Results obtained in this thesis highlight the important role of the neuronal monocarboxylate transporter MCT2 in conditions involving synaptic plasticity both *in vitro* and *in vivo*. Important implications for different areas that likely depend on neuroenergetics can be postulated and we present below a short discussion on some of these aspects.

6.1 Putative role of MCT2 in adapting energy supply in synaptic plasticity

Long-term potentiation (LTP) is a persistent, use-dependent increase in the efficiency of synaptic transmission that can be induced by the delivery of high-frequency synaptic stimulation (HFS) (Reviewed by Robert D. Blitzer 2005). It has been proposed as one of the mechanisms implicated in synaptic plasticity, which form the cellular basis of memory. LTP induction requires NMDA receptors activation with subsequent Ca^{2+} influx (Malenka et al., 1992) as well as translocation of AMPA receptors at the synapse (Yang et al., 2008). LTP maintenance requires de novo protein synthesis (Frey and Morris 1997, Barco et al., 2002). This protein synthesis-dependent phase is stable and is taking place as early as 20 minutes after the induction. A possible link between MCT2 and LTP is supported by the findings of Pierre and coworkers (Pierre et al., 2009). Indeed, they observed that the GluR2 subunit of AMPA receptors and MCT2 were colocalized and concomitantly introduced into the membrane following glutamate stimulation (Pierre et al., 2009). Because during LTP induction AMPA receptors are translocated into the postsynaptic membrane, it is possible that MCT2 plays also a role in LTP induction. During the maintenance phase, LTP-related proteins that have to be synthesized are CamKII, AMPA receptors and delta PKC (Tsokas et al., 2005; Ju et al., 2004; Lisman et al., 2002). Since LTP maintenance requires AMPA receptors synthesis and MCT2 expression influence AMPA receptor GluR2 subunit expression levels (Maekawa et al., 2009), it might be possible that MCT2 plays also a role in

LTP maintenance. Recent studies started to elucidate the mechanisms responsible for LTP-related protein synthesis. They showed that both MAPK and mTOR are necessary for LTP maintenance (Tsokas et al., 2005; Kelleher et al., 2004). Our results have shown that induction of MCT2 synthesis was regulated through PI3K/Akt/mTOR pathway as well as MAPK pathway following BDNF stimulation (Robinet and Pellerin 2010). Thus, the putative LTP-related MCT2 synthesis could occur via both PI3K/Akt/mTOR and MAPK pathways.

Although evidences suggest that MCT2 could be involved in synaptic plasticity, its possible role in well-described processes such as LTP remains to be determined. In order to study the role of MCT2 in synaptic plasticity, we should perform some electrophysiological recordings on hippocampal slices. We could first downregulate the expression of MCT2 (using lentiviruses injected in vivo, Chap 5) in hippocampal slices or organotypic hippocampal slice culture. Then, study the effect of MCT2 downregulation on long-term potentiation (LTP) or long-term depression (LTD), two phenomena implicated in synaptic plasticity which form the cellular basis of memory. For example, it would be interesting to know whether MCT2 is crucial only for induction or for the maintenance of LTP, the two components for LTP establishment.

6.2 Role of MCT2 in learning and memory: enhancing cognitive performance by overexpressing MCT2?

In the brain, it has been shown that during cognitive tasks, some metabolic changes occur. These include increased glucose consumption (Ros et al., 2006) and lactate production (Urrila et al., 2003, 2004). Another study showed that a physiological stimulation of neuronal activity produces an increase in both lactate and glucose in the rat brain (Fray et al., 1996). The increase in lactate level on stimulation coincides with the growing energy demand of increased neuronal activity (Magistretti and Pellerin, 1999). In parallel our results, and those of others, show that a downregulation of MCT2 in the hippocampus leads to memory deficits. Such deficits were proposed to be caused by disruption of lactate uptake and utilization by neurons (Suzuki et al., 2011). These findings suggest that energy supply, via monocarboxylate transporters, could be a necessary determinant of learning and memory processes, and in particular that neuronal lactate supply via MCT2 is important for the establishment of memory.

It would be interesting to investigate under which conditions memory could be enhanced by stimulating the expression of MCT2. In fact, it seems that cognitive performances can be influenced by different factors such as specific pharmacological treatments (e.g. cognitive enhancers), behavioral conditions, as well as lifestyle. Some of these might directly or indirectly target MCT2 expression (this aspect will be discussed in point 6.3). It is easy to imagine potential clinical applications. For example, in the context of neurogenerative diseases such as Alzheimer's disease, it would be interesting to investigate whether an upregulation of MCT2 would lead to an increase in cognitive performance. In fact, patient suffering of Alzheimer's disease progressively lose memory and develop a hypometabolism of glucose in specific brain regions, involving parieto-temporal and frontal areas (Mosconi et al., 2008). It might be worth investigating whether an upregulation of MCT2 would enhance memory in an Alzheimer's disease mice model (this aspect will be discussed later in point 6.4.2).

6.3 The putative role of MCT2 in special diet and in exercise

Some evidences indicate that BDNF expression is affected by diet. Indeed, caloric restriction or certain diets affect levels of BDNF. Under high-fat diet, mice exhibited reduced levels of BDNF mRNA and protein in the hippocampus compared to mice under normal diet following a spatial memory task (Molteni et al., 2002). In addition, high-fat diet impairs neurogenesis, reducing the level of BDNF expression in the hippocampus (Park HR et al., 2010). Since BDNF induced *in vitro* and *in vivo* an increase in the level of MCT2 expression in neurons (Robinet and Pellerin, 2010, 2011), and the high-fat diet reduces the level of BDNF, we could have expected to find a decrease of MCT2 expression on neurons. However it has been recently shown that high-fat diet induced expression of the three monocarboxylate transporter isoforms on neurons in different brain areas, suggesting that the exposure to a high fat diet sets a new metabolic environment for the brain that most likely leads to specific metabolic adaptations (Pierre et al., 2007). Another kind of high-fat diet with low carbohydrates, named ketogenic diet was shown to have an impact on neuronal metabolism as well as on astrocytic metabolism. Indeed, it was demonstrated that this diet decreased neuronal glucose metabolism and increased astrocytic metabolism (Melo et al., 2006). These changes may reflect adaptation of the brain to ketone bodies as a major source of energy.

These findings are consistent with the increase of MCTs under high-fat diet and further suggest that MCTs play a critical role under high-fat diet to fulfill neurons energy needs.

In contrast to high-fat diet, dietary restriction in rats induces BDNF expression in the dentate gyrus in parallel with an increase in the number of newly generated cells suggesting that this diet affect the process of neurogenesis as well as neurotrophic factor production (Lee et al., 2000). In addition, it was shown that a chronic caloric restriction was associated with an increase of BDNF in the prefrontal cortex, a reduction of tissue damages after traumatic brain injury and an improvement of spatial memory (Rich et al., 2010). Recent research has provided solid ground to the concept that limiting calorie intake slows down brain aging and protects from age-related neurodegenerative diseases (Reviewed by Contestabile A, 2009). For the moment, it is not known if caloric restriction modifies brain energetics or the expression levels of any energy substrate transporters including MCTs.

BDNF expression can be also affected by exercise. It was shown that physical exercise increases BDNF levels in the hippocampus and the cortex (Cotman et al., 2002; Neeper et al., 1996; Fuss et al., 2010), and this increase in BDNF may improve hippocampus-based learning, synaptic plasticity and neurogenesis (Cotman et al., 2002; Vaynman et al., 2004). Moreover, it was shown that forced exercise increased cerebral metabolism, including expression of GLUT1, GLUT3, phosphofructokinase (PFK), lactate dehydrogenase (LDH) and phosphorylated AMPA activity (Kinni H et al., 2011). Since exercise affects both BDNF expression level and increases cerebral metabolism, and because our results have shown that BDNF increased the level of MCT2 expression, it is likely that voluntary exercise will improve lactate utilization by neurons through MCT2.

Taken together, exercise and caloric restriction increase the level of BDNF in the brain. It was also demonstrated that during exercise, the systemic lactate produced is known to be a brain energy source (van Hall G et al., 2009) and this lactate production increases BDNF blood concentration in humans (Schiffer T et al., 2011). It would be interesting to measure the level of lactate, BDNF and MCTs in the brain under exercise or caloric restriction. We could expect that during these physiological states, the level of MCT2 expression would be enhanced via BDNF stimulation, increasing lactate uptake in neurons and its neuronal utilization as additional energy substrate to sustain their activity. However, this would require additional experiments.

6.4 Enhancing MCT2 expression as a neuroprotective mechanism

6.4.1 Study of signaling pathways

Acute neurological insults like hypoxia-ischemia, seizures and hypoglycemia share common characteristics. They all induce neurotoxicity by disrupting neuronal energy profiles and produce hyperexcitation at glutamatergic synapses which leads to an increased glutamate release. Excess of glutamate is highly deleterious for the brain and must be cleared from the synaptic cleft by astrocytes. Thus, glutamate excess and hyperexcitation impair greatly the energy status of brain cells.

A solution proposed to counteract this effect was to increase energy supply by overexpressing the glucose transporter in neurons, thus providing more glucose to the cell. This was done in neurons infected with a defective herpes simplex virus (HSP) vector overexpressing the rat brain glucose transporter gene (Ho et al., 1995). This gene therapy permitted to protect cultured hippocampal, septal and spinal neurons from various necrotic insults (i.e hypoglycaemia, glutamate). In order to further improve neuronal energy supply, Bliss and coworkers used a dual cell, dual gene therapy approach (Bliss et al., 2004). They overexpressed both GLUT1 in astrocyte and MCT2 in neurons in mix cultures. This technique permitted to enhance glucose uptake by astrocytes and lactate uptake by neurons, thus, favoring the astrocyte-neuron lactate shuttle. This double therapy provided more neuroprotection than either therapy alone. Thus it seems of considerable importance to increase both glucose supply to astrocytes and lactate supply to neurons to overcome brain insults. These results are consistent with lactate being preferentially used by neurons *in vitro* and *in vivo* under certain conditions. Indeed, lactate was shown to be able to sustain synaptic activity (Schurr et al., 1988) and to play a major neuroprotective role in cerebral ischemia (Schurr et al., 2001a,b ; Berthet et al., 2009)

Since MCT2 overexpression can provide neuroprotection after acute brain insults, the regulation of MCT2 is of particular importance. Our results demonstrated that MCT2 is in part controlled by the neurotrophic factor BDNF. The pathway induced by BDNF provides an interesting approach that could be further exploited to protect the brain against damages via increased MCT2 expression. In fact, apart from its effect on cellular differentiation and

synaptic plasticity, BDNF is one of the most important anti-apoptotic neurotrophin and its protective role has been demonstrated in several cell types (Hetman and Xia 2000). Our data have shown that BDNF activated PI3K/Akt and MAPK pathways. Each of these pathways was preferentially activated depending on the type of cellular injury (Hetman et al., 1999). In neural stem cells, BDNF acts as a neuroprotection agent against neurotoxin-exerted apoptotic death through PI3K/Akt and MAPK pathways (Nguyen et al., 2009). Indeed, mechanisms underlying the neuroprotective and functional effect of BDNF include the activation of neuronal pro-survival signaling pathways, including PI3K/Akt as well as MAPK (ERK) signaling pathway (Kaplan D.R. et al., 2000). Three other neuroactive substances (NA, Insulin and IGF-1) enhance MCT2 expression through activation of these pathways. All these substances were shown to have a role in neuroprotection. Indeed, *in vitro* experiments showed that insulin can reduce oxidative stress-induced apoptosis by activating the PI3K/Akt signaling pathway (Yu et al., 2006). Similarly IGF-1 was shown to protect cortical neurons from apoptosis via PI3K/Akt and ERK pathways (Willaime-Morawek et al., 2005) and noradrenaline (NA) has a neuroprotective effect on dopaminergic neurons by activation of MAPK signaling pathway (Troadec JD et al., 2002).

These data suggest that the activation of PI3K/Akt and MAPK signaling pathway are involved in neuroprotection. Since MCT2 was shown to be upregulated via these signaling pathways and can participate to neuroprotection, it could be one of factors involved in the protective effect of these substances. It could be interesting to investigate this possible protective role for example in neurodegenerative diseases

6.4.2 Neurodegenerative disorders

As described above, a link exists between dysregulation of BDNF signaling and some neurodegenerative pathologies. In fact, neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease and Huntington's disease, present an impairment in oxidative/energy metabolism, and thus altered glucose metabolism (Reviewed by Blass 2001). Two common points that they share is a decrease of cerebral glucose metabolism and a dysregulation of BDNF expression.

Alzheimer's disease is characterized by the progressive loss of synapses and, subsequently, of neurons themselves, which occurs within diverse cortical circuits, beginning in the entorhinal

cortex and the hippocampus, two structures essential for memory formation (Braak and Braak, 1991; Kordower et al., 2001; Masliah et al., 1994). BDNF is normally produced in the entorhinal cortex and the hippocampus in adulthood (Yan et al., 1997). Intriguingly, BDNF levels become deficient in the entorhinal cortex and the hippocampus in Alzheimer's disease (Connor et al., 1997; Hock et al., 2000; Narisawa-Saito et al., 1996). Thus, the idea of a therapeutic application of BDNF in AD has emerged. Indeed, BDNF gene delivery improved learning and memory in hippocampus-dependent tasks in aged rats and non-human primate models (Nagahara et al., 2009). Concerning the metabolic state in AD patients, a severe reduction of the cerebral metabolic rate of glucose was detected (Zakzanis KK et al., 2003, Mosconi et al., 2008). Moreover, some studies suggest that two opposite metabolic processes may occur in AD brains: a reduction of neuronal glucose metabolism and an increase of astroglial metabolism (Allaman et al., 2010, Blass, 2001; Gibson, 2002). In this context, if BDNF plays a role in regulating lactate metabolism in the brain, as suggested by its role in MCT2 expression upregulation, it is likely that a deficit of BDNF will negatively affect lactate supply to neurons. Thus, it is possible that the re-establishment of BDNF release/expression in AD patients would enhance the level of MCT2, increasing lactate uptake in neurons. However, some experiments must be performed to confirm this hypothesis. For example, it would be interesting to study the expression of MCTs in brain of AD mouse models or of old mice.

BDNF expression is also dysregulated in Parkinson's disease, a neurodegenerative disorder that impairs motor function and cognitive ability, affecting dopaminergic neurons from substantia nigra projecting to the striatum. Indeed, the expression of BDNF protein and its mRNA are reduced in nigral neurons (Mogi et al., 1999; Parain et al., 1999). Moreover, a BDNF treatment in rodent models prevented the loss of dopaminergic neurons in the substantia nigra after chemical-induced lesion (Levivier et al., 1995; Frim et al.,1994). Furthermore, in non-human primates, BDNF protein infusion exhibited beneficial anatomical and behavioural effect by reducing cell loss and enhancing striatal reinnervation (Tsukahara et al., 1995). It could be interesting to investigate whether MCT2 expression is also dysregulated in this neuropathology.

In the same manner, BDNF expression is dysregulated in Hungtinton's disease (HD). Huntington's disease is an autosomal dominant inherited neurodegenerative disease caused by the expansion of CAG triplicate repeats in the gene encoding the protein huntingtin (HTT).

