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The Effects of Running-induced Neuronal Activation on the Plastic Expression of Mouse Cerebral GLUT1

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Abstract

Glucose, the predominant energy substrate of the central and peripheral nervous system, is delivered to neurons via a family of facilitative glucose transporters (GLUTs). The majority of glucose is transported to the brain via GLUT1 located at the blood-brain barrier. Several types of memory and learning tasks leading to changes in neuronal activity have also been linked to increases in glucose demand and local cerebral glucose utilization (LCGU). Current research has indicated a corresponding change in GLUT1 expression in response to increased metabolic demand in operant tasks. However, concomitant neuronal structural plasticity has not been currently investigated. In addition to operant learning tasks, voluntary running has also been identified as a source of neuronal activation. The purpose of this study is to examine the effects of neuronal activation induced by voluntary running on the plastic expression of GLUT1 and the concomitant remodeling of neurons in the mouse brain.

Glucose Transporters and Neuronal Activation

Background

This thesis examines the relationship between structural plasticity in the brain, neuronal organization, and the possible plasticity of glucose transporters that allow the entry of glucose, the main metabolic fuel of the brain. The general hypothesis is that since neuronal structural plasticity must require metabolic energy to be achieved, there might be adaptations in the brain mechanisms to acquire glucose in order to meet these demands. The metabolic adaptations contemplated here go beyond the dynamic adjustment of blood vessels diameter to facilitate blood flow. Specifically, we are exploring the possibility that the expression of the main glucose transporter that shuttles glucose from the blood to the brain (GLUT1) increases to help the brain meet its metabolic requirements. This increase in GLUT1 would occur when a brain area is solicited by new behavioural activities such as learning a new task or performing a certain behaviour at a high level such as running (in the present experiment).

The general idea is that the brain metabolic supply and glucose transporter adapt to a certain level of activity when an equilibrium is reached between demand of glucose by neurons and supply of glucose mediated by glucose transporters. When the brain encounters a sufficiently different situation, neuronal demand increases which leads to a possible deficit if glucose transporters cannot fulfill demand. At that point, glucose transporter density would increase to allow increased glucose influx. Finally, as tested in the present thesis, the increased demand could also be related to the initiation of structural plasticity (for example, an increase in dendritic spines). The present hypothesis is based on a number of findings related to extracellular glucose and one previous

experiment that examined the impact of training on an operant task on glucose transporter density. In the next few paragraphs, we will describe these findings.

For the past 20 years, the brain was viewed as a single compartment that is freely permeable to glucose, with an overall steady-state glucose concentration of approximately 2mM (Lund-Andersen, 1979; Sokoloff, et al., 1977). Current studies on brain extracellular fluid (ECF) have identified a brain glucose level of approximately 1.3mM (Fellows, Boutelle, & Fillenz, 1992). Research conducted by McNay and colleagues (2001) explored the idea of glucose compartmentalization and regional distribution in the rat brain. They found that under resting conditions, extracellular glucose concentration is variable in different brain regions, with hippocampal glucose ECF level of ~1.3mM and striatal ECF value of ~0.7mM. Because of the characteristics of the blood brain barrier and glucose transporters, brain extracellular fluid glucose concentration is roughly 20-30% of the concentration observed in the blood (Abi-Saab, et al., 2002).

Researchers then measured extracellular glucose concentrations in the striatum and hippocampus following testing in a *hippocampus-dependent* but *striatum-independent*, spontaneous alternation task (Johnson, Olton, Gage, & Jenko, 1977; McNay & Gold, 1998; Ragozzino & Gold, 1994; Ragozzino, Parker, & Gold, 1992; Stevens & Cowey, 1973). During behavioural testing, they found a ~30% decrease in extracellular glucose in the hippocampus but no decrease of ECF glucose in the striatum. They also found that systemic administration of glucose improved performance of rats compared to rats tested without glucose administration. They also found that the rise in peripheral

blood glucose was also accompanied by a normalization of hippocampal extracellular glucose content (i.e., the 30% decrease was prevented) (McNay, et al., 2001).

McNay, Fries, & Gold (2000) perform a similar experiment in which they measured the changes in extracellular glucose as a function of two different tasks requiring different levels of cognitive demand. They also determined the effects of systemic glucose administration on performance and on hippocampal extracellular glucose levels. In this study, they found that with an increase in cognitive demand, there was a concomitant decrease in hippocampal extracellular glucose levels. The systemic administration of glucose prior to testing prevented extracellular glucose decrease and led to an improvement in performance on the more difficult task. These findings imply that hippocampal extracellular glucose is depleted by cognitive demand, and that exogenous glucose administration can counteract the depletion, thereby improving memory performance. Collectively, these findings support the idea that brain glucose is compartmentalized, with region-specific activity being associated with concomitant increases in glucose uptake. Under certain conditions, transport from blood to brain appears to be a limiting factor, both for the supply of glucose to neurons but also for the optimal functioning of brain systems when they are highly active and thus require high levels of glucose.

In the present study, we hypothesized that changes in axonal or dendritic morphology and remodeling of synapses induced by the running task should be accompanied by an upregulation of glucose uptake. Previous research has shown that the glucose transporter 1 (GLUT1) expression in the hippocampus is plastic and increases following training in a bar-pressing task (Choeiri, Staines, Miki, Seino, & Messier, 2005).

Thus, GLUT1 expression could serve as a general index of changes in glucose utilization associated with plastic changes occurring in the brain. As a first approximation, we hypothesized that the more extensive the morphological changes associated with training, the larger the increases in GLUT1 antibody upregulation. We used the changes in expression of beta-tubulin (a protein associated with microtubules) as a proxy measure of morphological changes since these changes are likely to be associated with cytoskeletal changes such as microtubule formation (Ginzburg, Behar, Littauer, & Griffin, 1989; Gu, Firestein, & Zheng, 2008; Kollins, Bell, Butts, & Withers, 2009). In the next sections, we will briefly summarize research describing the morphological changes that occur following various types of "experience" from sensory stimulation to memory and learning. We will then present an overview of glucose transporters in the brain and the likely path that glucose follows from blood to neuron.

Structural Plasticity

Neuronal plasticity, in general, refers to the changes in neuronal circuits that occur as a result of new experiences or learning. During development, these neuronal circuits form at a fast rate as the brain grows and matures. Although activity-dependent neuronal plasticity occurs more slowly into adulthood, these circuits are still plastic, such that they still have the capability to undergo changes (Holtmaat, De Paola, Wilbrecht, & Knott, 2008). Numerous studies indicate examples of plasticity including lesions, injuries, and various other types of new experiences that produce corresponding changes in size and location of cortical sensory maps (Darian-Smith & Gilbert, 1994; Daw, Fox, Sato, & Czepita, 1992; Frenkel, et al., 2006; Wang, Merzenich, Sameshima, & Jenkins, 1995). Structural changes such as dendritic spine formation and pruning, axonal

sprouting, synaptic formation and elimination are all examples of modifications of the neural network connections and thus, they may play significant roles in use-dependent functional plasticity (Xerri, 2008).

Structural Plasticity in Dendritic Spines

Dendritic spines are small protrusions from a neuronal dendrite that can form synapses with a nearby axon, and subsequently send electric signals to the cell body. Spines typically receive excitatory inputs, however, they can receive a combination of excitatory and inhibitory connections (Knott, Quairiaux, Genoud, & Welker, 2002). Changes in spine structure correlate with memory formation and synaptic plasticity, although this relationship is not proven to be causal (Lamprecht & LeDoux, 2004). Nevertheless, these structural changes may be involved in the persistence of plastic alterations in synaptic transmission, as common molecular constituents take part in spine formation and memory formation.

Neuroanatomical analyses of adult mammals have provided evidence for structural changes in the cortical connections related to housing in enriched environments. These complex environments consist of a variety of motor, sensory, and spatial learning (Segal, 2005). Changes related to the enrichment include increased dendritic branching (Greenough, Volkmar, & Juraska, 1973; Turner, Lewis, & King, 2003), greater number of synapses per neuron (Kolb, Gibb, & Gorny, 2003; Schapiro & Vukovich, 1970; Turner, et al., 2003), increase in dendritic length and number of spines, as well as a rise in size and number of synaptic junctions (Mollgaard, Diamond, Bennett, Rosenzweig, & Lindner, 1971; Vrensen & Cardozo, 1981).

Xerri (2008) suggested that structural plasticity can lead to enhanced synaptic efficacy based on observations that LTP is correlated with spine formation (Engert & Bonhoeffer, 1999) and expansion (Lang, et al., 2004) in the hippocampus. In addition, a transient increase in spine density in the dentate gyrus is observed following water maze training (Eyre, Richter-Levin, Avital, & Stewart, 2003). The number of labile spines appears to be region-dependent ranging from 50% of transient spines in the mouse barrel cortex (with half-lives of a few days) (Trachtenberg, et al., 2002) to only 4% in the mouse visual cortex (Grutzendler, Kasthuri, & Gan, 2002). These discrepancies in these studies may be due to different methodologies or distinct activity patterns in visual and somatosensory cortices (Segal, 2005). The barrel cortex study *in vivo* has recently drawn attention to dendritic remodeling as a manifestation of adult cortical plasticity (Holtmaat, Wilbrecht, Knott, Welker, & Svoboda, 2006).

Structural Plasticity in Axons

Axonal branch-sprouting is involved in target exploration and circuit modification, thus it is of great relevance to structural plasticity. Two types of axonal target exploration include formation and disappearance of en-passant boutons found along pre-existing branches, or modification of terminal boutons. Both types vary in the extent of their plasticity not only as a function of bouton type, but vary amongst the different types of axons (De Paola, et al., 2006; Stettler, Yamahachi, Li, Denk, & Gilbert, 2006). Like dendritic spines, en-passant boutons and terminal boutons play a role in synaptic plasticity as they also have the ability to extent and retract, appear and disappear, thereby leading to the formation or elimination of synapses (Holtmaat, et al., 2008).

Terminal boutons can span larger areas for samplings and are usually more plastic than en-passant boutons. Terminal boutons usually form synapses with dendritic shafts instead of spines, whereas en-passant boutons synapse with spines. Another plastic mechanism that is found in en-passant boutons is the modifications in active zone numbers in the en-passant boutons in response to formation and elimination of spine synapses (McAllister, 2007). Axons vary in their densities of terminal boutons (De Paola, et al., 2006). Some terminal boutons exhibit local terminal arborizations that allow sampling of tens of micrometers of postsynaptic area by way of multiple synapses and branchlets, or short individual branches that have one synapse at their tip. High remodeling rates have been reported, indicating that many terminal boutons can undergo remodeling many times during an animal's lifetime (De Paola, et al., 2006; Gogolla, Galimberti, & Caroni, 2007; Stettler, et al., 2006).

Reorganization of synaptic contacts and formation or elimination of entire synaptic structures may implicate structural remodeling as a basis for learning and memory processing. For example, mice that were housed in an enriched environment exhibited an increase in local complexity of hippocampal mossy fiber terminal complexes in stratum lucidum as a function of synaptic activity and release of transmitter from the terminal (Galimberti, et al., 2006). Likewise, there was an expansion of mossy fibers terminals in the CA3 region of the hippocampus in rodents that was stimulated by spatial learning (Holahan, Rekart, Sandoval, & Routtenberg, 2006).

Another study reported a rise in synaptic densities and significant reorganization of receptive fields in the motor cortex when monkeys learned a new complex motor task requiring the hand (Kleim, Barbay, et al., 2002). These findings propose that a learning

experience can lead to changes in axonal connections in the adult CNS, although the precise remodeling mechanisms are still unclear.

Remodeling of Cytoskeletal Components: Microtubules and Actin

The spine and axon cytoskeletons are responsible for their plasticity. Cell cytoskeleton is predominantly composed of actin, which permits dynamic alterations in number, shape, and volume in short time interval. Actin polymerizes similarly to microtubules and hydrolyzes ATP. There is growing evidence that the dynamic characteristics of microtubules (MTs) are dramatically influenced by actin filaments and that this relationship is also reciprocal. These interactions are crucial for axon elongation and specification (Abbott, 2002; Arimura & Kaibuchi, 2007; Dent & Gertler, 2003).

Microtubules, another cytoskeletal component, are dynamically unstable as they characteristically experience repeated cycles of growth and disassembly. Microtubules serve multiple roles in neurons (Heidemann, 1996). Besides acting as the substrate for the transport of membrane-bound organelles, MTs are necessary for the extension of neurites during development; they provide the scaffolding for maintaining neurites after extension, and they help maintain the definition and integrity of intracellular compartments. They contribute to essential neuronal processes such as serving as tracks along which organelles, vesicles, and other components in the cytoplasm are transported. Microtubules apparently contribute to spine plasticity since their inactivation reduces spine plasticity (Gu, et al., 2008). In addition, synaptic transmission relies on the ability of neurons to polarize, an action due to the dynamic assembly and organization of the neuron's microtubules (Conde & Caceres, 2009).

A microtubule is composed of tubulin subunits, with each subunit consisting of a tubulin dimer of two similar and noncovalently bound proteins, α-tubulin and β-tubulin. These subunits stack together to form 13 linear chains with alternating α-tubulin and β-tubulin subunits. These chains, known as protofilaments, align into a hollow cylinder form. Each protofilament has either α-tubulin at one end, known as the minus end, and β-tubulin at the other end.

Class III β-tubulin is a component of microtubules that is found exclusively in neurons (Lippert-Gruener, Maegele, Garbe, & Angelov, 2007), mostly in axons or dendrites (Kleiman, Banker, & Steward, 1990). Activity-prompted dendritic and axonal remodeling leads to calcium-influx-dependent activation of signaling pathways within minutes and gene transcription within hours. Dendrite growth continues for days and requires extension and stabilization of the cytoskeleton leading to an increase in microtubules and microtubule-associated proteins (MAPs) (Szebenyi, et al., 2005). β-tubulin is incorporated in mature microtubules in developing neuronal processes and thus a class III β-tubulin antibody (Tuj) is a good marker of existing and functional microtubules in neurons (Ferreira & Caceres, 1992).

In the present experiment, we examined changes in Tuj expression as an indirect measure of neuronal remodeling activity. In the next section, I review the few experiments that have examined neuronal plasticity associated with running in rodents.