The disease is characterized by involuntary choreiform movements, cognitive impairment and psychiatric dysfunction and has an onset in mid-adult life. Interestingly, BDNF levels decline in the striatum and the cerebral cortex in HD patients (Walker 2007). A BDNF infusion into the striatum of HTT-mutant mice increases the survival of striatal neurons and improves motor function (Canals et al., 2004). Recently, it was shown that astrocytes engineered to release BDNF can constitute a therapeutic approach for HD (Giralt A et al., 2010). But until now, BDNF therapy has not still been studied in non-human primate HD models.

Thus, considering that BDNF regulates MCT2 expression, it could be a useful agent in the treatment of this neurodegenerative disease by promoting also lactate uptake into neurons coming from astrocytes. However, to confirm this hypothesis, many experiments remain to be performed.

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Annexe: Publications

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Brain-derived neurotrophic factor enhances the expression of the monocarboxylate transporter 2 through translational activation in mouse cultured cortical neurons

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MCT2 is the predominant neuronal monocarboxylate transporter allowing lactate use as an alternative energy substrate. It is suggested that MCT2 is upregulated to meet enhanced energy demands after modifications in synaptic transmission. Brain-derived neurotrophic factor (BDNF), a promoter of synaptic plasticity, significantly increased MCT2 protein expression in cultured cortical neurons (as shown by immunocytochemistry and western blot) through a translational regulation at the synaptic level. Brain-derived neurotrophic factor can cause translational activation through different signaling pathways. Western blot analyses showed that p44/p42 mitogen-activated protein kinase (MAPK), Akt, and S6 were strongly phosphorylated on BDNF treatment. To determine by which signal transduction pathway(s) BDNF mediates its upregulation of MCT2 protein expression, the effect of specific inhibitors for p38 MAPK, phosphoinositide 3-kinase (PI3K), mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK), p44/p42 MAPK (ERK), and Janus kinase 2 (JAK2) was evaluated. It could be observed that the BDNF-induced increase in MCT2 protein expression was almost completely blocked by all inhibitors, except for JAK2. These data indicate that BDNF induces an increase in neuronal MCT2 protein expression by a mechanism involving a concomitant stimulation of PI3K/Akt/mTOR/S6, p38 MAPK, and p44/p42 MAPK. Moreover, our observations suggest that changes in MCT2 expression could participate in the process of synaptic plasticity induced by BDNF.
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Keywords: energy metabolism; lactate; MAPK; mTOR; PI3K; synaptic plasticity

Introduction

Brain-derived neurotrophic factor (BDNF) is a widely expressed neurotrophin in the central nervous system (Skup, 1994). Acting through specific tyrosine kinase receptors, BDNF affects neuronal survival, differentiation, and synaptic plasticity after the activation of multiple intracellular signal transduction mechanisms, such as phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) signaling pathways (Bramham and Messaoudi, 2005). Presynaptically, BDNF potentiates depolarization-evoked Ca^{2+}-dependent glutamate release while causing direct glutamate release through Ca^{2+} mobilization from Ins(1,4,5)P_3-sensitive stores, whereas the postsynaptic actions of BDNF include changes in glutamate receptor phosphorylation, subcellular localization and synthesis, and local alterations in protein synthesis, as well as long-term changes in gene expression (Carvalho *et al*, 2008). These effects of BDNF contribute not only to synaptic plasticity but also to modifications in spine density and morphology (Carvalho *et al*, 2008). However, one aspect that has not been explored in the context of synaptic plasticity concerns putative changes in neuroenergetics. Indeed, it is likely that as a consequence of alterations in synaptic efficacy, the supply of energy substrates must be adapted to meet the energy needs imposed by new levels of synaptic response.

In recent years, the role of monocarboxylates such as lactate as additional energy substrates for neurons has attracted increasing attention (Pellerin, 2003), raising interest for the identification of specific

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transporters in the central nervous system. MCT2 was shown to be the predominant monocarboxylate transporter expressed by neurons (Pierre *et al*, 2002). It belongs to a family of proton-linked carriers involved in the transport of lactate, pyruvate, and ketone bodies (Garcia *et al*, 1994, 1995). MCT2 immunoreactivity was found to be abundant in the neuronal processes of various brain regions, including the cortex, hippocampus, and cerebellum (Bergersen *et al*, 2002; Pierre *et al*, 2002). At the subcellular level, MCT2 is expressed on axons and dendrites (Pierre *et al*, 2002, 2009). Moreover, MCT2 is present at glutamatergic synapses and exclusively on postsynaptic elements (Bergersen *et al*, 2002, 2005; Pierre *et al*, 2009). It is particularly enriched in the postsynaptic density as well as in an intracellular pool within the spines (Bergersen *et al*, 2005). Recent observations have shown that MCT2 expression can be regulated in cultured neurons. Thus, it was found that noradrenaline, insulin, and insulin-like growth factor-1 (IGF-1) increase MCT2 protein expression in neurons through a translational mechanism (Chenal and Pellerin, 2007; Chenal *et al*, 2008). Interestingly, each of these neuroactive substances is known to induce long-term changes in synaptic transmission (Kobayashi and Yasoshima, 2001; Trejo *et al*, 2007; van der Heide *et al*, 2006). Therefore, it became of interest to investigate whether BDNF could regulate MCT2 expression in cultured neurons and to characterize the signal transduction pathways involved in this effect. In addition, the nature of the mechanism (transcriptional or translational) by which MCT2 expression is regulated by BDNF has been investigated.

Materials and methods

Neuronal Cultures and Pharmacological Treatments

Primary cultures of mouse cortical neurons were prepared from embryonic day 17 OF1 mice (Charles River, Lyon, France). As described previously (Debernardi *et al*, 2003), after decapitation and brain dissection, cortices were mechanically dissociated in phosphate-buffered saline (PBS) supplemented with glucose (NaCl, 150 mmol/L; KCl, 3 mmol/L; KH_2PO_4, 1.5 mmol/L; Na_2HPO_4, 7.9 mmol/L; glucose, 33 mmol/L; penicillin, 0.006 g/L; streptomycin, 0.1 g/L; pH 7.4). Cells were plated on poly L-ornithine (15 mg/L)-precoated dishes and cultured in neurobasal-B27 medium (Brewer *et al*, 1993) supplemented with 0.5 mmol/L L-glutamine. All experiments were carried out on day 7 *in vitro*. At this stage, cultures contained $<5\%$ of glial cells (Debernardi *et al*, 2003). Neuronal treatments with pharmacological agents were carried out without changing the medium before or during the incubation time. Brain-derived neurotrophic factor (CYT-207; Brunschwig, Basel, Switzerland) was added directly into the culture medium at various concentrations and cells were incubated for the indicated times. Rapamycin, 20 ng/mL (mammalian target of rapamycin (mTOR) inhibitor), SB202190 HCl, 10 μmol/L and SB203580 HCl, 10 μmol/L (p38 MAPK inhibitors), LY294002, 10 μmol/L (PI3K inhibitor), PD98059, 50 μmol/L (MAPK/ERK kinase (MEK inhibitor), UO126, 10 μmol/L (p44/p42 MAPK (ERK, extracellular signal-regulated kinase) inhibitor), and AG490 25 μmol/L (JAK2 (Janus kinase 2) inhibitor) were added directly to the medium 30 mins before BDNF. All these inhibitors were purchased from Alexis Biochemicals (Lausen, Switzerland), except LY294002 (L9908; Sigma, Buchs, Switzerland) and SB203580 (S8307; Sigma). Transcription and translation inhibitors (5 μmol/L actinomycin D (ActD) and 10 μmol/L cycloheximide, respectively) were added 30 mins before pharmacological agents. All other chemicals were purchased from Sigma. Data represent mean ± s.e.m. of '*n*' determinations, which are independent measurements (from different culture plates) obtained from at least three separate neuronal cultures.

Immunocytochemistry and Related Quantification

After removal of the culture medium, cells were carefully rinsed in PBS at 37°C and directly postfixed in an ice-cold paraformaldehyde fixative (4% in PBS for 30 mins at 20°C). Fixed cells were treated with casein (0.5% in PBS) for 1 h at room temperature to block nonspecific sites. For immunostaining, cultures were incubated overnight at 4°C in 50 μL of freshly prepared MCT2 antibody solution (anti-MCT2 diluted 1:500 in PBS containing 0.25% bovine serum albumin) (Pierre *et al*, 2000). After carefully rinsing in PBS, cultures were incubated in a solution containing Cy3-conjugated anti-rabbit Igs (diluted 1:500 for 2 h at room temperature; Jackson Immunoresearch, Baltimore, MD, USA). After rinsing in PBS twice and a final rinsing in water, coverslips were mounted with Vectashield (Reactolab SA, Burlingame, CA, USA). Coverslips were examined and photographed with an Axioplan2 microscope (Zeiss, Hallbergmoos, Germany) using epifluorescence with an appropriate filter.

To quantitatively assess the influence of different treatments on MCT2 protein expression, a quantitative analysis of images obtained by epifluorescence with a $\times 20$ objective and acquired using a cooled CCD camera (Axiocam, Zeiss), together with the 4.6 Axiovision software (Zeiss) was carried out. Three fields were chosen randomly on each coverslip; they contained at least 20 MCT2-labeled neurons per field. All pictures were acquired and presented as different levels of gray with identical acquisition time for all. Pictures were then analyzed using NIH software (National Institutes of Health Image program, version 1.62, Rockville Pike, MD, USA). The fluorescence intensity of eight isolated cells taken randomly in each of the three captured areas was

assessed. The average fluorescence intensity representing neuronal MCT2 expression was obtained by calculating the average of 24 measurements per coverslip. Measurements were obtained in a blinded manner with the investigator unaware of the culture treatments. Mean and s.e.m. for a particular condition were calculated from average fluorescence intensity values of distinct coverslips representing independent determinations (numbers indicated on each bar of the graph). Data were statistically analyzed with an ANOVA (analysis of variance), followed by Dunnett's or Bonferroni's test.

Western Blot and Related Quantification

Neurons in each culture dish were homogenized in 50 μL of buffer containing the following: Tris-HCl, pH 6.8, 20 mmol/L; sucrose, 0.27 mol/L; EGTA (ethylene glycol tetraacetic acid), 1 mmol/L; EDTA (ethylene diamine tetraacetic acid), 1 mmol/L; NaF, 50 mmol/L; Triton X-100, 1%; β-glycerophosphate, 10 mmol/L; DTT (dithiothreitol), 10 mmol/L; 4-nitrophenylphosphate, 10 mmol/L; and a mixture of protease inhibitors (Complete, Roche Molecular Biochemicals, Mannheim, Germany). Each stimulated condition was examined in duplicate and the contents of the two Petri dishes were pooled. Protein samples were sonicated and heated at 95°C for 5 mins in half the final volume of SDS-PAGE sample buffer (Tris-HCl, 62.5 mmol/L; DTT, 50 mmol/L; SDS, 2%; glycerol, 10%; and bromophenol blue, 0.1%). Samples were loaded onto polyacrylamide gels composed of a 10% or 6% acrylamide-bisacrylamide running gel and a 4.5% acrylamide-bisacrylamide stacking gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes (Trans-Blot Transfer Medium 162-0115; Bio-Rad, Reinach, Switzerland) using a Transblot semi-dry transfer cell (Bio-Rad). For protein detection, membranes were incubated in a blocking solution of Tris-buffered saline supplemented with Tween-20 (TBST; Tris-HCl, pH 7.5, 50 mmol/L; NaCl, 150 mmol/L; and Tween-20, 0.1%) containing 5% nonfat milk for 1 h at room temperature. Membranes were incubated overnight at 4°C with the antiphospho-serine/threonine protein kinase from AKT virus (Akt)-Ser473 (1:700), antiphospho-p44/p42 MAPK-Thr202/Tyr204 (both 1:1,000), antiphospho-mTOR-Ser1448 (1:1,000), anti-mTOR (1:1,000), antiphospho-S6-Ser235/236 ribosomal protein (1:1,000), and anti-β-actin (A5441; Sigma). All primary antibodies were purchased from Cell Signalling (BioConcept, Allschwil, Switzerland), except anti-β-actin (A5441; Sigma). After three washes in TBST, membranes were incubated with the secondary antibodies Alexa Fluor 680 goat anti-IgG (Juro, Lucerne, Switzerland) and IRDye 800 goat anti-mouse IgG (BioConcept), diluted at 1:5,000 in TBST containing 1% nonfat milk, for 2 h at room temperature, and protected from light. After three washes in TBST, membranes were scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA), which permits detection and quantification of proteins of interest. β-Actin, revealed in green, was used for normalization and the proteins of interest were revealed in red. As phospho-mTOR has a very high molecular weight (289 kDa), actin was not visible on the same gel. To normalize western blots for phospho-mTOR, samples were loaded in duplicate onto a 6% running gel and proteins were transferred onto a nitrocellulose membrane using a 10% methanol transfer buffer. The membrane was then cut into two identical pieces and probed either with the phospho-mTOR antibody (1:1,000) or with the mTOR antibody (1:1,000). Thus, quantifications were performed on samples resolved on the same gel and transferred onto the same membrane, and normalization was conducted against mTOR (instead of β-actin). The remaining of the procedure was unchanged.

Quantitative Real-Time Reverse Transcriptase-PCR

Quantitative determination of MCT2 mRNA expression levels was performed by quantitative reverse transcriptase-PCR according to Heid *et al* (1996) using an ABI Prism 7000 sequence detection system from Applied Biosystems (Rotkreuz, Switzerland). The following sets of oligonucleotides were used: $5' \rightarrow 3'$; ActinFo, GCTTCTTTGCAGCTCCTTCGT; ActinRe, ATATCGTCATCCATGGCGAAC (Embl: X03672); MCT2Fo, CAGCAACAGCGTGATAGAGCTT; MCT2Re, TGGTTGCAGGTTGAATGCTAAT (Embl: NM_011391); NPYFo, ACCAGACAGAGATATGGCAAGAGA; NPYRe, GGCGTTTTCTGTGC (produced by Microsynth, Balgach, Switzerland).