Plasticity & Running

Enriched environment. An enriched environment (EE), also known as an enriched condition (EC) is composed of a combination of play toys, social interaction, and possibly running wheel activity that stimulate and improve social, cognitive, sensory, or motor processes in comparison to standard housing conditions (Nithianantharajah &

Hannan, 2006). This enrichment has led to alterations in neuronal structural changes and function (van Praag, Kempermann, & Gage, 2000).

Many studies have reported an increase in synapse number, spine density, or dendritic arborization in cortical areas of animals exposed to an EE. For example, an increase in hippocampal dendritic spine number following spatial learning was observed in rats (Moser, Trommald, & Andersen, 1994). Another study observed experience-dependent increase in neuron number (Kaplan, 1981) and an increase in dendritic length in the visual cortex of rats (Wallace, Kilman, Withers, & Greenough, 1992).

Neuronal plasticity has been identified primarily in brain regions that are associated with the processing of changes in environmental stimuli (Markham & Greenough, 2004). These areas include the visual cortex (Diamond, et al., 1966; Volkmar & Greenough, 1972) auditory cortex (Greenough, et al., 1973), amygdala (Nikolaev, Kaczmarek, Zhu, Winblad, & Mohammed, 2002), cerebellar cortex (Greenough, McDonald, Parnisari, & Camel, 1986), hippocampus (Fiala, Joyce, & Greenough, 1978; Moser, Trommald, Egeland, & Andersen, 1997), and primary somatosensory cortex (Coq & Xerri, 1998).

One of the challenges of the EE paradigm is that the relative contribution of motor-skill learning and motor activity cannot be separated for EE that include running wheels. Thus, an initial study was conducted by Black and colleagues (1990) in order to distinguish the relative contributions of motor skill training and motor activity on neuronal structure. Subsequent studies have also given support to specific effects of running on brain plasticity. In addition to neuronal morphologies, cerebrovasculature

constituents may also exhibit structural changes as a result of experience, which will be discussed in the following section.

Effects of chronic motor activity on vascular plasticity. In a study by Black and colleagues (1990), the number of synapses in the paramedian lobule (PML) of the cerebellar cortex was observed in rats subjected for 30 days to an acrobatic training task in comparison to rats exposed to running only and an inactive group. Rats in the acrobatic condition were subjected to a series of increasingly difficult sets of rope bridges, balance beams, and other objects. The PML is stimulated in response to limb movements that occur during acrobatic training and in running exercise. They observed a 25% increase in the number of synapses per Purkinje cell in the PML of the acrobatic animals (Black, et al., 1990). There was no significant difference in synapse number between the exercised and inactive groups. However, animals in the repetitive exercise group exhibited a greater blood vessel density than the other groups, and no significant difference in blood vessel density between the acrobatic and inactive groups. The results of this study show that the repetitive motor activity stimulated angiogenesis while the learning process, independent of the motor activity associated with the acrobatic task, led to synaptogenesis. Interestingly, there was no significant correlation between the distance run and number of synapses per Purkinje cell or PST length, thus, the size and formation of synapses are not dependent on repetitive activity in the PML.

A subsequent study (Faherty, Kerley, & Smeyne, 2003) examined morphological changes such as cell volume and dendritic length in response to enriched environment with running wheels compared to a running wheel only group. The running group with increased motor activity alone, independent from other enriched environment

components such as cognitive stimulations and social interactions, did not lead to changes in cortical dendritic length or neuronal volume in the hippocampus, striatum, or motor cortex (Faherty, et al., 2003). In addition, the only morphological changes in neurons were found in the CA1 hippocampal neurons of the enriched environment group. Both groups had exposure to a running wheel, and thus, it was concluded that the social and learning aspects of the enriched environment were responsible for neuronal structural changes in the hippocampus (Faherty, et al., 2003). The absence of gross neuronal morphological changes in the motor-associated regions such as the striatum and motor cortex suggested that neuronal alterations may not occur in response to exercise alone (Faherty, et al., 2003).

Interestingly, a recent study by Eadie and colleagues (2005) revealed not only an increase in neurogenesis, but also an increase in dendritic complexity, dendritic length, and spine density in the dentate gyrus as a result of voluntary running in rats. Thus, this suggests that voluntary exercise, not just an enriched environment, can promote structural plasticity, however, this has been shown specifically in the dentate gyrus (Eadie, et al., 2005; Redila & Christie, 2006).

A study by Kleim and colleagues (2002) examined the effect of 30 days of voluntary exercise on blood vessel density as well as on the topography of movement within the forelimb motor cortex in rats. The results of this study suggested that chronic motor activity that enhances angiogenesis does not promote changes in the functional organization of the motor cortex (Kleim, Cooper, et al., 2002).

Another similar study examined the effect of 30 days of exercise on the capillary structure in the motor cortex of adult rats (Swain, et al., 2003). Their purpose was to

determine whether capillary changes similar to those observed in the cerebellum (Black, et al., 1990) would occur in the primary motor cortex (MC) in response to chronic repetitive exercise. In order to ascertain whether changes are specific to the motor regions, they examined the effect of prolonged exercise on the capillary density and structure in other motor regions such as the frontoparietal cortex and subcortical motor region (the striatum), as well as non-motor regions of the cerebral cortex such as the medial septal nucleus (Swain, et al., 2003). They found a 28% increase in blood flow in exercised animals compared to the inactive ones (Swain, et al., 2003). This is an indication of cerebral vasculature plasticity in the motor cortex in response to glucose and oxygen requirements of active neurons during chronic exercise (Swain, et al., 2003).

They did not find evidence of angiogenesis or capillary morphologies in other regions other than the motor cortex (Swain, et al., 2003). However, in another study, exercise-induced angiogenesis was found in other motor regions such as frontoparietal cortex and dorsolateral striatum after 21 days of 30min/day exercise in older rats (Ding, et al., 2006). Nevertheless, this study in combination with the Black study (1990) suggests that vascular plasticity such as angiogenesis and capillary blood flow occur predominantly in forebrain motor regions of the cerebral cortex in response to exercise. It also supports previous findings that synaptic and vascular plasticity are region-specific and task-specific such that motor activity stimulates angiogenesis and learning induces synaptogenesis (Black, et al., 1990; Kleim, Lussnig, Schwarz, Comery, & Greenough, 1996).

Although exercise does not increase synaptogenesis in the motor cortex, it leads to increases in levels of BDNF (Klintsova, Dickson, Yoshida, & Greenough, 2004),

which is responsible for neuronal differentiation and survival (Vaynman & Gomez-Pinilla, 2005). Thus, increase in blood flow, blood vessel growth, and growth factors induced by repetitive exercise may lead to a more supportive and trophic environment for neurons (Adkins, Boychuk, Remple, & Kleim, 2006). BDNF is involved in angiogenesis as it increases the release of vascular endothelial growth factor (VEGF) (Nakamura, et al., 2006), as well as by its actions on endothelial cells expressing its TrkB receptors (Kermani, et al., 2005).

Until recently, most research on experience-dependent plasticity has focused on neuronal plasticity including neurogenesis, dendritic remodeling, and synaptogenesis. However, non-neuronal elements of the nervous system also undergo experience-dependent plasticity such as angiogenesis, astrocytic hypertrophy, and increases in myelination and astrocytic ensheathement of synapses (Markham & Greenough, 2004).