Preparation and Stimulation of Synaptoneurosomes

Synaptoneurosomes were prepared from the forebrains of 8- to 14-day-old mice pups (10 to 15 pups per preparation) according to a previously published protocol (Rao and Steward (1991), as modified by Schratt *et al* (2004)). Briefly, the total forebrains were dissected in 20 mL of homogenization buffer (0.32 mol/L sucrose, 0.1 mmol/L EDTA, 0.25 mmol/L DTT, and 3 mmol/L HEPES, pH 7.4) and disrupted using a Teflon-coated Dounce-Potter homogenizer (B. Braun, Crissier, Switzerland) by eight up-and-down strokes. Nuclei and cell debris were pelleted by 2 mins of centrifugation at 2,000 *g*. The supernatant was collected and centrifuged for an additional 10 mins at 14,000 *g* to pellet a crude synaptoneurosome-containing fraction (P2). The pellet was then brought up to 8 mL total volume (with a solution of 0.32 mol/L sucrose and 1 mmol/L $NaHCO_3$). This suspension (4×2 mL) was layered onto three different discontinuous sucrose gradients (0.85, 1, 1.2 mmol/L) that had been equilibrated at 4°C for 1 h. The gradient was centrifuged at 45,000 *g* for 45 mins in a Centrikon T-1075 ultracentrifuge using a

SW 41.14Ti swinging bucket rotor. Synaptoneurosomes were collected from the 1/1.2 mol/L interface (500 μL for 2 mL of P2), washed twice in 1 × PBS (in a 2-mL tube), with a centrifugation at 7,000 *g* for 2 mins. Synaptoneurosomes were resuspended in 500 μL of synaptoneurosome incubation buffer (10 mmol/L Tris, pH 7.5, 2.2 mmol/L $CaCl_2$, 0.5 mmol/L Na_2HPO_4, 0.4 mmol/L KH_2PO_4, 4 mmol/L $NaHCO_3$, and 80 mmol/L NaCl). Synaptoneurosomes were centrifuged at 7,000 *g* for 2 mins, the supernatant was discarded, and synaptoneurosomes in the lower fraction were either used immediately or stored at −80°C. No significant differences were observed between results from freshly prepared or frozen synaptosomes such that results were pooled. Enrichment of the synaptosomal fraction was controlled by performing western blotting for the postsynaptic protein PSD-95 (data not shown).

The synaptoneurosome pellet was thawed on ice for 30 mins and diluted in a prewarmed (37°C) synaptoneurosome incubation buffer containing a mixture of antiproteases (Complete 11257000; Roche Molecular Biochemicals) to yield a protein concentration between 2 and 9 mg/mL. Synaptoneurosome samples (100 μL) were exposed to either ActD (5 μmol/L) or cycloheximide (10 μmol/L) for 30 mins before application of 100 ng/mL BDNF for 6 h. The reaction was stopped by adding 20 μL of SDS buffer (5 ×), and samples were boiled for 5 mins and served as starting material for western blotting.

Results

Effect of Brain-Derived Neurotrophic Factor on MCT2 Protein Expression in Cultured Neurons

To assess the effect of BDNF on neuronal MCT2 protein expression, cultured cortical neurons were treated with BDNF for 6 h at various concentrations up to 100 ng/mL. Experiments conducted with BDNF concentrations of 10, 50, and 100 ng/mL followed by immunocytochemistry led to a striking enhancement of fluorescence intensity corresponding to higher levels of MCT2 immunoreactivity (IR) at 100 ng/mL (Figure 1A). Western blot analysis showed that the maximal increase of MCT2 expression was found with 100 ng/mL. At lower concentrations, MCT2 expression had a tendency to be increased, but the effect was not significant statistically (Figure 1B).

Changes in the levels of MCT2 IR induced by BDNF were also studied as a function of time (1, 6, 12, and 24 h). It was observed that 100 ng/mL BDNF caused a 40% increase in MCT2 IR 6 h after the beginning of the treatment that remained elevated at 12 and 24 h (Figure 2A). Western blot analysis showed that the increase of MCT2 expression was already found significant as early as after 1 h of BDNF stimulation (100 ng/mL) and that this significant increase was still present up to 24 h (Figure 2B).

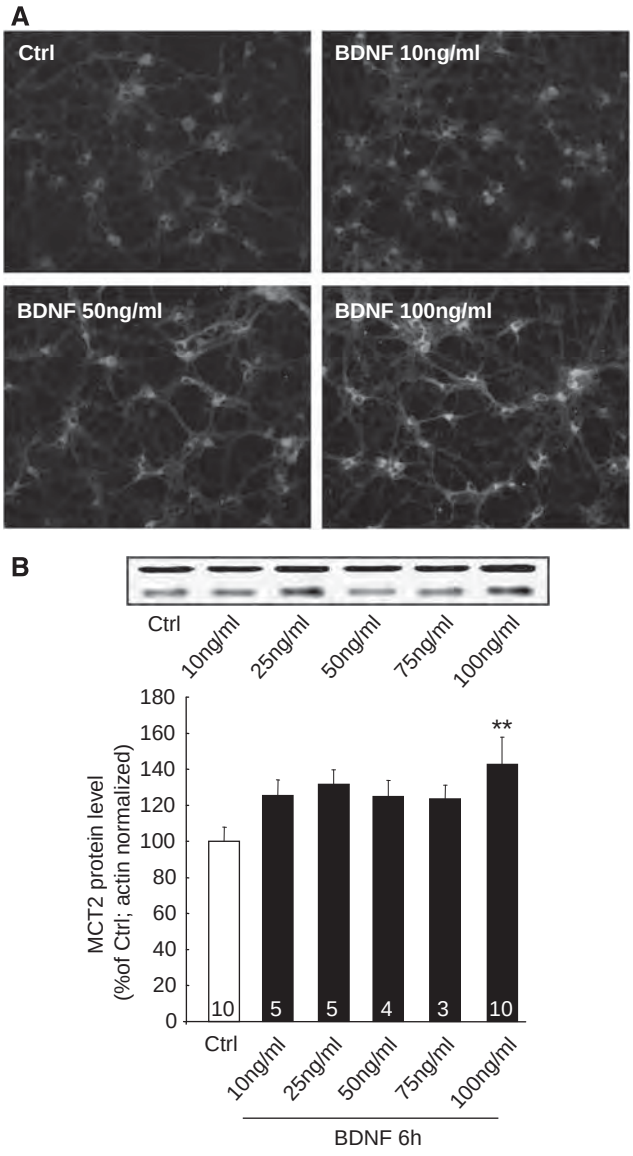

Figure 1 Concentration-dependent effect of BDNF on MCT2 expression in primary cultures of mouse cortical neurons. (**A**) Immunocytochemical stainings for MCT2 in untreated cultures (Ctrl) and in cultures treated with BDNF for 6 h at different concentrations. (**B**) Western blot analysis of MCT2 expression in primary cultures of mouse cortical neurons treated with BDNF for 6 h at various concentrations up to 100 ng/mL. Western blots were quantified using Odyssey software (LI-COR Biosciences, Lincoln, NE, USA). Results were expressed as percentage of control after the values were normalized using β-actin signal as the reference. Statistical analysis was performed using ANOVA followed by Dunnett's test. ** indicates MCT2 protein levels significantly different from control with $P < 0.01$. Numbers in the graph bars represent the number of independent experiments for each condition.

The effect of BDNF was found to be as robust as the previously described effect of noradrenaline (100 μmol/L) on MCT2 expression after 6 h of treatment (Figure 2C).

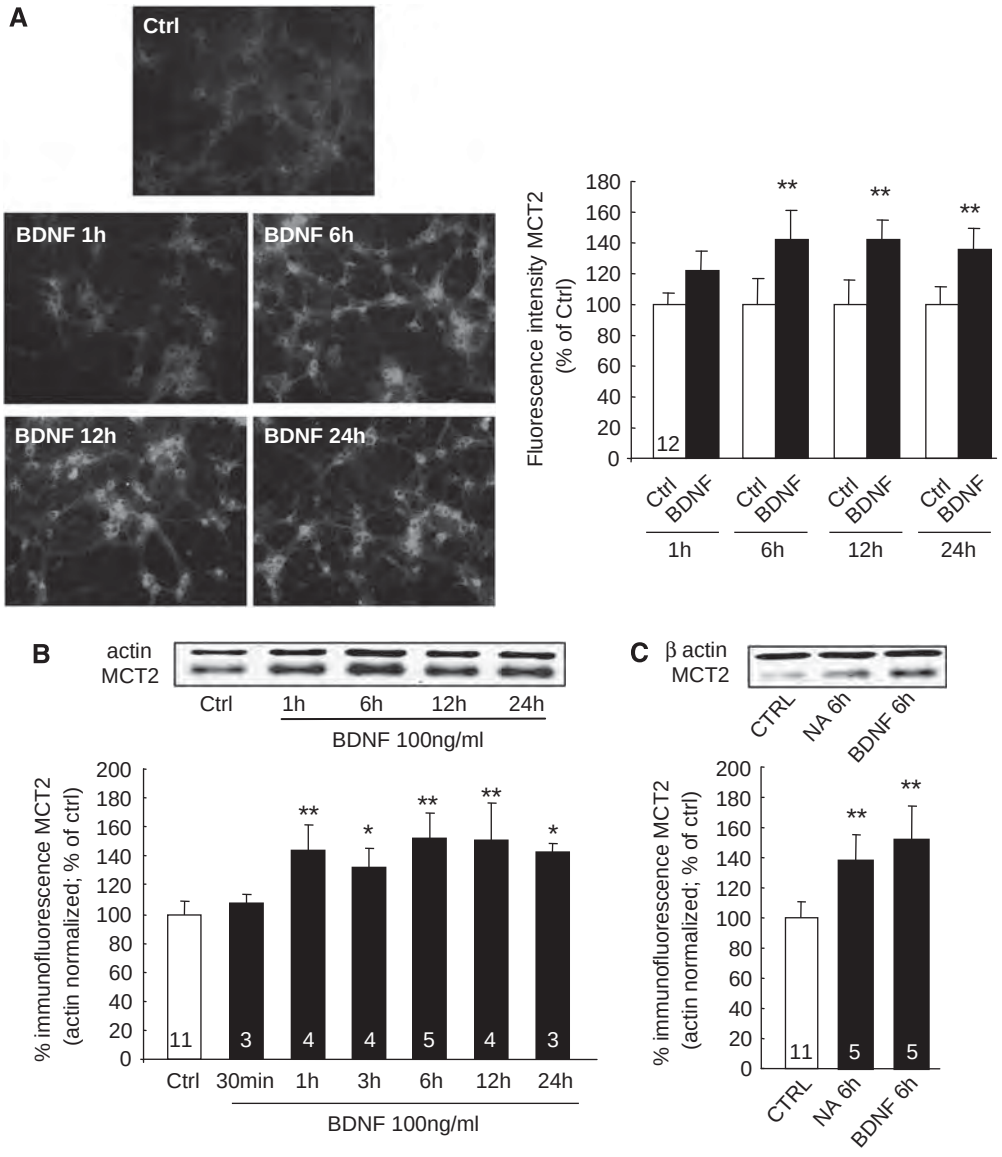

Figure 2 Time course of the effect of BDNF on MCT2 protein expression in primary cultures of mouse cortical neurons. (**A**) MCT2 IR and related quantification of primary cultures of mouse cortical neurons treated with BDNF at a final concentration of 100 ng/mL for various periods of time up to 24 h (1, 6, 12, and 24 h). Left panels represent immunocytochemical stainings for MCT2 in untreated cultures (Ctrl) or cultures treated with BDNF for 6 h. The bar graph represents the quantitative determination of fluorescence intensity corresponding to MCT2 IR in cultured neurons treated with BDNF for various periods of time up to 24 h. Results are expressed as percentage of control fluorescence intensity and are represented as the mean ± s.e.m. of independent determinations (numbers indicated on bars) from four distinct experiments. The value of fluorescence intensity for each determination represents the average level from 24 cells on the same coverslip. (**B** and **C**) Western blot analysis of MCT2 protein expression in cultures of mouse cortical neurons treated with BDNF 100 ng/mL or noradrenaline 100 μmol/L (NA 6 h) for the indicated times as compared with untreated cells (Ctrl). Western blots were quantified using Odyssey software (LI-COR Biosciences, Lincoln, NE, USA). Results are expressed as percentage of control (mean ± s.e.m.) after the values had been normalized using β-actin signal as the reference. Statistical analysis was performed using ANOVA followed by Dunnett's test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control (Ctrl) for MCT2 protein levels. Numbers in the graph bars represent the number of independent experiments for each condition.

Putative Involvement of Distinct Signal Transduction Pathways in Brain-Derived Neurotrophic Factor-Induced MCT2 Expression

Involvement of specific signal transduction mechanisms involved in the effect of BDNF on MCT2 expression was investigated with a series of inhibitors. Cultured cortical neurons were pretreated with five different inhibitors (LY294002, a specific PI3K inhibitor; rapamycin, a specific mTOR inhibitor; PD98058, a specific MEK inhibitor; SB202190, a specific p38 MAPK inhibitor; and AG490, a specific JAK2 inhibitor) 30 mins before the addition of BDNF (100 ng/mL) for 6 h. The six panels on the left of Figure 3A show that a 30-min pretreatment of cultured mouse cortical neurons with LY294002

(10 μmol/L), rapamycin (20 ng/mL), PD98058 (50 μmol/L), or SB202190 (10 μmol/L) blocked the BDNF-induced increase in MCT2 IR after 6 h of treatment. In contrast, AG490 (25 μmol/L) had no effect (not shown). The quantification of MCT2 IR shows that all inhibitors (with the exception of AG490) prevented the induction of MCT2 protein expression by BDNF, reducing the overall expression by $\sim$40% (Figure 3A). Similar results were obtained by western blot, confirming the MCT2 IR data (Figure 3B).

The effect of all five inhibitors alone (without posttreatment with BDNF) was tested previously on cultured cortical neurons and no change in MCT2 IR was observed compared with the control condition (data not shown). To further validate our observations, two more inhibitors were tested for their effect on BDNF-induced MCT2 expression (UO126, a specific p44/p42 MAPK inhibitor, and SB203580, another specific p38 MAPK inhibitor). Cultured cortical neurons were pretreated with UO126 (10 μmol/L) and SB203580 (10 μmol/L) 30 mins before the addition of BDNF (100 ng/mL) for 6 h. Western blot analysis shows that UO126 and SB203580 significantly reduced MCT2 protein expression induced by BDNF (Figure 3C).

Activation by Brain-Derived Neurotrophic Factor of the Different Signal Transduction Pathways Implicated in MCT2 Upregulation

It became necessary to assess the effect of BDNF on each signal transduction pathway putatively implicated in MCT2 upregulation and to verify the efficacy of each inhibitor used. Cultured cortical neurons were treated with BDNF (100 ng/mL) for three time periods (5 mins, 30 mins, and 1 h). First, to evaluate the activation of the PI3K/Akt/mTOR/S6 pathway, the phosphorylation levels of Akt on Ser473, mTOR on Ser2448, and S6 ribosomal protein on Ser235/236 were determined by western blot. Brain-derived neurotrophic factor induced the phosphorylation of Akt after 5 mins of treatment (Figure 4A). The level of phosphorylation was increased by $\sim$300% above control (set at 100%) after 5 mins of treatment. Maximal activation was reached after 30 mins. Phosphorylation of Akt was sustained for 1 h of BDNF treatment. To obtain the confirmation that Akt signaling can be inhibited in a manner similar to MCT2 upregulation, cultured cortical neurons were pretreated with 10 μmol/L LY294002 for 30 mins before the addition of BDNF (100 ng/mL) for 5 mins, 30 mins, and 1 h. Indeed, LY294002 pretreatment prevented the phosphorylation of Akt induced by BDNF in cultured cortical neurons (Figure 4A).

Activation of mTOR by BDNF treatment was investigated by monitoring its level of phosphorylation. Brain-derived neurotrophic factor induced the phosphorylation of mTOR within 5 mins after the beginning of the treatment (Figure 4B). Phosphorylation of mTOR was further increased by more than 50% above the control level after 1 h of BDNF treatment. To determine whether mTOR activation can be prevented in the same manner as MCT2 upregulation, cultured cortical neurons were pretreated with 20 ng/mL rapamycin for 30 mins before adding BDNF (100 ng/mL) for 5 mins, 30 mins, and 1 h. Rapamycin pretreatment completely prevented the phosphorylation of mTOR induced by BDNF in cultured cortical neurons at 30 mins (Figure 4B).

S6 phosphorylation represents one of the late steps in the activation of the PI3K/Akt/mTOR/S6 pathway. Phosphorylation levels of S6 were assessed after BDNF treatment. Brain-derived neurotrophic factor induced the phosphorylation of S6 within 5 mins after the beginning of the treatment (80% above control; Figure 4C). Maximal activation was reached after 1 h with an increase of 350% above control. As the S6 protein is known to be a downstream effector of the Akt/mTOR signaling pathway, cultured cortical neurons were pretreated with 20 ng/mL rapamycin (inhibitor of mTOR) for 30 mins before the addition of BDNF for 5 mins, 30 mins, and 1 h. It was observed that rapamycin pretreatment completely prevented the phosphorylation of S6 ribosomal protein induced by BDNF in cultured cortical neurons (Figure 4C).

Mitogen-activated protein kinase activation is purported to be involved in the effect of BDNF on MCT2 expression. Phosphorylation levels of p44 and p42 MAPK (phospho-ERK) on Thr202/Tyr204 were determined by western blot after treatment of cultured cortical neurons with BDNF (100 ng/mL) for three different time periods (5 mins, 30 mins, and 1 h). Brain-derived neurotrophic factor induced the phosphorylation of p44 and p42 MAPK after 5 mins of treatment (Figure 5A). The level of phosphorylation was increased by more than 500% (for both p44 and p42) above control (set at 100%) at that time. Phosphorylation of p44 and p42 MAPK was sustained for 1 h of BDNF treatment. To ascertain whether MAPK activation can be prevented similar to MCT2 upregulation, cultured cortical neurons were pretreated with 50 μmol/L PD98059 for 30 mins before the addition of BDNF (100 ng/mL) for 5 mins, 30 mins, and 1 h. As expected, PD98059 pretreatment attenuated the phosphorylation of p44 and p42 MAPK induced by BDNF in cultured cortical neurons (Figure 5A).

Activation of p38 MAPK is another possible signaling mechanism involved in BDNF-induced MCT2 upregulation. Phosphorylation levels of p38 MAPK on Thr180/Tyr182 were determined by western blot. Brain-derived neurotrophic factor induced the phosphorylation of p38 MAPK that reached a maximum after 1 h with an increase of $\sim$150% above control (set at 100%; Figure 5B). To investigate whether p38 MAPK activation can be prevented using the inhibitor previously used, cultured cortical

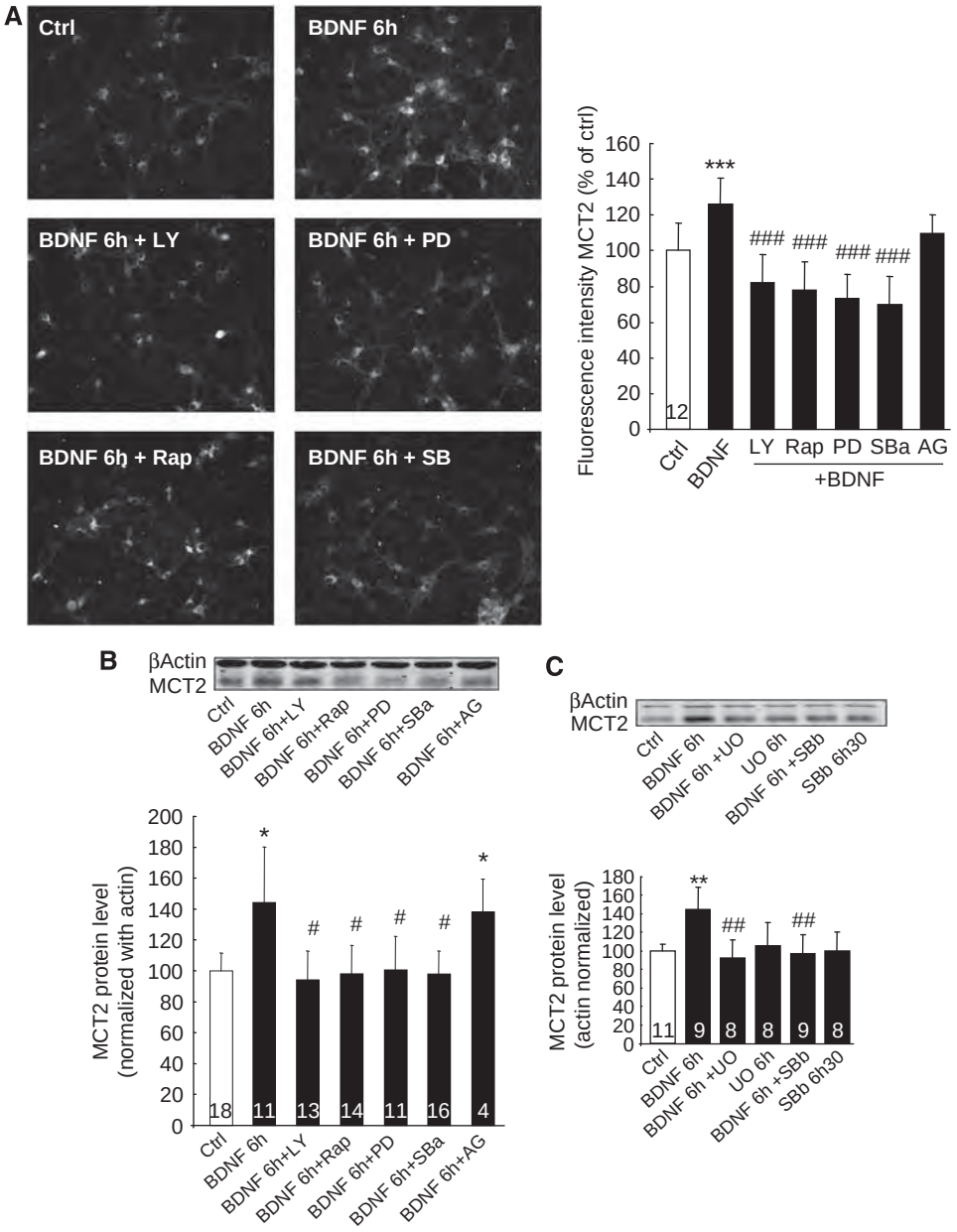

Figure 3 Effect of different signaling pathway inhibitors on BDNF-stimulated MCT2 expression in primary cultures of mouse cortical neurons. Primary cultures of mouse cortical neurons were pretreated for 30 mins with specific signaling pathway inhibitors (PD98095, 50 μmol/L (PD); SB202190, 10 μmol/L (SB_a); LY294002, 10 μmol/L (LY); rapamycin, 20 ng/mL (Rap); AG490 25 μmol/L (AG); UO126, 10 μmol/L (UO); SB203580, 10 μmol/L (SB_b)) before stimulation with BDNF 100 ng/mL for 6 h (BDNF 6 h). (**A**) Left panels represent immunocytochemical stainings for MCT2 in untreated cells (Ctrl), cultures treated with BDNF alone for 6 h (BDNF), or pretreated for 30 mins with each inhibitor (LY, Rap, PD, SB_a) before the addition of BDNF. The bar graph represents the quantitative determination of fluorescence intensity corresponding to MCT2 IR in cultured neurons treated with the specific signaling pathway inhibitors followed by exposure to BDNF for 6 h. Results are expressed as percentage of control fluorescence intensity and represent the mean ± s.e.m. of independent determinations (numbers indicated on bars) from four distinct experiments. The value of fluorescence intensity for each determination represents the average level from 24 cells on the same coverslip. (**B** and **C**) Western blot analysis of MCT2 expression in cultures of mouse cortical neurons treated with specific signaling pathway inhibitors (+ LY, Rap, PD, SB_a, AG, UO, SB_b) before stimulation with BDNF 100 ng/mL for 6 h (BDNF 6 h). Western blots were quantified using Odyssey software (LI-COR Biosciences, Lincoln, NE, USA). Results are expressed as percentage of control (mean ± s.e.m.) after the values had been normalized using β-actin signal as the reference. Statistical analysis was performed using ANOVA followed by Bonferroni's test. $^*P < 0.05$, $^{**}P < 0.01$ versus control (Ctrl). $^{\#}P < 0.05$ versus 6 h BDNF treatment. Numbers in the graph bars represent the number of independent experiments for each condition.

neurons were pretreated with 10 μmol/L SB202190 for 30 mins before the addition of BDNF (100 ng/mL) for 5 mins, 30 mins, and 1 h. SB202190 pretreatment was found to prevent phosphorylation of p38 MAPK induced by BDNF in cultured cortical neurons (Figure 5B).

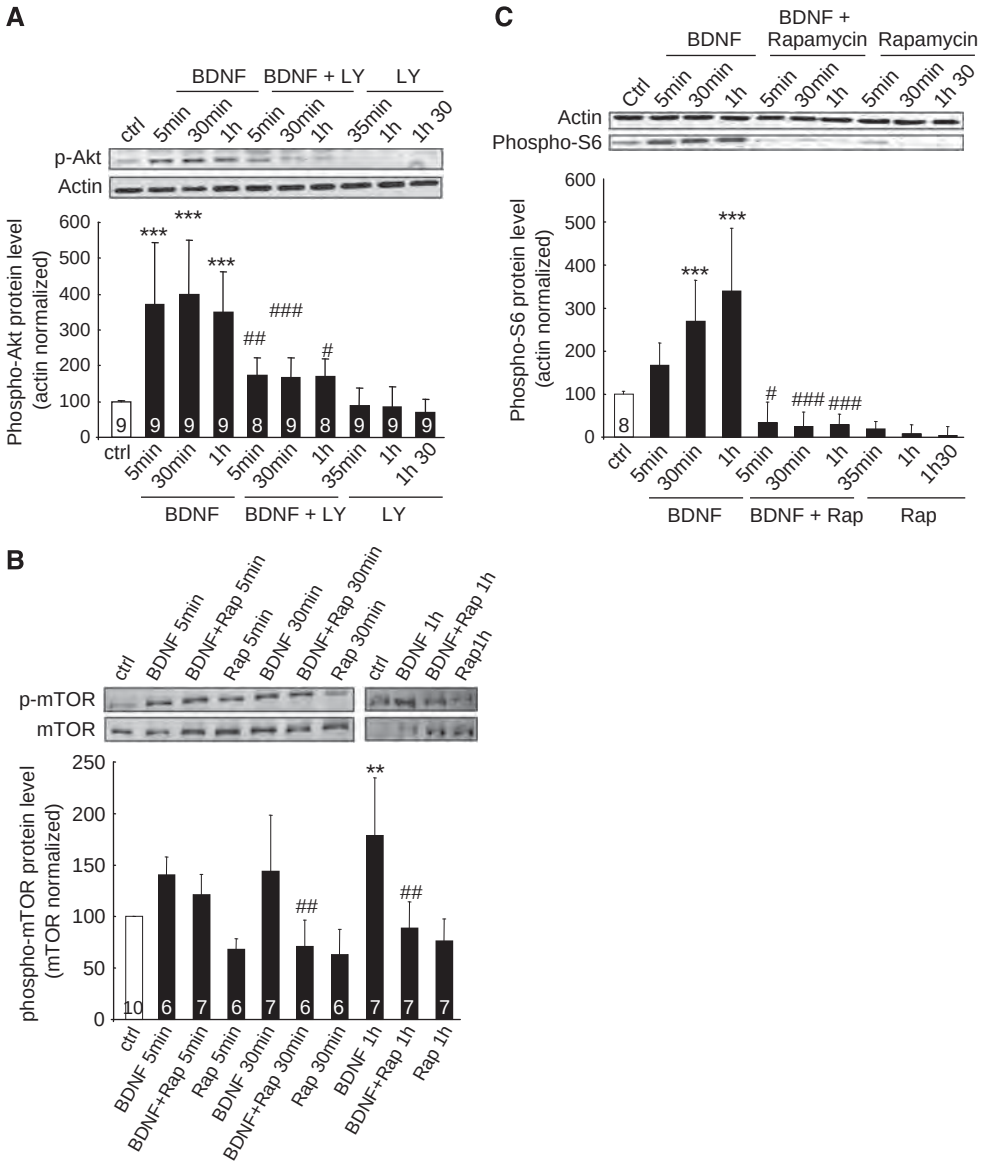

Figure 4 Effect of BDNF on the phosphorylation levels of Akt, mTOR, and S6 in cultured mouse cortical neurons. Western blot analysis of phospho-Akt (**A**), phospho-mTOR (**B**), and phospho-S6 (**C**) levels in cultures of mouse cortical neurons treated with BDNF 100 ng/mL for the indicated times as compared with untreated cells (ctrl). (Panel A) LY294002, a specific PI3K inhibitor, was added to the culture medium at the concentration of 10 μmol/L, 30 mins before incubation with BDNF 100 ng/mL during 5 mins, 30 mins, and 1 h. Western blots were quantified using Odyssey software (LI-COR Biosciences, Lincoln, NE, USA). Results are expressed as percentage of control after the values had been normalized using β-actin signal as the reference. (Panels B and C) Rapamycin, a specific mTOR inhibitor, was added to the culture medium at a concentration of 20 ng/mL, 30 mins before incubation with BDNF 100 ng/mL. Results are expressed as percentage of control after the values had been normalized using either β-actin signal (panel C) or mTOR signal (panel B) as the reference. Statistical analysis was performed using ANOVA followed by Bonferroni's test. **, *** indicates phospho-Akt, phospho-mTOR, or phospho-S6 protein levels significantly different from control with $P < 0.01$, $P < 0.001$ respectively. $^{\#, \#\#, \#\#\#}$ indicates phospho-Akt, phospho-mTOR, or phospho-S6 protein levels significantly different from the BDNF-treated condition with $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Numbers in the graph bars represent the number of independent experiments for each condition.