Certain forms of experience determine the types of plastic changes that will take place. The learning of a motor skill is linked to alterations in synaptic strength (Rioult-Pedotti, Friedman, & Donoghue, 2000; Rioult-Pedotti, Friedman, Hess, & Donoghue, 1998), neuron structure (Greenough, Larson, & Withers, 1985; Kleim, et al., 1996), and topography of movement representations within the motor cortex (Kleim, Barbay, & Nudo, 1998; Plautz, Milliken, & Nudo, 2000). Learning also influences astrocytic hypertrophy, synaptogenesis, and neuronal survival (Markham & Greenough, 2004).

The effects of motor activity alone, such as access to running wheels, include increases in BDNF levels (Farmer, et al., 2004; Neeper, Gomez-Pinilla, Choi, & Cotman, 1995; Tong, Shen, Perreau, Balazs, & Cotman, 2001), stimulation of angiogenesis (Black, et al., 1990; Isaacs, Anderson, Alcantara, Black, & Greenough, 1992; Swain, et

al., 2003), the formation of new cortical microglia (Ehninger & Kempermann, 2003), cell proliferation and survival in the hippocampus (van Praag, Kempermann, & Gage, 1999). Furthermore, an increase in motor activity without motor learning does not change neuron morphology in the motor cortex (Kleim, Barbay, et al., 2002; Kleim, et al., 1996) or topography of movement representations (Kleim, et al., 1998; Plautz, et al., 2000). However, physical exercise without skill learning promotes increases in neurogenesis (Eadie, et al., 2005; Ra, et al., 2002; van Praag, et al., 1999), spine density, and dendritic length in the dentate gyrus (Eadie, et al., 2005). Although the various types of cerebral plasticity appear to be regulated by independent processes, there are interactions between neuronal and non-neuronal elements which allow for the behavioral adaptation to the environment (Markham & Greenough, 2004).

Much research has focused on structural neuronal plasticity but little on metabolic changes within the brain. In the following sections, I briefly describe glucose transporters in the brain, and some of the observations that indicate that these transporters are plastic.

Metabolic Plasticity

Glucose Transporters

Glucose uptake is the rate-limiting step for cerebral glucose utilization (Klip, Tsakiridis, Marette, & Ortiz, 1994). Glucose is mainly consumed aerobically, and is converted to CO_2 and water, however, it may also be used in anaerobic glycolysis (R. C. Vannucci & Vannucci, 2000). Glucose is phosphorylated into glucose-6-phosphate by hexokinase enzymes once it enters into the neuronal cytoplasm (Fields, Rinaman, & Devaskar, 1999). A family of membrane-embedded, facilitative glucose transporters (GLUTs) proteins have been identified that serve to transport glucose in the brain.

Transport occurs in an energy independent fashion as the GLUTs deliver glucose down its concentration gradient, into the intracellular region.

Generally, the GLUTs are highly homologous and composed of approximately 500 amino acids with one asparagine-linked site of glycosylation. The arrangement of the GLUTs has been identified as a pore structure defined by 12 transmembrane helices, with both external and internal ligand binding sites and N- and C-terminal cytoplasmic domains (Mueckler, et al., 1985; Olson & Pessin, 1996). Studies suggest that transmembrane helices fold and unravel to allow the movement of glucose through the pore during transport (Dwyer, 2001).

Many GLUTs have been identified, however, only a few have been detected in the CNS in substantial amounts (McEwen & Reagan, 2004). The nomenclature of the family of GLUTs has been organized using DNA sequence resemblances. The brain GLUTs are identified as Class I (GLUTs 1 to 4) and Class II (GLUT5). More recently discovered GLUTs form Class III, including GLUT6, GLUT8, and GLUT10 that are detected in the brain (McEwen & Reagan, 2004). Very little is known about GLUT6 and GLUT10 in the brain so these will not be further discussed. GLUT8 (originally known as GLUTx1) mRNA had been identified in a large number of neurons (Ibberson, Uldry, & Thorens, 2000; Reagan, et al., 2001; Sankar, Thamotharan, Shin, Moley, & Devaskar, 2002). However, in the CNS, GLUT8 glucose transport activity only occurs within the cytosol and the endoplasmic reticulum (ER) because it does not contain the dileucine motif necessary for the inclusion of this GLUT into the plasma membrane and thus do not participate in glucose transport into neurons (Piroli, et al., 2002; Reagan, et al., 2001).

GLUT2 and GLUT5 have specialized functions not directly involved in glucose transport in neurons and will only be briefly addressed here. GLUT2 is functionally different from the other GLUTs, such that it predominantly monitors and regulates glucose levels in peripheral tissue, rather than transporting glucose to cells for consumption. GLUT2 has a low affinity for glucose, with a high Km of approximately 40 mmol/L (G. K. Brown, 2000). This means that at high levels of glucose, GLUT2 is not saturated, allowing it to work as a signaling device, for example, in the pancreatic β-cells to release insulin. (Dwyer, Vannucci, & Simpson, 2002). GLUT2 is found to have the lowest expression in the brain of all the GLUTs. Immonhistochemistry and PCR methods have indicated that GLUT2 in the arcuate nucleus, nucleus tractus solitaris, motor nucleus of the vagus, paraventricular nucleus and lateral area of the hypothalamus, olfactory bulbs, and in a subpopulation of astrocytes (Leloup, et al., 1994). The brain regions expressing GLUT2 appear to be regions that are specifically sensitive to glucose level fluctuations (Leloup, et al., 1994) which suggests that GLUT2 has a glucose-sensing role in the brain (McEwen & Reagan, 2004).

GLUT5 is not a very efficient transporter of glucose and is mainly a fructose transporter. In the rat and human brain, GLUT5 has been identified in low levels. It has been located predominantly in microglial cells, the resident macrophages in the brain (Maher, Vannucci, & Simpson, 1994). In the next sections, we review the characteristics of the three main glucose transporters in the brain (GLUT1, GLUT3 and GLUT4) and present two competing views of how glucose-based energy is brought to neurons.

The supply of glucose-based energy to neurons. Glucose uptake across the blood-brain barrier (BBB) occurs mainly as the result of an extensively glycosylated, 55-kDa

isoform of GLUT1 found predominantly in the endothelium cells that make up the walls of the blood vessels in the brain. GLUT1 protein distribution is thought to be asymmetrical, with a three-four times higher concentration in the abluminal (brain-side) surface compared to the luminal (blood-side) surface (Dobrogowska & Vorbrodt, 1999; Farrell & Pardridge, 1991). It is likely that a smaller concentration of GLUT1 on the luminal surface maintains the intracellular endothelial glucose concentration lower than that of the blood plasma, by allowing the flow of glucose into the cell. Moreover, the quick delivery of glucose into the parenchyma is thought to depend on the higher concentration of GLUT1 protein on the abluminal surface of the endothelial cells (Choeiri, Staines, & Messier, 2002). Following this first step in which glucose enters the brain extracellular fluid, two pathways for the further distribution of glucose-based energy have been proposed and the relative contribution of each pathway is still hotly debated. The first pathway is quite straightforward. Once glucose has entered the extracellular compartment of the brain, it is taken up by neurons through GLUT3 (mainly found on pre- or post-synaptic sites) and by GLUT4 (mainly found on neuronal cell bodies). This simple model is still widely accepted but has been challenged by a number of observations from which a new hypothesis, the *astrocyte-neuron lactate shuttle* has been developed by Pellerin and Magistretti (2003b). In the next section, we describe briefly this hypothesis.