Brain-Derived Neurotrophic Factor Enhances MCT2 Expression by a Translational Mechanism

The effect of BDNF on MCT2 mRNA expression was investigated by quantitative reverse transcriptase-PCR on total RNA from mouse cortical neurons. Neurons were treated with BDNF (100 ng/mL) for various time points up to 24 h. Figure 6A shows that BDNF exerted no enhancing effect on MCT2 mRNA levels at any time point, whereas NPY mRNA levels (a peptide known to be transcriptionally regulated) showed a significant increase after 12 and 24 h. A small but significant decrease in MCT2 mRNA levels was observed at 3 h. To ascertain whether the effect

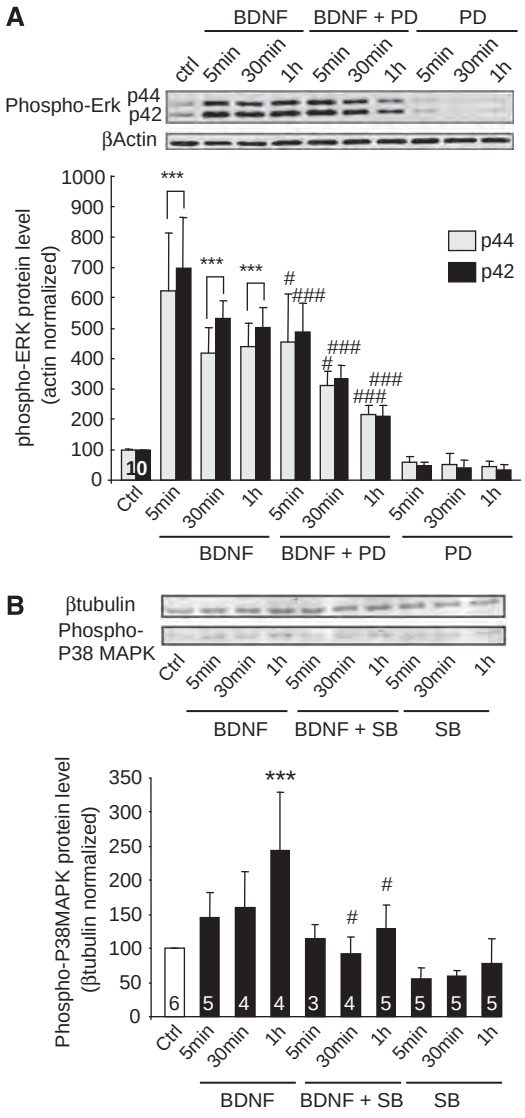

Figure 5 Effect of BDNF on the phosphorylation levels of ERK and p38 MAPK in cultured mouse cortical neurons. Western blot analysis of phospho-ERK (**A**) and phospho-p38 MAPK (**B**) levels in cultures of mouse cortical neurons treated with BDNF 100 ng/mL for the indicated times as compared with untreated cells (ctrl). (Panel A) PD98058, a specific MEK inhibitor, was added to the culture medium at a concentration of 50 μmol/L, 30 mins before incubation with BDNF 100 ng/mL during 5 mins, 30 mins, and 1 h. (Panel B) SB202190, a specific p38 MAPK inhibitor, was added to the culture medium at a concentration of 10 μmol/L, 30 mins before incubation with BDNF 100 ng/mL. Western blots were quantified using Odyssey software (LI-COR Biosciences, Lincoln, NE, USA). Results are expressed as percentage of control after the values had been normalized using β-actin signal as reference. Statistical analysis was performed using ANOVA followed by Bonferroni's test. *** indicates phospho-ERK or phospho-p38 MAPK levels significantly different from control with $P < 0.001$. ##, ### indicates phospho-ERK or phospho-p38 MAPK levels significantly different from BDNF-treated condition with $P < 0.01$ and $P < 0.001$, respectively. Numbers in the graph bars represent the number of independent experiments for each condition.

of BDNF on MCT2 expression requires the activation of translation but not of transcription, cultured neurons were treated with inhibitors for these two processes. Application of cycloheximide (10 μmol/L), a protein synthesis inhibitor, 30 mins before BDNF stimulation (100 ng/mL), prevented the enhancement of MCT2 protein expression (Figure 6B). Quite unexpectedly, the mRNA synthesis inhibitor ActD (5 μmol/L) also blocked the effect of BDNF on MCT2 protein expression. To determine whether the enhancement in MCT2 protein synthesis occurs at the synaptic level and requires transcriptional activation, an experiment was conducted on synaptoneurosomes (Figure 6C). Brain-derived neurotrophic factor (100 ng/mL) induced an increase in MCT2 protein expression after 6 h in this preparation. The effect of BDNF was prevented by cycloheximide (10 μmol/L) but not by ActD (5 μmol/L).

Discussion

In recent years, BDNF has emerged as a major regulator of synaptic transmission and plasticity at adult synapses in many regions of the central nervous system (Bramham and Messaoudi, 2005). Among the mechanisms subserving synaptic plasticity, enhancement of localized protein synthesis constitutes a critical process for long-term adaptation of synaptic efficacy. Brain-derived neurotrophic factor is one of the major activity-dependent modulators of dendritic protein synthesis and it is known to activate specific components of the translational machinery in neurons (Bramham and Messaoudi, 2005; Steward and Schuman, 2003). Previously, it has been shown that the rapid induction of protein synthesis by BDNF is mediated both through the PI3K pathway, as it involves the activation of PI3K and mTOR (Bramham and Messaoudi, 2005; Schratt *et al*, 2004; Yoshii and Constantine-Paton, 2007), as well as through the MAPK signaling pathway (Kelleher *et al*, 2004). Interestingly, BDNF was shown in this study to enhance MCT2 protein expression in cultured cortical neurons through a stimulation of translation. This is supported by the observations that no changes in MCT2 mRNA levels were detected, whereas MCT2 protein expression was enhanced by BDNF in synaptoneurosomes, a preparation that can sustain translation but not transcriptional activation. The observation that the mRNA synthesis inhibitor ActD can prevent BDNF-induced enhancement of MCT2 protein expression in intact cells but not in synaptoneurosomes is intriguing. It cannot be excluded that as a general transcription inhibitor, ActD indirectly interferes with the translation process in intact cells by reducing the expression of some essential components. The effect of BDNF on MCT2 protein expression was shown to involve the activation of three distinct signaling pathways, all classically implicated in the regulation of translation initiation (Hay

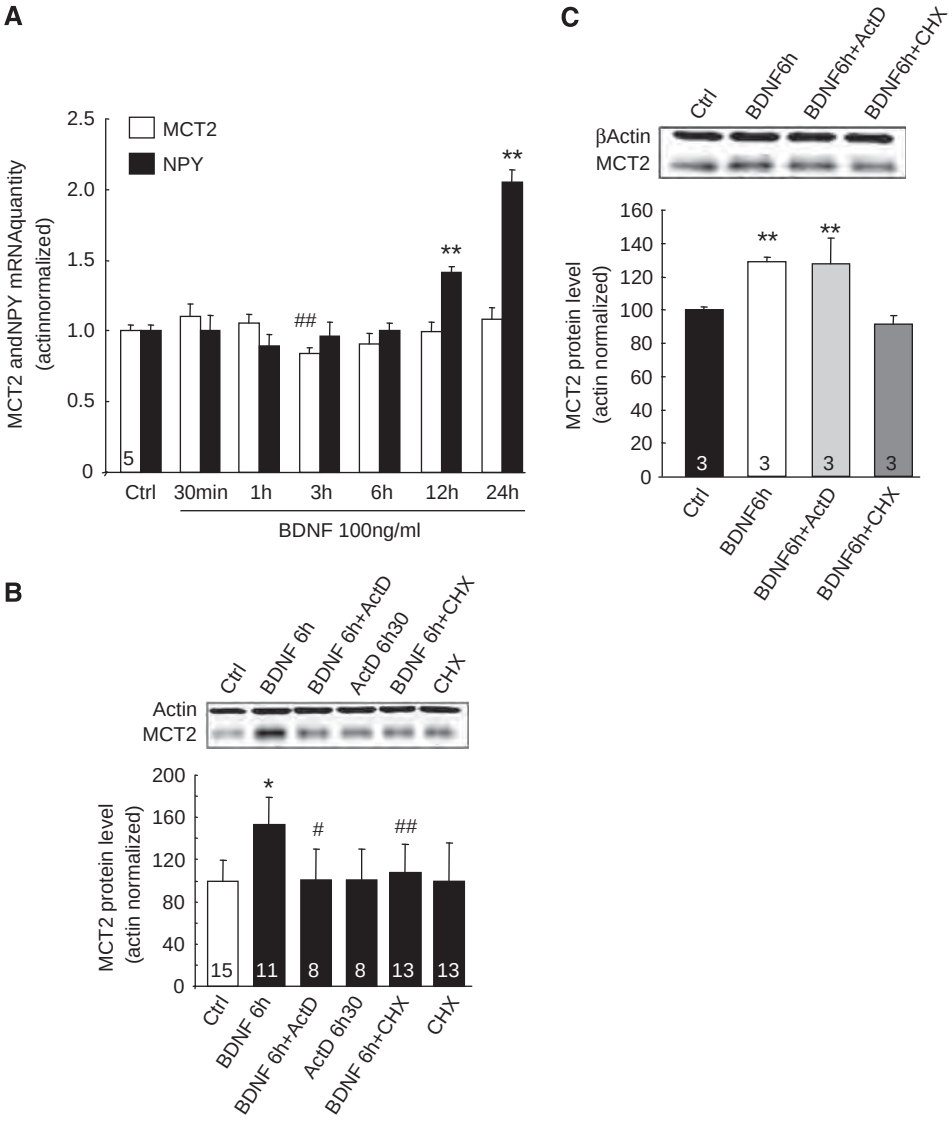

Figure 6 Synaptic regulation of MCT2 protein synthesis by BDNF. (**A**) Primary cultures of mouse cortical neurons were treated with BDNF 100 ng/mL for various periods of time. Neuronal cultures were harvested, and total RNA was extracted and analyzed for MCT2 and NPY mRNA expression by quantitative real-time RT-PCR. Results are expressed as percentages of control (mean ± s.d.) after the values had been normalized using β-actin gene as the internal reference and were expressed as a relative mRNA quantity compared with control. Five independent experiments were pooled after performing quantitative RT-PCR. (**B**) Western blot analysis of MCT2 expression in primary cultures of mouse cortical neurons exposed to either actinomycin D (ActD) or cycloheximide (CHX) for 30 mins before the application of 100 ng/mL BDNF for 6 h. (**C**) Western blot analysis of MCT2 expression in synaptoneurosomes treated with BDNF 100 ng/mL in the presence or absence of ActD or CHX. Western blots were quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Results are expressed as percentage of control after the values had been normalized using β-actin signal as the reference. Statistical analysis was performed using ANOVA followed by Bonferonni's test. *,** indicate MCT2 protein levels significantly different from control with $P < 0.05$ and $P < 0.01$. $^{\#,\#\#}$ indicate MCT2 protein levels significantly different from BDNF-treated condition with $P < 0.05$ and $P < 0.01$, respectively. Numbers in the graph bars represent the number of independent experiments for each condition.

and Sonenberg, 2004). Thus, a concomitant activation of PI3K/Akt/mTOR, p38 MAPK as well as MEK/ERK kinases was found to be necessary for the enhancement of MCT2 protein expression by BDNF (Figure 7). Such an observation suggests a putative cross talk between the different signaling pathways activated by BDNF to regulate translational activation, a possibility previously proposed by others (Almeida *et al*, 2005; Bramham and Messaoudi, 2005). It is noted that noradrenaline (a neurotransmitter), insulin (a hormone), and IGF-1 (a neurotrophic factor) were also previously shown to regulate MCT2 protein expression at the translational level, notably by activating the PI3K/Akt/mTOR/S6 kinase pathway in cultured cortical neurons (Chenal and Pellerin, 2007; Chenal *et al*, 2008). As each of these neuroactive substances, similar to BDNF, has been implicated in different forms of synaptic

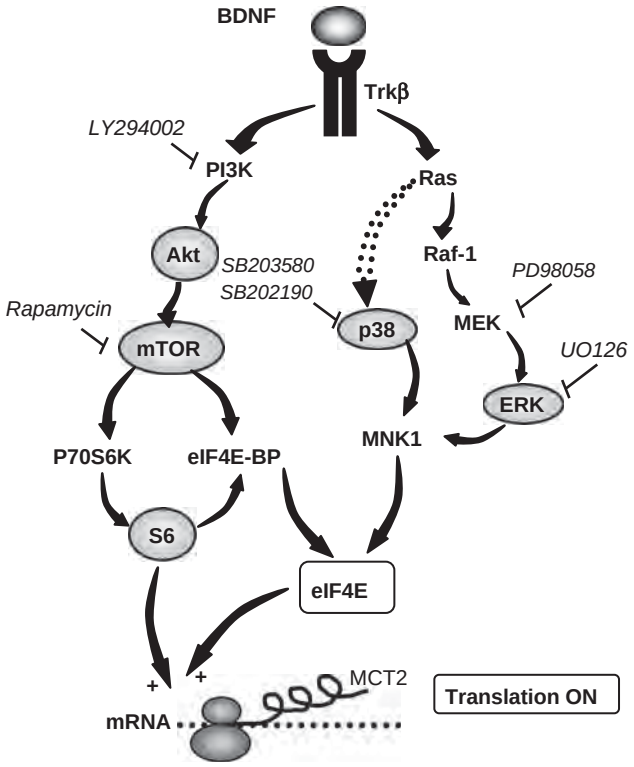

Figure 7 Putative signaling pathways leading to translational activation and enhanced MCT2 protein synthesis after BDNF treatment in cultured cortical neurons. BDNF and TrkB can activate distinct signal transduction pathways involving specific kinases leading to translation initiation. Two pathways investigated in this study are illustrated herein (PI3K/Akt/mTOR/S6 and MAPK signaling pathways). The surrounding proteins were directly investigated for their level of phosphorylation. First, phosphorylation of PI3K can cause the phosphorylation and activation of Akt, which then directly phosphorylates mTOR, which in turn phosphorylates p70S6K. The target of p70S6K, the ribosomal S6 protein, once phosphorylated, participates in the translational machinery as part of the 40S complex. Second, the MAPK cascade is also activated by BDNF requiring the activation of MEK. MEK phosphorylates the p44 and p42 MAPK, which can activate, among others, MNK1. When activated, MNK1 phosphorylates eIF4E on Ser209 that correlates with enhanced rates of translation of capped mRNA. Specific inhibitors for some kinases have been used to distinguish the implication of each pathway in the effect of BDNF: LY294002, PI3K inhibitor; rapamycin, mTOR inhibitor; PD98058, MEK inhibitor; SB202190 and SB203580, p38 MAPK inhibitors.

plasticity, it suggests that MCT2 represents a common target for such signals, pointing to a putatively important role of MCT2 in relation to synaptic modifications.

In view of the current results, it appears that MCT2 belongs to a group of neuronal proteins specifically regulated at the translational level. Until now, more than 100 different mRNAs coding for proteins involved in neurotransmission and in the modulation of synaptic activity have been identified in dendrites. Local protein synthesis from these mRNAs is postulated to provide the basic mechanism of long-term changes in the strength of neuronal connections (Skup, 2008). Postsynaptic proteins that undergo enhancement of their synthesis locally include calmodulin kinase II (Ouyang *et al*, 1999; Wu and Cline, 1998), MAP2 (Blichenberg *et al*, 1999), Arc/Arg 3.1 (Steward and Schuman, 2001), and GluR1, as well as GluR2 AMPA receptor subunits (Ju *et al*, 2004). Although the presence of MCT2 mRNA has not been described yet in dendrites, it seems likely that the translational regulation of MCT2 expression would occur locally. Our results obtained with synaptoneurosomes support this conclusion. The MCT2 protein is not only present in dendrites but it is also specifically associated with spines, where it was found to be expressed in the postsynaptic density as well as on vesicle-like structures forming an intracellular pool (Bergersen *et al*, 2005). MCT2 was recently found to interact with a specific subset of postsynaptic proteins (Pierre *et al*, 2009). This is particularly the case with GluR2, a subunit of the glutamatergic AMPA receptor subtype. It was shown that MCT2 seems not only to determine the subcellular localization of GluR2 in neural cells but also to regulate its expression levels (Maekawa *et al*, 2009). In addition, it was observed that MCT2 together with GluR2 undergoes a trafficking process between the plasma membrane and an intracellular pool under conditions inducing synaptic plasticity (Pierre *et al*, 2009). Translocation of GluR2 to and from the plasma membrane has been implicated in synaptic plasticity mechanisms, such as long-term potentiation and long-term depression (Kessels and Malinow, 2009; Malenka, 2003; Sheng and Kim, 2002). Thus, MCT2 localization and interaction with specific synaptic proteins involved in plasticity mechanisms strengthen the view that its expression may be regulated in a manner similar to its partners. In this regard, increased MCT2 protein expression by BDNF may be part of a coordinated mechanism of local synthesis for various postsynaptic proteins involved in the long-term regulation of glutamatergic transmission.

Although a major effort has been devoted to decipher the molecular events involved in synaptic plasticity, including those induced by BDNF, few studies have explored the concomitant changes in neuroenergetics that could take place with alterations in synaptic transmission. Recently, the concept that energy metabolism might be coupled to synaptic plasticity has been proposed together with the implication of BDNF in such interactions (Vaynman *et al*, 2006). Indeed, BDNF was shown to enhance mitochondrial activity (El Idrissi and Trenkner, 1999). Regarding the energy substrates that might be concerned, lactate has attracted much attention recently. Lactate has been shown to be a preferential oxidative substrate for neurons both *in vitro* (McKenna *et al*, 1993, 1994) and *in vivo* (Hyder *et al*, 2006; Serres *et al*, 2005). Lactate is not only able to sustain synaptic vesicle turnover and synaptic transmission

(Morgenthaler *et al*, 2006; Rouach *et al*, 2008; Schurr *et al*, 1988) but it is also shown to allow, at least in part, the establishment of long-term potentiation (Izumi *et al*, 1997; Yang *et al*, 2003). In addition, glutamatergic activity increases the production and release of lactate by astrocytes (Pellerin and Magistretti, 1994), and it has been purported that such an effect participates in a mechanism to provide lactate as an additional energy substrate to neurons to sustain their activity (Pellerin *et al*, 2007). As the primary role of MCT2 is to supply neurons with nonglucose energy substrates, e.g., lactate, changes in MCT2 expression might facilitate the utilization of alternative substrates. Indeed, overexpression of MCT2 in neurons was shown to enhance lactate consumption by neurons stimulated with kainate (Bliss *et al*, 2004). Our observation that MCT2 can be upregulated by BDNF is consistent with the possibility of a coupling between lactate utilization and synaptic plasticity. It is hypothesized that to meet higher energy demands caused by enhanced synaptic transmission after synaptic plasticity, MCT2 expression is increased to facilitate lactate supply at potentiated synapses.

In conclusion, we have shown that BDNF enhances the expression of the monocarboxylate transporter MCT2 in cultured cortical neurons through a translational regulation. A possible role for such an effect could be to enlarge the local lactate transporter pool to allow potentiated synapses to meet higher energy demands on activation.

Conflict of interest

The authors declare no conflict of interest.

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BRAIN-DERIVED NEUROTROPHIC FACTOR ENHANCES THE HIPPOCAMPAL EXPRESSION OF KEY POSTSYNAPTIC PROTEINS *IN VIVO* INCLUDING THE MONOCARBOXYLATE TRANSPORTER MCT2

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Abstract—Brain-derived neurotrophic factor (BDNF) promotes synaptic plasticity via an enhancement in expression of specific synaptic proteins. Recent results suggest that the neuronal monocarboxylate transporter MCT2 is a postsynaptic protein critically involved in synaptic plasticity and long-term memory. To investigate *in vivo* whether BDNF can modulate the expression of MCT2 as well as other proteins involved in synaptic plasticity, acute injection of BDNF was performed in mouse dorsal hippocampal CA1 area. Using immunohistochemistry, it was found that MCT2 expression was enhanced in part of the CA1 area and in the dentate gyrus 6 h after a single intrahippocampal injection of BDNF. Similarly, expression of the immediate early genes *Arc* and *Zif268* was enhanced in the same hippocampal areas, in accordance with their role in synaptic plasticity. Immunoblot analysis confirmed the significant enhancement in MCT2 protein expression. In contrast, no changes were observed for the glial monocarboxylate transporters MCT1 and MCT4. When other synaptic proteins were investigated, it was found that postsynaptic density 95 (PSD95) and glutamate receptor 2 (GluR2) protein levels were significantly enhanced while no effect could be detected for synaptophysin, synaptosomal-associated protein 25 (SNAP25), αCaMKII and GluR1. These results demonstrate that MCT2 expression can be upregulated together with other key postsynaptic proteins *in vivo* under conditions related to synaptic plasticity, further suggesting the importance of energetics for memory formation.

Key words: energy metabolism, hippocampus, lactate, synapses/dendrites, trophic factors.

The neurotrophin brain-derived neurotrophic factor (BDNF) plays an important role in activity-dependent synaptic plasticity mechanisms such as long-term potentiation (LTP) (Bramham and Messaoudi, 2005). Moreover, it has been proposed to participate in processes leading to memory formation (Yamada and Nabeshima, 2003). Indeed, several studies have shown an increase in BDNF mRNA expression and TrkB activation during memory acquisition and consolidation (reviewed in Yamada and Nabeshima, 2003). Application of BDNF to hippocampal slices facilitates the induction of synaptic potentiation and enhances basal synaptic transmission in CA1 pyramidal neurons (Ji et al., 2010). Similarly, acute intrahippocampal infusion of BDNF leads to a long-lasting enhancement of synaptic transmission in the rat dentate gyrus (Messaoudi et al., 1998) and induces LTP in intact adult hippocampus (Ying et al., 2002). The BDNF-induced long-lasting potentiation requires extracellular signal-regulated protein kinase (ERK) pathway activation and the upregulation of Arc and Zif268 synthesis (Rosenblum et al., 2002; Ying et al., 2002). *Arc* and *Zif268* are two immediate early genes (IEGs) transiently and rapidly activated during LTP as well as along the process of learning and memory formation (Davis et al., 2003; Plath et al., 2006; Lonergan et al., 2010). Ultimately, it is purported that BDNF exerts its effect on synaptic function by regulating local translation of a specific subset of synaptic proteins (Santos et al., 2010). While a few of these proteins have been described, more remain to be identified. Moreover, BDNF-induced enhancement in expression of several key synaptic proteins still awaits confirmation *in vivo*.

Apart from classical proteins involved in synaptic transmission, BDNF possibly regulates the expression of proteins involved in other cellular functions. In this regard, putative changes in energetics occurring upon synaptic plasticity and possible regulation of associated proteins have not been explored. Recently, the role of monocarboxylates, and particularly lactate, as additional energy substrates for neurons has attracted attention (Pellerin, 2003). Release and uptake of these substrates are dependent on the presence of specific carriers known as monocarboxylate transporters (MCTs) that are expressed by both neurons and glial cells (Pierre and Pellerin, 2005). Possible BDNF-mediated alteration in expression of these transporters *in vivo* has never been investigated. In the present study, the level of expression of six synaptic proteins, two IEGs, as well as three monocarboxylate transporters were determined in mouse hippocampus 6 h after a single intrahippocampal infusion of BDNF. Results clearly indicate that the neuronal monocarboxylate transporter MCT2 is one of the key postsynaptic proteins upregulated as part of the synaptic changes induced by BDNF.

EXPERIMENTAL PROCEDURES

Animals

Seven-week-old C57Bl/6Rj male mice (~20 g) were obtained from the Janvier Breeding Center (Le Genest Saint-Isle, France) and received 1 week prior to the experiments to acclimatize them to

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Abbreviations: Arc, activity-regulated cytoskeleton-associated protein; BDNF, brain derived neurotrophic factor; GluR, glutamate receptors; IEGs, immediate early genes; LTP, long-term potentiation; MCTs, monocarboxylate transporters; PBS, phosphate buffered saline; PSD95, postsynaptic density 95; SNAP25, synaptosomal-associated protein 25; TBST, Tris-buffered saline supplemented with Tween-20; Zif268, zinc-finger proteins; αCamKII, acalmodulin kinase II.

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the animal facility. They were housed in a temperature-controlled environment with 12:12 h. light-dark cycle and given free access to food and water. Animal experiments were performed in accordance with the Animal Care and Use Committee guidelines (Service vétérinaire du Canton de Vaud, Switzerland).

Surgery and intrahippocampal injection of BDNF

Mice were weighed and anesthetized by i.p. injection with a mixture of ketamine (24 μl) and xylazine (10 μl) diluted in 100 μl NaCl 0.9%, and positioned in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). Rectal temperature was maintained at 36 °C with a thermostatically controlled electric heating pad. Stereotaxic coordinates relative to bregma were anteroposterior (AP): −1.85; mediolateral (ML): −1.25; dorsoventral (DV): −1.4. A single intrahippocampal injection was performed using a 5 μl Hamilton syringe with a 33-gauge needle. The needle was inserted into the dorsal hippocampal CA1 area and 2 μl of phosphate buffered saline (PBS) or 2 μg of BDNF in 2 μl PBS were injected slowly during 20 min (brain-derived neurotrophic factor human, recombinant expressed in *E. coli*, Sigma, Buchs, Switzerland). Mice completely recovered 1 h after the surgery. Mice were sacrificed 6 h after the end of surgery.

Western blotting and related quantification

Once mice had been sacrificed, the brain was rapidly removed and put into a cold solution of phosphate buffered saline (PBS 1×). Hippocampi were rapidly dissected on ice, directly freezed in liquid nitrogen and stored at −80 °C.

For Western blotting, hippocampal tissue was homogenized and sonicated in a 0.32 M sucrose solution containing HEPES 1 mM, $MgCl_2$ 1 mM, $NaHCO_3$ 1 mM, phenyl-methyl-sulfonyl fluoride 0.1 mM, pH 7.4, in presence of a complete set of protease inhibitors (Complete; Roche, Mannheim, Germany). Protein concentrations were determined by the BCA method. Ten micrograms of protein were heated at 95 °C in SDS-PAGE sample buffer (62.5 mM Tris–HCl, 50 mM DTT, 2% SDS, 10% glycerol and 0.1% Bromophenol Blue) and loaded onto 10% polyacrylamide gels. After electrophoresis, samples were transferred on nitrocellulose membranes (Bio-Rad Laboratories, Reinach, Switzerland). For protein detection, membranes were incubated in a blocking solution of Tris-buffered saline supplemented with Tween-20 (TBST; Tris–HCl 50 mM pH 7.5, NaCl 150 mM, and Tween-20, 0.1%) containing 5% nonfat milk for 1 h at room temperature. Membranes were incubated overnight at 4 °C with a mixture containing the primary antibodies: anti-MCT4 (Santa Cruz Biotechnology, Heidelberg, Germany), anti-GluR1 and anti-GluR2/3 (Chemicon, CA, USA) diluted 1:500; anti-MCT1 and anti-MCT2 antibodies (developed and characterized by our group, as described in Pierre et al., 2000), anti-synaptophysin (Sigma, Buchs, Switzerland), anti-SNAP25 (Alomone Labs, Jerusalem, Israel), anti-PSD95 and anti-CamKIIα (Cell Signaling, Allschwil, Switzerland) diluted 1:1000; anti-βactin (Sigma, Buchs, Switzerland), diluted 1:10000; anti-βtubulin (Sigma, Buchs, Switzerland), diluted 1:1000. These antibodies were diluted in TBS, 0.1% Tween, 5% bovine serum albumin (BSA). After three washes in TBST, membranes were incubated with the secondary antibodies Alexa Fluor 680 goat anti IgG (Juro, Luzern, Switzerland) and IRDye_800 antimouse IgG (BioConcept, Allschwil, Switzerland), diluted at 1:5000 in TBST containing 1% nonfat milk, for 2 h at room temperature, and protected from light. For anti-MCT1, peroxydase-conjugated donkey anti-rabbit IgG was used as secondary antibodies (Amersham, Piscataway, NJ, USA) diluted 1:10000. After three washes in TBST, membranes were scanned and quantified with the Odyssey®Infrared Imaging System (LI-COR® Biosciences, Lincoln, NE, USA). For MCT1 detection, Enhanced Chemiluminescence (ECL) was required for detection and ImageJ software (http://rsbweb.nih.gov/ij/) was used for the quantification. β-actin or β-tubulin was used for normalization of proteins of interest.

Immunohistochemistry

Under urethane anaesthesia (1.5 g/kg, i.p.), animals were perfused intracardially with a heparinized solution of saline (25 IU/ml in 0.9% NaCl, during 2 min) followed by a freshly prepared solution of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4, for 15 min). Brains were removed and fixed in 4% paraformaldehyde for 2 h, then cut on a vibratome (Leica VT1000M; Leica Microsystems, Heerbrugg, Switzerland) to obtain coronal sections (40 μm thick), which were collected in PBS. Sections were rinsed several times in PBS and incubated with casein (0.5% in PBS) for 1 h to block non-specific sites. They were incubated overnight at 4 °C in freshly prepared primary antibody solution diluted in PBS containing 0.25% BSA: anti-MCT2 (1:500), anti-MAP2 (1:200; Sigma, Buchs, Switzerland), anti-Arc (1:200; Santa Cruz Biotechnology, Heidelberg, Germany), anti-EGR1 (1:500; Cell Signaling, Allschwil, Switzerland).

After careful rinsing in PBS, sections were incubated in a solution containing Cy3-conjugated anti-rabbit Igs (diluted 1:500, 2 h, room temperature; Jackson Immunoresearch, West Grove, PA, USA) and/or anti-mouse FITC-conjugated Igs (diluted 1:200, 2 h, room temperature; Jackson Immnoresearch, West Grove, PA, USA). After rinsing in PBS twice and a final rinsing in water, slices were mounted with Vectashield-DAPI (Reactolab SA, Servion, Switzerland).

Microscopy

Brain slices were examined and photographed with an Eclipse 80i microscope (Nikon, Kingston, England) using epifluorescence with an appropriate filter together with the NIS-Elements Microscope Imaging 5.1 software (Nikon, Kingston, England). They were also examined under a Zeiss LSM 710 Quasar confocal microscope using the DPSS 561-10, 561 nm laser illumination. Stacks were made of images taken with 0.5–0.8 μm step size. Pictures were treated with Imaris software, version 7.0.0.

Statistical analysis

All values are presented as mean±SEM. Mann–Whitney's test, or one-way ANOVA followed by Dunnett's post hoc test were used for data analysis with GraphPad Prism® software, version 5.0 (La Jolla, CA, USA). *P*-values <0.05 were considered as statistically significant.