Astrocyte-neuron lactate shuttle. A major player in the shuttle is the astrocyte. Astrocytes have numerous processes called endfeet that wrap on blood capillaries on the one hand and on synaptic processes on the other hand. One of their roles, among others, is to provide a feedback link between synaptic activity and blood vessels, in which higher

synaptic activity will lead to capillary dilatation which in turn increases blood volume and ultimately allows more oxygen (and possibly more glucose) to enter the brain parenchyma. The second role of interest for us is linked to the presence of a 45-kDa, non-vascular isoform of GLUT1 on astrocytes (Ardizzone, Lu, & Dwyer, 2002; Dwyer, et al., 2002).

By some estimates, approximately 95% of the energy consumed by the brain is done by active neurons and 5% by astrocytes (Attwell & Laughlin, 2001). If glucose were the sole energy substrate of neurons, then we would expect the amount of glucose used by the neuron to be also 95% of all glucose used. Some data suggest that this is not the case. There is still controversy over the relevance of the following data to what is happening in the brain *in vivo* even though there is good support from *in vitro* experiments (Waagepetersen, Sonnewald, Larsson, & Schousboe, 2000; Zwingmann, Richter-Landsberg, Brand, & Leibfritz, 2000). Pellerin and Magistretti (2003b) tested this idea by measuring, after electrical stimulation, the relative distribution of 2-deoxyglucose and lactate in Schwann cells and axons in an isolated vagus nerve preparation. They found that approximately 80% of glucose uptake occurred in glial cells, indicating that these cells probably use the most glucose. However, because ultimately neurons are the largest users of energy, it was postulated that astrocytes release a metabolic intermediate, lactate, that would be used by neurons during periods of increased energy demand.

The first quantitative *in vivo* study that investigated the compartmentalization of glucose uptake by neurons and astrocytes used immunohistochemistry in combination with a novel microautoradiographic imaging procedure (Nehlig, Wittendorp-Rechenmann, & Lam, 2004). This imaging technique localizes the individual tracks of

electrons emitted during the disintegration of 2-deoxyglucose in the resting brain of rats (Nehlig, et al., 2004). According to this study, approximately half of the glucose uptake occurs in neurons and the other half is taken up and phosphorylated by astrocytes. These findings suggest that glucose is used by both neurons and astrocytes, however, the exact metabolic fate of glucose following phosphorylation is uncertain.

According to the astrocyte-neuron lactate shuttle hypothesis, glutamate is released by neurons when they are more active, which in turn, stimulates an increase in glycolysis and glucose transport in astrocytes (Loaiza, Porras, & Barros, 2003). This induces astrocytes to release lactate, which is taken up by neurons then becomes an energy source for neurons (Akaoka, et al., 2001; Pellerin, Bonvento, Chatton, Pierre, & Magistretti, 2002; Pellerin & Magistretti, 2003a). Interestingly, one study found a strong correlation between neuronal activation and the expression of the astrocytic 45-kDa isoform of GLUT1 but not of the 55-kDa GLUT1 vascular isoform or the GLUT3 neuronal transporter (Choeiri, et al., 2005) indicating that the astrocytic GLUT1 is the most plastic. Although this is beyond the scope of the present experiment, one prediction that can be derived from these results is the idea that proteins involved in the transport of energy-bearing molecules (e.g. glucose, lactate) would also be plastic. Lactate is transported into neurons by monocarboxylate transporters (MCTs). Four variants of such transporters have been identified.

MCT1 is widespread in all brain regions, however is abundant in endothelial cells of microvessels, ependymocytes (Gerhart, Enerson, Zhdankina, Leino, & Drewes, 1998; Hanu, McKenna, O'Neill, Resneck, & Bloch, 2000; Pierre, Pellerin, Debernardi, Riederer, & Magistretti, 2000) as well as in astrocytes (Ainscow, Mirshamsi, Tang,

Ashford, & Rutter, 2002; Debernardi, Pierre, Lengacher, Magistretti, & Pellerin, 2003; Hanu, et al., 2000; Pierre, et al., 2000). MCT4 is located exclusively in astrocytes and is widespread throughout the brain and has high expression in hippocampus, cortex and in the cerebellum (Bergersen, Rafiki, & Ottersen, 2002; Rafiki, Boulland, Halestrap, Ottersen, & Bergersen, 2003). The principal neuronal transporter is MCT2 (Bergersen, et al., 2002; Pierre, et al., 2000; Rafiki, et al., 2003).

Astrocytes release lactate via MCT1 or MCT4, thereby forming an extracellular pool that can be subsequently taken up by active neurons via the MCT2 transporters located on dendrites and axons (Bergersen, et al., 2001; Pierre, Magistretti, & Pellerin, 2002; S. J. Vannucci & Simpson, 2003). This is in accordance with studies that found that lactate functions in combination with glucose to maintain neuronal activation such as action potential propagation (A. M. Brown, Tekkok, & Ransom, 2003; Sakurai, Yang, Takata, & Yokono, 2002; Schurr, West, & Rigor, 1988; Takata, Sakurai, Yang, Yokono, & Okada, 2001). There is a certain amount of resistance to accept the astrocyte-neuron lactate shuttle hypothesis partly because some of its strongest evidence comes from *in vitro* experiments in which cells notoriously do not behave as they would *in vivo*. The alternative model is presented in the next section.

Neuronal glucose uptake. GLUT3, is known as the main neuronal glucose transporter. The greatest intensity of GLUT3 immunohistochemical staining has been identified in neuropil, and is mostly absent from cell bodies. High concentrations of GLUT3 were identified primarily in pre- and post-synaptic nerve endings, suggesting that GLUT3 provides for transport of glucose for synaptic energy metabolism in these areas where the majority of the brain's energy is spent (Leino, Gerhart, van Bueren, McCall, &

Drewes, 1997). GLUT3 is absent from the vascular fraction (Maher, Vannucci, & Simpson, 1993) and is not expressed in glial cells (Mantych, James, Chung, & Devaskar, 1992; Nagamatsu, et al., 1993). While GLUT1 and GLUT3 both have a high affinity for glucose (Km for GLUT1 is ~20mmol/L, Km for GLUT3 is ~10mmol/L), GLUT3 has a higher turnover rate than GLUT1 (853 sec^{-1} vs. 123 sec^{-1}). Thus, on that basis, neurons could transport a significantly greater amount of glucose than glial cells with the same number of glucose transporters at similar glucose concentrations (Carruthers, 1990; Maher, Davies-Hill, & Simpson, 1996; S. J. Vannucci, Maher, & Simpson, 1997). GLUT3's elevated rate of glucose transport has been taken as a demonstration that neurons can meet their high metabolic requirements without a significant contribution of the astrocyte-neuron lactate shuttle.

The final neuronal glucose transporter is GLUT4 but its expression is limited in most neurons even if expression and mRNA is larger in the cerebellum, some small population of neurons, and in ependymal cells of cerebral ventricles (Apelt, Mehlhorn, & Schliebs, 1999; Kobayashi, Nikami, Morimatsu, & Saito, 1996). GLUT4 neuronal expression has been found predominantly in the cerebellum in the granule and Purkinje cells, as well as in granule cells of the hippocampus and olfactory bulb (Kobayashi, et al., 1996; Leloup, et al., 1996). GLUT4 protein colocalizes significantly with its mRNA in regions such as the hippocampus, hypothalamus, thalamus, striatum, midbrain, medulla oblongata, olfactory bulb, cerebral cortex, and the cerebellum (Campbell, Dominiczak, Livingstone, & Gould, 1995; Leloup, et al., 1996; Rayner, Thomas, & Trayhurn, 1994; S. J. Vannucci, Koehler-Stec, et al., 1998). GLUT4 appears in neuronal processes, especially at the synaptic thickening regions, as well as in neuronal cell bodies and

cytoplasmic vesicles (El Messari, et al., 1998; Leloup, et al., 1996). In the periphery, GLUT4 is known as the insulin-sensitive glucose transporter. The presence of insulin produces massive translocation of GLUT4 from intracellular stores to the cellular membrane in the heart, skeletal muscle, as well as in brown and white adipose tissue (Dwyer, et al., 2002) which leads to a 20 fold increase in glucose uptake (Pessin & Bell, 1992; Simpson & Cushman, 1986). There is some evidence that insulin in the brain could have the same effect on GLUT4 (Grillo, Piroli, Hendry, & Reagan, 2009). Insulin-stimulated translocation of GLUT4 to the plasma membrane in rat hippocampus is PI3-kinase dependent (Grillo, et al., 2009).

In summary, glucose enters the brain from the blood through the 55-kDa GLUT1. It makes its way into the brain and then can be either taken up by neurons directly through the GLUT3 and the more sparsely distributed GLUT4. The observation that GLUT3 has a faster turnover rate gives it an advantage and its efficiency may be greater than its absolute number suggests. Other observations suggest that glucose is taken up by astrocytes through the 45-kDa GLUT1 transporter, transformed into lactate and then released to be picked up and used as metabolic fuel by neurons. As we will see in the next section, one *in vivo* experiment found that the only glucose transporter that increased following brain activation was the 45-kDa astrocytic glucose transporter which gives some support to the lactate shuttle hypothesis.

Known Examples of GLUT Plastic Expression in the Brain

We now turn to some conditions under which glucose transporter expression changes. The largest set of evidence comes from studies that have examined changes in glucose transporters in animals made diabetic by the toxic administration of

streptozotocin. Previous studies have shown that streptozotocin-induced chronic hyperglycemia caused a significant decline in glucose transport in the BBB of rats (Gjedde & Crone, 1981; McCall, Millington, & Wurtman, 1982). On the other hand, other studies revealed a minimal decrease or no effect on the expression of GLUT1 in cerebral microvessel and GLUT3 in neuronal cell populations of hyperglycemic rats (Duelli, et al., 2000; Simpson, et al., 1999). In contrast, other experiments found an increase in cerebral GLUT1 expression in response to diabetes (Choi, Boado, & Pardridge, 1989; Lutz & Pardridge, 1993). Another study observed increases in GLUT3 mRNA and protein in the hippocampus of diabetic animals, however, with no change in GLUT3 mRNA in the cortex (Reagan, et al., 1999). Similarly, a decrease in GLUT4 expression in the brain may occur in response to diabetes. It has been shown that trafficking of insulin-sensitive GLUT-4 from the cytosol to the cell membrane is hindered in diabetics in the hippocampus and the cerebellum (Piroli, et al., 2002; Reagan, et al., 1999). In summary, the regulation of GLUT expression in the brain associated with diabetes is uncertain, however, regional variations in GLUT expression are potentially responsible for these contradictory findings (McEwen & Reagan, 2004).

GLUT5 has been identified mainly as a fructose transporter and is found predominantly in skeletal muscle, small intestine, and microglia as mentioned previously. Using real-time polymerase chain reaction (PCR), Shu and colleagues (2006) examined the effects of feeding 20% fructose solution on GLUT5 mRNA levels in the brain, jejunum, and other tissues, along with hippocampus GLUT5 protein expression in the rat brain. They observed a 2.5 fold-increase in jejunum GLUT5 mRNA levels after 7 days of feeding which was in accordance with previous studies (Inukai, et al., 1993; Mavrias &

Mayer, 1973). The changes in GLUT5 expression are most likely specific to GLUT5 since changes in GLUT3 and GLUT4 expression were unaltered. Messier and colleagues (2007), did not find any significant effect of fructose feeding on GLUT5 expression in the brain of mice but this absence of effect may have been due to the overall lower amount of fructose ingested. Finally, in the rat brain, cerebral hypoxia-ischemia produces an activated microglial response (scavenger/cytotoxic). It was found that this process was concomitant with a rise in GLUT5 mRNA and protein expression (Koehler-Stec, et al., 2000).

In summary, there are a number of examples of brain glucose transporter plasticity in response to demand or substrate availability. In the final section of this introduction, I will briefly present data that shows that glucose transporter density is correlated with glucose use by the brain. This link is important as it forms the logical background to postulate that glucose transporters density would change in response to increase glucose demand.

Glucose Transporter Density and Glucose Demand

There is an association between metabolic activity and GLUT1 and GLUT3 levels in the brain (S. J. Vannucci, Clark, et al., 1998; Zeller, Duelli, Vogel, Schrock, & Kuschinsky, 1995), mainly resulting from neuronal activation. For example, studies have shown an increase in levels of GLUT1 and GLUT3 corresponding to increase in LCGU in osmoregulatory structures in response to water deprivation (Duelli, Maurer, Heiland, Elste, & Kuschinsky, 1999). GLUT1's density coincides with LCGU in numerous brain areas (Duelli & Kuschinsky, 2001; S. J. Vannucci, Clark, et al., 1998; Zeller, Rahner-Welsch, & Kuschinsky, 1997). GLUT1 vascular density was also found to be significant

greater in the frontal and motor cortex (Choeiri, et al., 2002). This appears to correspond with the detection of relatively high LCGU as opposed to non-cortical areas of the brain, which coincides with the high neuronal activity in these brain regions (Sokoloff, 1985; Zeller, et al., 1997).

The link between cerebral glucose uptake and GLUTs is also observed in the developing brain. In the newborn rat brain, cerebral glucose uptake and utilization are only approximately 20% of normal adult levels, however, they increase quickly by about 21-30 days old. GLUT1 and GLUT3 protein levels in the newborn rat brain closely follow these CGU and utilization changes (S. J. Vannucci, 1994). Low levels of the 55-kDa isoform GLUT1 are observed at birth. However, they rapidly increase after suckling. In comparison, the increase in the 45-kDa GLUT1 was more progressive, possibly due to global brain maturation.

Conversely, developmental studies have also shown that monocarboxylates are important energy substrates in the foetus and newborn. Lactate is present in high concentrations in the blood immediately upon birth (Dombrowski, Swiatek, & Chao, 1989). Lactic acid can support up to 60% of the total cerebral energy metabolism in the perinatal brain, thus, illustrating the ability of the young brain to substitute glucose with other fuels in situations of low circulating glucose levels (R. C. Vannucci & Vannucci, 2000). During weaning, there is a decrease in circulating ketone bodies and CGU increases with an increased dependence on glucose as the main fuel source (R. C. Vannucci & Vannucci, 2000).