RESULTS

Intrahippocampal injections of BDNF (or PBS) were made in the right (or left) hippocampus of 7 week-old C57/Bl6 mice. Six hours later, hippocampal expression of MCT2 was examined by immunohistochemistry on coronal sections taken at different levels along the anteroposterior axis. Results showed that MCT2 immunofluorescence was stronger in a portion of the pyramidal cell layer of the CA1 area as well as in the granule cell layer of the dentate gyrus for the BDNF-injected hippocampus compared to the contralateral, PBS-injected structure, as indicated by arrows (Fig. 1). No sign of lesion was detected in the surroundings of the needle tract. Confocal microscopy showed at high magnification a visible increase of MCT2 staining in neurons after BDNF treatment compared to PBS control (Fig. 2). Under the same conditions, the expression of two immediate early genes, *Arc* and *Zif268*, known to be impli-

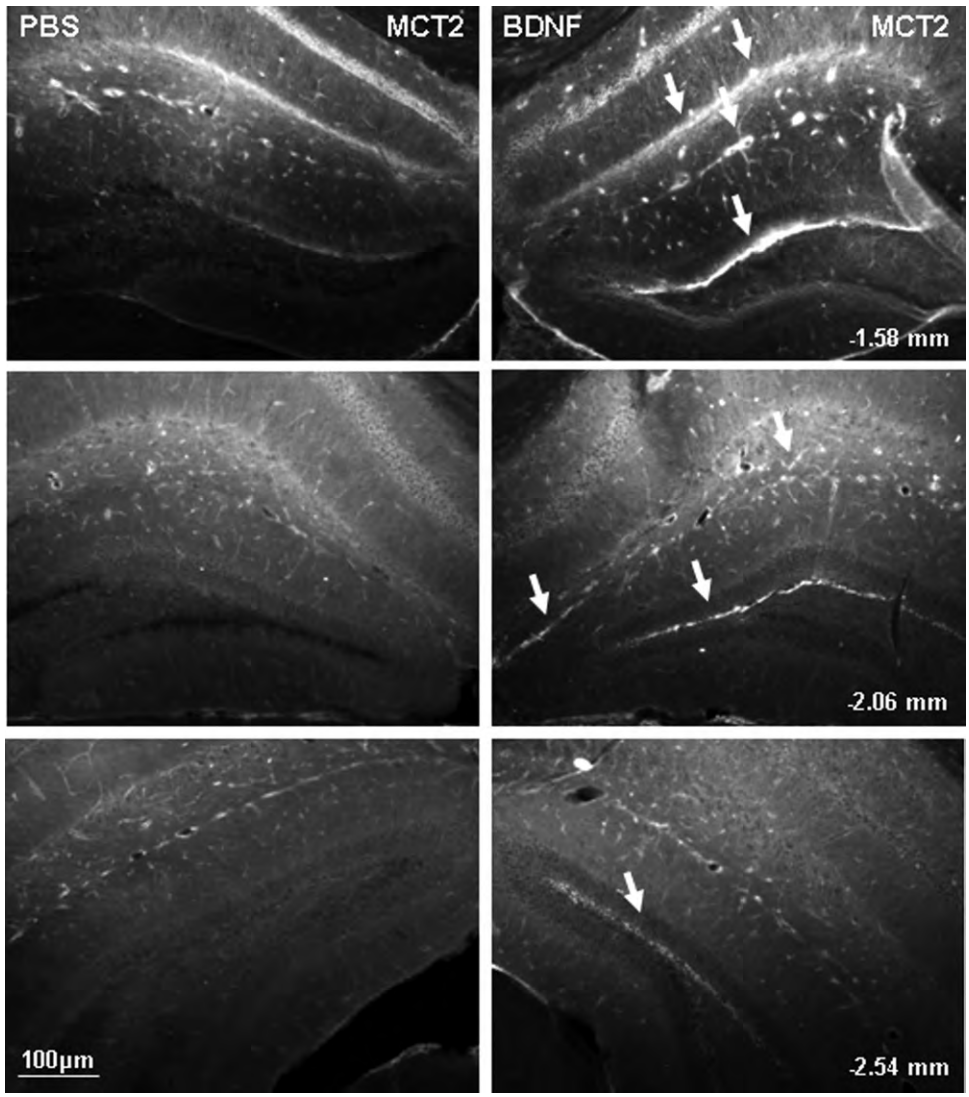

Fig. 1. Effect of acute intrahippocampal injection of BDNF *in vivo* on MCT2 protein expression. MCT2 immunolabelling in coronal brain sections at three different hippocampal levels 6 h after injection of either PBS (left column) or 2 μg BDNF (right column). As pointed out by white arrows, stronger immunolabelling is visible in portions of the hippocampus injected with BDNF compared to PBS, particularly in the CA1 area and in the dentate gyrus. Magnification, 10×; calibration bar, 100 μm. Numbers at the bottom right on right column images refer to position from bregma.

cated in synaptic plasticity, was evaluated by immunohistochemistry. Double immunostainings for Arc or Zif268 proteins with the neuronal marker MAP2 revealed an enhancement in neuronal expression of these two immediate early gene products after BDNF injection compared to PBS control in the same hippocampal area as for MCT2 (Fig. 3). Based on the comparison of all immunostainings performed in the present study, BDNF did not systematically and significantly altered the level of MAP2 expression (nor its co-localization with MCT2), in agreement with observations reported in the literature (Labelle and Leclerc, 2000; Schäbitz et al., 2004).

In order to further characterize the extent and nature of the changes induced by BDNF, expression levels of several synaptic and extra-synaptic proteins, including MCT2, were determined by immunoblot on hippocampal extracts. Western blot analysis of hippocampal extracts from BDNF-injected tissue showed a significant increase (125±12% vs. control) of MCT2 expression compared to control (no injection, contralateral side) or PBS-injected animals (Fig. 4A). Basal hippocampal MCT2 expression levels were not different between anesthetized (Keta/Xyla+) and unanesthetized (Keta/Xyla−) mice, excluding a confounding effect of anesthesia (Fig. 4B). Expression levels of MCT1 and MCT4, two other monocarboxylate transporters present in the brain but predominantly on glial cells, were not altered (Fig. 4C, D, respectively).

In parallel to monocarboxylate transporters, the effect of BNDF on the hippocampal expression of several pre- and postsynaptic proteins was determined by Western blot (Fig. 5A). Quantitative analysis of immunoblots for synaptophysin and synaptosomal-associated protein 25 (SNAP25), two presynaptic proteins, showed no expression change 6 h after BDNF injection (Fig. 5B, C, respectively). A similar analysis

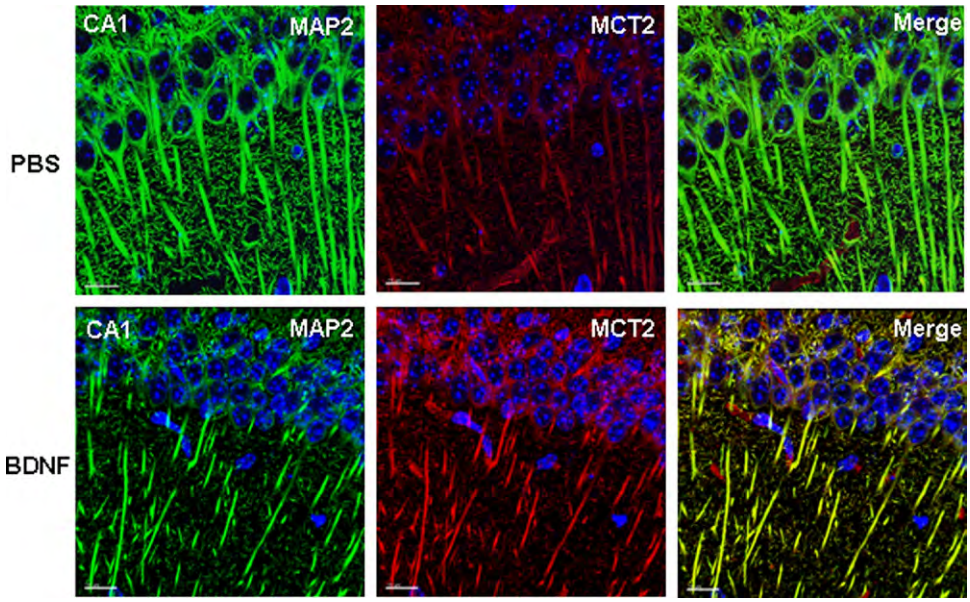

Fig. 2. Effect of acute intrahippocampal injection of BDNF *in vivo* on neuronal MCT2 expression in the hippocampal CA1 area. Double immunofluorescent labellings for the neuronal marker MAP2 (green) and MCT2 (red) in coronal hippocampal sections (−2.06 mm from bregma) obtained after 6 h from animals injected with either PBS (top row) or 2 μg BDNF (bottom row). A stronger MCT2 immunolabelling is clearly visible in CA1 pyramidal neurons after BDNF injection compared to control (PBS). Pictures were obtained with a confocal microscope and images correspond to a projection of 18 sections of 0.5 μm. Magnification, 63×; calibration bar, 15 μm.

on some postsynaptic proteins gave differential results. No expression change was observed for the protein kinase αCamKII and the glutamate receptor subunit GluR1 (Fig. 5D, F). In contrast, a significant increase of the postsynaptic density scaffold protein postsynaptic density 95 (PSD95) (118±9%) and of the glutamate receptor subunit GluR2/3 (124±7%) compared to control (no injection) was observed 6 h after BDNF injection (Fig. 5E, G).

DISCUSSION

As it is proposed to provide the underlying mechanism subserving learning and memory processes, synaptic plasticity is the subject of intense attention. Brain-derived neurotrophic factor represents an essential signal released under learning-associated conditions that contributes to the modifications of synaptic transmission taking place in this context (Gottmann et al., 2009; Yoshii and Constantine-Paton, 2010). Then, activation of TrkB receptors by BDNF leads to a cascade of events including transcriptional and translational steps that will result in the synthesis of a specific subset of synaptic proteins (Santos et al., 2010). However, relatively few informations exist *in vivo* about the specific proteins that are subject to regulation by BDNF. Moreover, evidence for a possible modulation of proteins essential in energy metabolism are scarce and limited to *in vitro* (Burkhalter et al., 2007; Robinet and Pellerin, 2010). This question was addressed here by investigating the putative changes in expression of several key synaptic proteins including monocarboxylate transporters, a group of proteins involved in energy substrate transfer and supply to neurons, following an intrahippocampal BDNF injection.

MCT2 is the predominant monocarboxylate transporter expressed by neurons whereas MCT1 and MCT4 are expressed by glial cells (Pellerin et al., 2005). At the subcellular level, MCT2 is expressed on axons and dendrites (Pierre et al., 2002, 2009). Moreover, MCT2 is present at glutamatergic synapses and exclusively on postsynaptic elements (Bergersen et al., 2002; Pierre et al., 2009). It is particularly enriched in the postsynaptic density of asymmetric synapses as well as in an intracellular pool within spines (Bergersen et al., 2005). Recently, it was demonstrated in cultured neocortical neurons that BDNF induces the expression of MCT2 via a regulation of local translation (Robinet and Pellerin, 2010). Data obtained in the present study showing increased MCT2 protein levels after BDNF injection further extend this observation to the hippocampus *in vivo*.

Interestingly, this increased neuronal expression of MCT2 in the hippocampus following BDNF injection is matched in terms of distribution by a similar enhancement in neuronal expression of both Arc and Zif268 proteins. Induction of the IEGs *Arc* (also known as *Arg3.1*) and *Zif268* (also known as *EGR1*) is required for late long-term potentiation (l-LTP) and long-term memory formation (Guzowski et al., 2000; Jones et al., 2001). In parallel, BDNF-induced long-lasting potentiation leads to an upregulation of Arc and Zif268 synthesis (Rosenblum et al., 2002; Ying et al., 2002). *Arc* mRNA is rapidly transported to dendrites and translated locally where it plays a critical role in local translation as well as in trafficking of AMPA receptors (Bramham et al., 2008). In parallel, Zif268 regulates the transcription of some late response genes and is involved in the degradation of synaptic proteins as well as in recep-

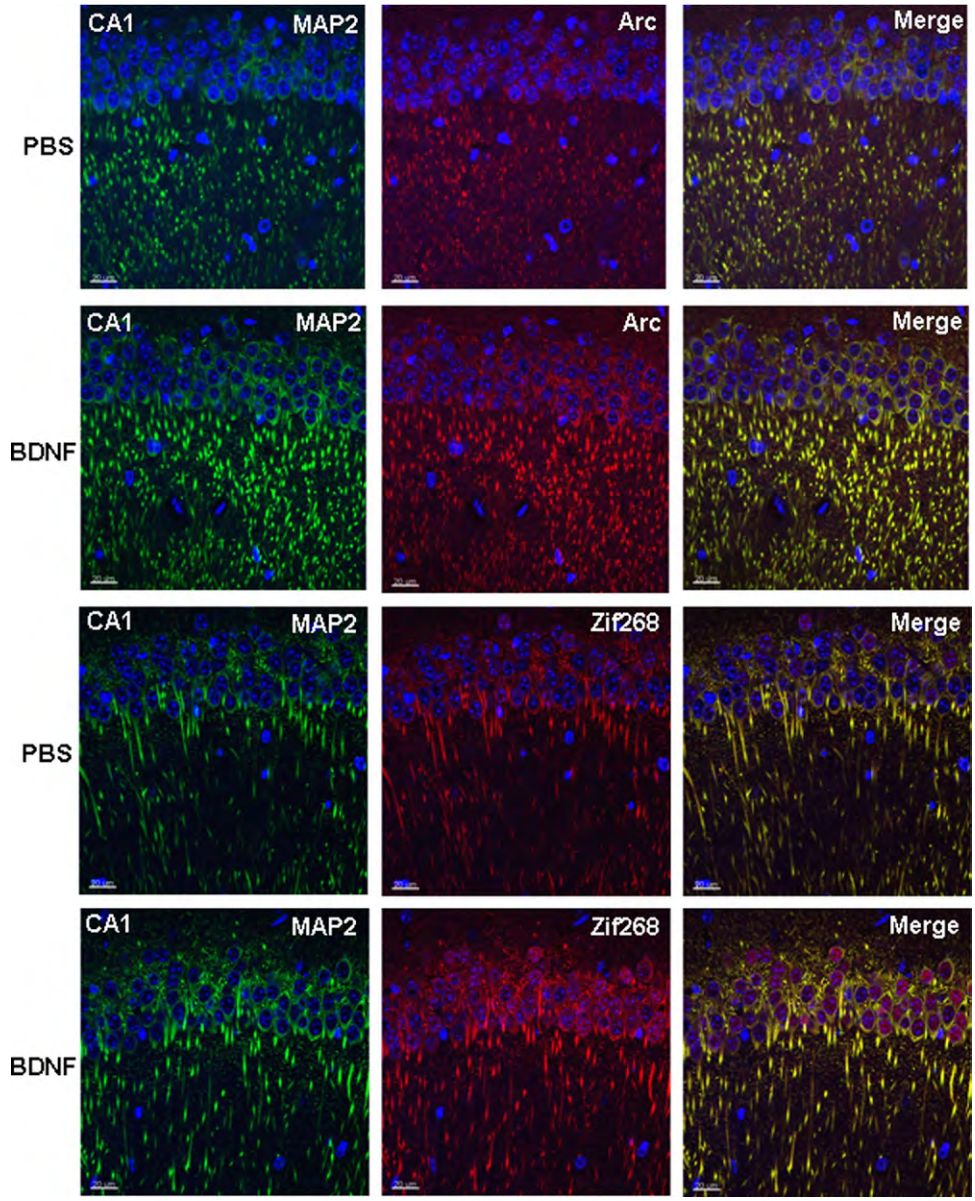

Fig. 3. Effect of acute intrahippocampal injection of BDNF *in vivo* on neuronal Arc and Zif268 expression in the hippocampal CA1 area. Double immunofluorescent labellings for the neuronal marker MAP2 (green) and either Arc or Zif268 (red) in coronal hippocampal sections (−2.06 mm from bregma) obtained after 6 h from animals injected with either PBS (top rows) or 2 μg BDNF (bottom rows). A strong immunolabelling for both Arc and Zif268 is observed in CA1 hippocampal neurons after BDNF injection compared to control (PBS). Pictures were obtained with a confocal microscope and images correspond to a projection of 18 sections of 0.5 μm. Magnification, 40×; calibration bar, 20 μm.

tor trafficking (McDade et al., 2009). Considering their spatially and timely related appearance, it is not excluded that Arc and Zif268 could be involved in the regulation of MCT2 expression and subcellular distribution induced by BDNF. Indeed, it was previously shown that BDNF-induced upregulation of MCT2 protein expression in cultured neurons requires a transcriptional step although MCT2 mRNA levels were not affected (Robinet and Pellerin, 2010). Thus, transcriptional activation of *Arc* and *Zif268* might be critical to allow a translationally regulated enhancement of MCT2 protein levels. Such a possibility will require to be further investigated both *in vitro* and *in vivo*.