In this introduction, we have presented the following arguments. Glucose transporters are a major mechanism by which the brain acquires glucose as an energy

substrate. The two isoforms of GLUT1 are the main routes by which glucose enter the brain. The 55-kDa vascular isoform brings glucose inside the brain parenchyma from blood while the astrocytic 45-kDa isoform shuttles glucose in and out of astrocytes. Neuronal energy comes mainly from glucose transported by GLUT3 and GLUT4 and quite possibly from lactate produced by astrocytes. However, the contribution of each process to the total energy uptake of neurons is still hotly debated. Glucose transporter density appears plastic in response to blood or brain glucose levels or neuronal demand. Functionally, there is only one convincing demonstration of *in vivo* glucose transporter plasticity in response to increased neuronal activity associated with learning (Choeiri, et al., 2005). The present thesis represents an extension of this experiment by examining the glucose transporter plasticity following 48 hours of access to a running wheel. Because extensive running was a new activity for the mice in the present experiment, we hypothesized that the motor regions of the brain would undergo plastic remodeling (as estimated by the microtubule constituent Tuj) associated with a new behavior (or new level of the running behavior) and that plastic remodeling would be correlated with changes in GLUT1 in the same regions.

Method

Animals and Running Task

Thirty-three 7-week old CD-1 male mice were used. The mice were supplied by Charles River and were placed in individual cages with food and water freely available. Each animal was given a 7-day acclimatization period before the start of the experiment. Animals in a first control group (n=11) were exposed to standard housing conditions without a running wheel. A second control group (n=11) had access to a stationary wheel for 48 hours to control for the possible effects of novelty so that any differences between that control group and the experimental one could be attributed only to running.

Animals in the experimental group (n=11) had free access to a moveable running wheel for 48 hours. This time period was chosen based on preliminary data. The distance run during the 48-hour period was recorded onto a dB2L cycle computer (FILZER Enterprises, Inc) fitted to each wheel. The plastic wheels had a 4.5 inch diameter and were purchased at a local pet store.

Immunohistochemistry

Each mouse was anaesthetized by an intraperitoneal injection of euthansol (65mg/mL) and perfused first with saline followed by Lana's fixative (4% paraformaldehyde, 0.2% picric acid in 0.16M sodium phosphate buffer, pH 7.1). The brains were postfixed for 4 hours at 4°C and then immersed in 10% sucrose dissolved in 0.1M sodium phosphate buffer, pH 7.1. The sucrose solution was changed 3 times over a 20 hour period before freezing the brain with CO_2. Sagittal sections (14μm) between lambda 1.40 and 2.00 (Paxinos and Franklin, 2001) were cut using a cryostat and transferred onto coated glass slides and stored in -80 °C.

Immediately before conducting immunohistochemistry, sections were immersed in PBS (10mM phosphate, 0.9% saline) for 15 minutes at room temperature. They were then incubated overnight at 4°C with a rabbit anti-GLUT1 (dilution 1:1000, Alpha Diagnostic International, San Antonio, TX, USA) and a mouse anti β-tubulin (Tuj) primary antibody (1:50, obtained from Dr. A. Frankfurter, University of Virginia, Charlottesville, Virginia).

A previous study that used the same GLUT1 antibody found that the antibody recognizes both the astrocytic 45-kDa and the vascular 55k-Da isoforms because it is raised against the C-terminus of the protein that is found in both isoforms (Choeiri, et al., 2005). So, the majority of the GLUT1 staining corresponded to blood vessels and the associated astrocytic endfeet (Choeiri, et al., 2005).

The following day, sections were rinsed and immersed in PBS for 15 minutes, then incubated with the respective secondary antibodies for 1 hour at room temperature. The secondary antibody coupled with GLUT1 was an Alexa594 donkey anti-rabbit (1:300, Molecular probes) and an Alexa488 donkey anti-mouse (1:400, Invitrogen) was coupled with Tuj. All antibodies were diluted in 0.3% triton-X100-PBS. Following incubation, the slides were rinsed in PBS for 15 minutes at room temperature and coverslipped using a noncommercial antifade solution (50mg p-phenylamine diamine in glycerol, pH 8).

Black and white pictures (8 bit) of the sections were taken using a QImaging Retiga 2000R camera and the QCapture program (QImaging) attached to an Olympus BX51 microscope at 20x magnification and a fixed 400ms exposure time. Pictures of the

following structures CA1, CA2, CA3, motor cortex, sensorimotor cortex, striatum and cerebellum were taken from each animal.

Data Analysis/Quantification

Quantification of the pictures was carried out using the ImageJ program from the National Institute of Health (http://rsb.info.nih.gov/ij/). Quantification consisted of the thresholding technique (King, Brey, Youssef, Johnston, & Patrick, 2002; Ruifrok, 1997) in which the mean grey value (optical density) was measured for the intensely labeled areas, as well as for the background. Thresholding is used to segment an image into objects of interest (blood capillaries and neuronal processes in the present case) and background on the basis of gray level. Each pixel of the picture analyzed has a certain gray level value (optical density). In general, the pixels with the highest gray levels correspond to the immunolabeled areas while the lower gray levels are associated with background fluorescence.

Image J allows you to visualize which parts of the picture are selected when a certain percentage of the range of gray levels is included. The general procedure is to find through visual inspection what the percentage is of the highest gray levels that have to be included to delineate the objects of interest and also the percentage of the lowest gray levels that has to be included to include most of the background fluorescence. The emitted fluorescence can either come from the top surface of the brain slice (clear, intense and focused) or from deeper layers (less clear and more subject to diffraction).

Thresholding will be more effective when restricting the analysis to the emitted fluorescence of the uppermost surface layers because the structures of interest can be more clearly identified. In the present project, the difference between the mean gray

values of the objects of interest and background was taken as an estimate of the staining intensity and taken as an estimate of the density of GLUT1 or β-tubulin. It is important to note that these estimates are relative measures used to compare the three groups and are not a quantification of GLUT1 or Tuj. Figure 8 illustrates black-filled boxes representing the approximate regional localization of GLUT1 and Tuj optical density measurements.

Thresholds were higher for β-tubulin than for GLUT1. For β-tubulin, the top and bottom 15-20% were measured. For GLUT1, top and bottom 5-10% were measured. Top 5-10% refers to the mean grey value of the most intensely-immunostained structures occupying 5-10% of the total area (i.e. GLUT1), whereas bottom 5-10% refers to the mean grey value of the least intensely-immunostained area occupying 5-10% of the total area photographed (background).

The cerebellum had consistently more intense staining than the other brain regions, thus a higher threshold % was used. For Tuj staining in cerebellum, we used top and bottom 20%. For GLUT1 staining in cerebellum, we used top and bottom 15-20%. Because the GLUT1 antibody used does not differentiate between the astrocytic and capillary location of GLUT1, the measures represent a global estimate of GLUT1 in both compartments.