BDNF was shown previously *in vitro* to enhance the expression of a few synaptic proteins. PSD95 is a scaffold protein of the postsynaptic density that plays an important role in synaptic plasticity (Gardoni et al., 2009). BDNF can induce the transport of PSD95 to dendrites and it was suggested that activation of the PI3K–Akt pathway by BDNF could lead to enhanced PSD95 synthesis (Yoshii and Constantine-Paton, 2007). Indeed, it was reported that activation of the PI3K–Akt pathway causes an increase of PSD95 expression in hippocampal slices (Lee et al., 2005). In cultured cortical neurons, BDNF was shown to enhance PSD95 protein expression (Matsumoto et al.,

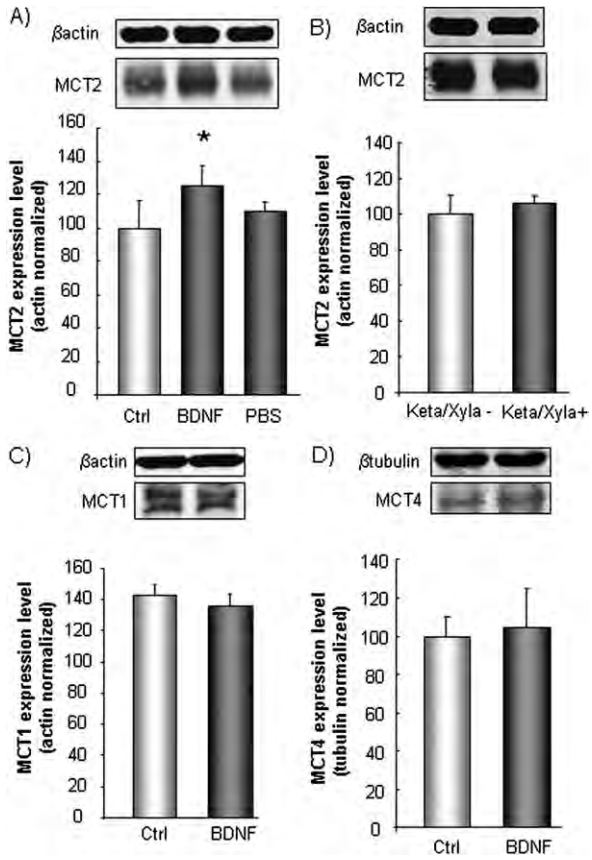

Fig. 4. Effect of intrahippocampal injection of BDNF *in vivo* on MCT1, MCT2 and MCT4 protein expression levels. Western blot analysis of MCT2 (A, B), MCT1 (C) and MCT4 (D) expression in hippocampal protein extracts. Right hippocampi were injected with 2 μg BDNF and collected after 6 h. Left hippocampi were used as control. Effect of anesthesia (Ketamine/xylazine) on hippocampal MCT2 protein expression was also performed as control (B). Western blots were quantified using Odyssey software (LI-COR Biosciences) or ImageJ software (http://rsbweb.nih.gov/ij/). Results are expressed as percentage of control (mean±SEM) after the values were normalized using β-actin or β-tubulin signal as reference. Statistical analysis was performed using one-way ANOVA followed by Dunnett's test (A) or performed using a Mann–Whitney's test (B–D). Asterisk indicates MCT2 protein levels significantly different from control (Ctrl) with $P<0.05$. $n=6$ mice for each condition.

2006). Our data provide a confirmation that BDNF can enhance PSD95 protein levels *in vivo*, supporting the idea that more PSD95 proteins are necessary to sustain BDNF-induced synaptic modifications. PSD95 colocalizes with MCT2 both *in vitro* and *in vivo* (Pierre et al., 2009). As PSD95 is a necessary component of the postsynaptic density, it might be required to ensure proper insertion of newly formed MCT2 in the postsynaptic membrane.

AMPA-type glutamate receptors are composed of distinct subunits known as GluR1 to GluR4. The synaptic distribution and levels of GluR1 and GluR2 subunits have been shown to be modulated as part of the mechanism of synaptic plasticity (Sprengel, 2006; Keifer and Zheng, 2010). It was demonstrated in both neocortical and hippocampal neurons in culture that BDNF treatment causes an enhancement of GluR1 and GluR2 protein expression levels (Narisawa-Saito et al., 1999; Matsumoto et al., 2006). Our observations *in vivo* that BDNF raises GluR2/3 protein levels partly confirm these data. It also emphasizes the important role that the GluR2 subunit appears to play in synaptic plasticity (Isaac et al., 2007; Bassani et al., 2009). MCT2 was shown previously to not only colocalize but also directly interact with GluR2 (Pierre et al., 2009). Moreover, changes in the expression levels of MCT2 have been shown to modify both the expression levels and the subcellular distribution of GluR2 subunits (Maekawa et al., 2009). Thus, it appears that MCT2 and GluR2 must be concomitantly regulated in order to fulfill their respective roles in the adaptation to changes in synaptic transmission.

In contrast to PSD95 and GluR2, no change could be detected in the expression of several other proteins. This is the case for the other monocarboxylate transporters MCT1 and MCT4. Since these transporters are rather expressed by glial cells, regulation of their expression might be under the control of other signals that remain to be identified. The protein kinase αcalmodulin kinase II (αCamKII) is a postsynaptic protein that has been involved in the process of synaptic plasticity (Miyamoto, 2006). BDNF has been reported to enhance αCamKII protein levels, notably in synaptosomes (Schratt et al., 2004). Surprisingly, no change in αCamKII protein levels could be evidenced *in vivo* under our experimental conditions. Similarly, the expression of the GluR1 subunit was not modified despite being enhanced by BDNF *in vitro* (Matsumoto et al., 2006). A possible explanation for these differences could be related to our experimental protocol. Since a single dose of BDNF

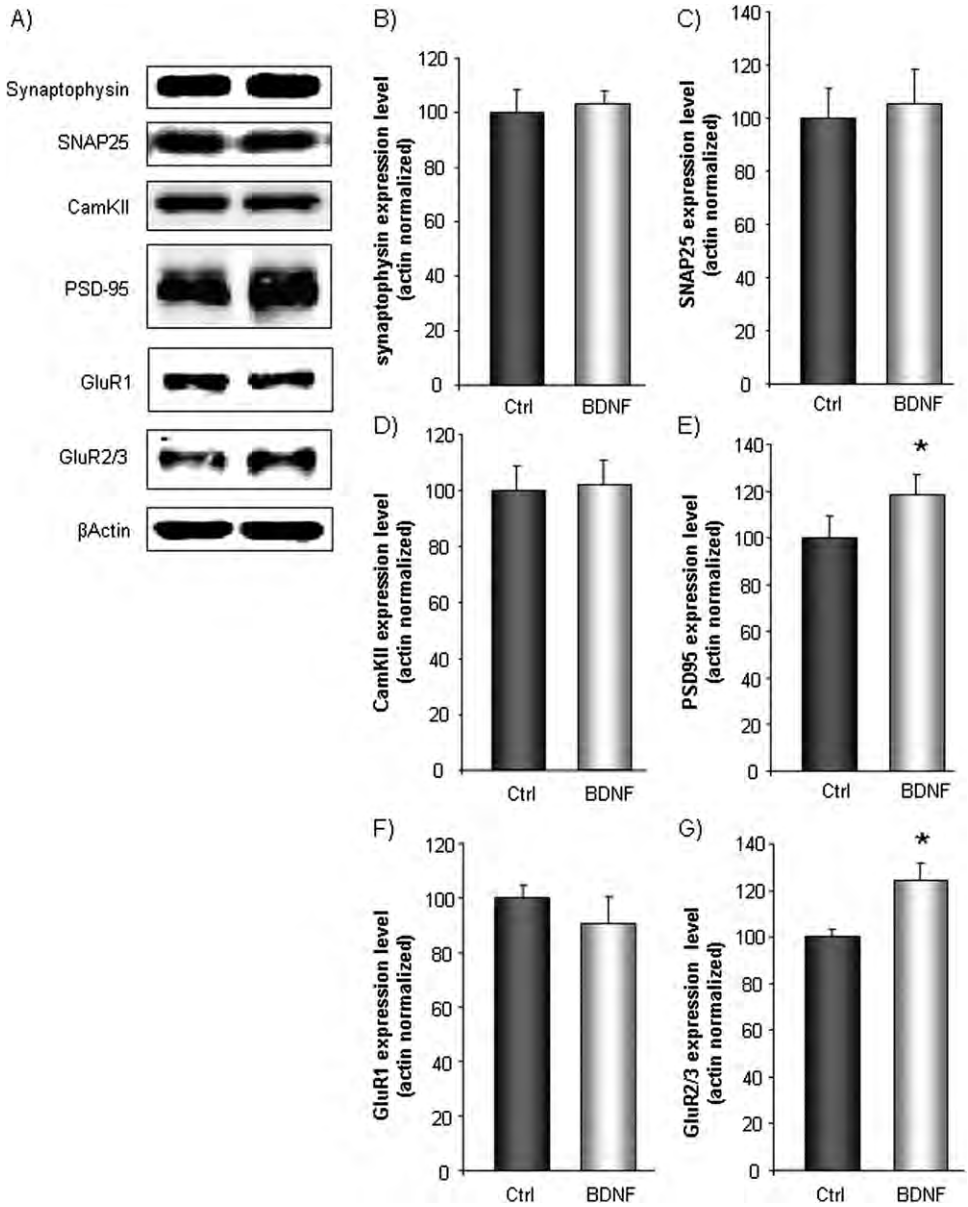

Fig. 5. Effect of intrahippocampal injection of BDNF *in vivo* on expression levels of several presynaptic and postsynaptic proteins. Western blot analysis of synaptophysin, SNAP25, αCamKII, PSD95, GluR1 and GluR2/3 expression on hippocampal protein extracts (A). Right hippocampi were injected with 2 μg BDNF and collected after 6 h. Left hippocampi were used as control. Western blot quantification for synaptophysin (B), SNAP25 (C), αCamKII (D), PSD95 (E), GluR1 (F) and GluR2/3 (G), performed with Odyssey software (LI-COR Biosciences). Results are expressed as percentage of control (mean±SEM) after the values were normalized using β-actin signal as reference. Statistical analysis was performed using a Mann–Whitney's test. Asterisk indicates PSD95 and GluR2/3 protein levels different from control (Ctrl) with $P<0.05$. $n=6$ mice for each condition.

was used and observation was made at only one time-point, possible changes in GluR1 and αCamKII protein expression might have been overlooked. Moreover, since hippocampal extracts were prepared from the entire hippocampus while the effect of injected BDNF was restricted to only portions of the tissue, putative differences might have been diluted in Western blot analyses. Further experiments investigating changes in expression at different times after BNDF injection (and at different doses), as well as using more restricted portions of the hippocampus, will be needed to clarify this point.

The presynaptic proteins synaptophysin and SNAP25 are involved in the mechanism of vesicle-dependent neurotransmitter release (Valtorta et al., 2004). BDNF was reported to enhance the expression of synaptophysin but not SNAP25 in neocortical neuronal cultures (Matsumoto et al., 2006). In our study, protein levels of synaptophysin and SNAP25 were not altered by intrahippocampal infusion of BDNF. The difference between *in vitro* and *in vivo* results might come from the developmental stage of each preparation. Since BDNF is known to promote synaptic maturation (Gottmann et al., 2009), involving synthesis of pre- and post-

synaptic proteins, such an effect might be more prominent in cultured cells prepared from embryonic animals than in adult mice.

Our data emphasize the fact that neuronal expression of MCT2, in parallel with other proteins known or highly suspected to participate in the mechanism of synaptic plasticity such as the immediate early genes *Arc* and *Zif268*, the postsynaptic scaffold protein PSD95 as well as the AMPA receptor GluR2 subunit, is upregulated by BDNF in the hippocampus *in vivo*. Considering the critical role of MCT2 in lactate uptake and utilization by neurons, our results point to the intriguing possibility of a coupling between neuronal lactate utilization and synaptic plasticity. Indeed, the concept that energy metabolism might be coupled to synaptic plasticity has been proposed together with the implication of BDNF in such interactions (Vaynman et al., 2006; Gomez-Pinilla et al., 2008). In addition, a recent study has shown that lactate import into neurons through MCT2 is necessary for long-term memory formation (Suzuki et al., 2011). In this context, it is tempting to hypothesize that to meet higher energy demands caused by enhanced synaptic transmission after synaptic plasticity, increased MCT2 expression induced by BDNF would facilitate lactate supply at potentiated synapses.

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Year	Course	Credits
06-07	Neurodevelopment, Plasticity and Critial Periods - T.Hensch	2
07-08	Introductory Course in Laboratory Animal Science - Module 1	2
07-08	Introduction to fluorescence imaging for the analysis of living cells - J-Y.Chatton	1
07-08	Practical course on Molecular Neurobiology - B.Boda	3
07-08	Seminars	1
07-08	Techniques d'investigation cerebrale - C.Michel	2
08-09	Seminars	1
08-09	Topics and Methods in Brain Development - C.Lebrand	3
09-10	BENEFRI "Animal Models for Human Nervous Diseases"	2
09-10	FENS 2010 Amsterdam	1

TOTAL **18**

Number of credits left to obtain **0** **ECTS**

Lausanne, le 07.08.2011

Prof. Jean-Pierre Hornung