For the optical density measurements for GLUT1, 5-10% of the area with the most intensely-labeled cells was measured for most brain regions. This included the top surface as well as some of the deeper layers since blood vessels that are less intense or out of focus may contain GLUT1 and should thus be included in the mean grey value. However, for the measure of blood vessel area, we used a stricter criterion and included only a mean area of approximately 1-2% (see Table 3, discussed later). This stricter

criterion was used in order to only include the blood vessels from the top surface of the brain slice to ensure that only clearly delineated blood vessels were included. Visual inspection of the area selected using this criterion revealed that only surface blood vessels were included and not non-identifiable structures or artifacts due to penetration of the GLUT1 antibody.

Analyses of variance (ANOVAs) were performed with one independent variable of group with three levels; control group 1, control group 2 (stationary wheel), and experimental group (runners). There were 14 ANOVAs run for each of the 14 dependent variables. The dependent variables were the measures of mean grey value after subtracting the background for each of the seven regions of interest stained with either the Tuj or the GLUT1 antibody. Structures were not entered in a higher level ANOVA as a factor because the comparisons across structures were not of interest, on the one hand and there was good a priori evidence that the vascularization and neuropil distribution in the various structures were not equivalent rendering comparisons uninformative. The Tuj and GLUT1 were also not entered as a factor because the immunohistological results using two different primary and secondary antibodies are not meaningfully comparable directly.

Using Image J thresholding technique, the area clearly immunostained by GLUT1 and that had clearly the characteristics of a capillary was selected and the portion (in %) of the image occupied by this staining was measured to obtain an estimate of blood vessel density. Since the background staining for the GLUT1 was relatively low, the area selected and measured contained blood vessels almost exclusively. However, because GLUT1-containing astrocyte endfeet surround capillaries and that they cannot be

separated at this magnification, they were included in the surface measure and may have slightly inflated the measures.

Seven ANOVAs were run for each brain region with the % area occupied by GLUT1 immunostained cells as the dependent variable. An alpha level of .05 was used for all statistical tests including Tukey post hoc tests.

Results

Mean Grey Value (Optical Density) of Tuj and GLUT1 Immunostaining

Table 1 presents the Tuj and GLUT1 immunohistochemical mean grey value (optical density) and associated probabilities for the seven regions of interest following the running task. The ANOVAs indicated a significant increase in Tuj staining of the cerebellum, $F(2,30)=8.1$, $p<.05$. Tukey HSD post hoc tests indicate a significant mean difference for control 1 compared to runners ($p=.004$) and for control 2 compared to runners ($p=.004$) in Tuj staining of the cerebellum.

There was also an overall difference in GLUT1 staining of the motor cortex, $F(2,30)=4.3$, $p<.05$. There were significant differences between the means of control 1 and runners ($p=.044$) and between control 2 and runners in GLUT1 staining for the motor cortex ($p=.040$). Trends toward increases were observed in GLUT1 staining of the sensorimotor cortex, $F(2,30)=3.1$, $p=.058$, of the striatum, $F(2,30)=3.0$, $p=.064$, and of the cerebellum, $F(2,30)=2.7$, $p=.083$. Tukey's tests indicated trends for a difference between the control 1 group in comparison to the runners for the GLUT1 staining of the sensorimotor cortex ($p=.068$) and between the control 2 group in comparison to the runners for the GLUT1 staining of the striatum ($p=.057$). In general, there were only

small and insignificant differences in the mean grey values between the two control groups and no consistent pattern emerged.

Figures 1 to 7 show the comparison of Tuj and GLUT1 staining for a control 2 and a running animal for each brain region. The few artifacts (extremely bright spots) found in the pictures were not included in the grey value measurements. In addition, only pictures from a control 2 animal were used in the figures because there was no significant difference between control group 1 and 2 on any of the measures. The staining from the same control 2 and running animal was used for all the regions with the exception of the motor and sensorimotor cortex of the control animal due to uncharacteristically bright staining in that particular region that was not representative of the mean staining of the control group.

Tuj and GLUT1 antibodies measure different antigens and thus are not directly comparable statistically. However, we calculated in Table 2 the percent difference in optical density between the running group in comparison to each of the control groups for each region. From Table 2, it can be seen that there was a parallel increase in staining for both Tuj and GLUT1 for the cerebellum while an increase in staining in the motor cortex was much smaller for Tuj than GLUT1. Smaller changes in Tuj and GLUT1 staining were seen in the sensorimotor cortex and striatum.

In contrast to GLUT1 that appears to be widespread, Tuj neuronal staining was limited to specific areas within the brain regions examined. The brightest Tuj staining was found in the molecular layer of the cerebellum, particularly in the dendrites and cell bodies of Purkinje cells. In addition, intense Tuj staining was also found in pyramidal neurons of the motor and sensorimotor cortex. Moderate Tuj staining was found in the

pyramidal neurons and mossy fibers of the hippocampus, as well as in the medium spiny neurons of the striatum. The more restricted expression of Tuj within each region may have led to overall smaller differences.

Blood Vessel Density of GLUT1 immunostaining

Mean percent area of GLUT1 immunohistochemical-stained capillaries and the F value and corresponding probabilities are reported in Table 3. In the seven regions examined, the majority of the regions (except CA1 and CA2) had a significant increase in the area of GLUT1-containing blood vessels in runners compared to each control group. This suggests that there was an increase in the number of GLUT1-containing blood vessels for most of the regions as a result of running. Tukey post hocs indicate a significant difference between control 1 and runners for the areas of GLUT1 staining in the sensorimotor cortex ($p=.009$) as well as in the cerebellum ($p=.014$). There were significant differences in areas of GLUT1 staining between control 1 and runners ($p=.007$) and near significance between control 2 and runners ($p=.050$) in the striatum. There were significant differences in areas of GLUT1 staining between control 1 and runners ($p=.002$) and between control 2 and runners in the motor cortex ($p=.020$).

Discussion

The main hypothesis of this experiment was that performance of a new motor activity would induce metabolism-related plastic changes (GLUT1 expression, angiogenesis) in brain regions associated with this activity and these changes would also be associated with dendritic and axonal growth/reorganization as estimated by an increase in the microtubule-associated protein Tuj.

While there was only a significant increase in GLUT1 expression in the motor cortex, other motor related structures showed a similar trend (sensorimotor cortex, striatum and cerebellum). Tuj expression followed a reversed pattern in which Tuj expression was highly increased in the cerebellum while much smaller increases were found in the other motor regions. These changes cannot be attributed to non-specific increases of GLUT1 and Tuj throughout the brain since they were not observed in the hippocampus (and other brain regions not reported here). When considering the amplitude of change, it is notable that GLUT1 increased by about 200% in the motor cortex while both Tuj and GLUT1 were increased by over 150% in the cerebellum (with the caveat that the increase in GLUT1 remained not statistically significant because of variability). In addition, there were no significant correlations between GLUT1 and Tuj expression in relation to the distance traveled following 48 hours of running. One possible explanation is that the effect of running on GLUT1 and Tuj expression may have reached a maximum after a certain distance run at the beginning of the 48 hour period.

Finally, we used thresholding to measure the surface of GLUT1 labeling closely associated with identifiable blood vessels. Using this approximate measure of blood vessel density, we found that running appeared to produce a significant increase in blood vessel density in all motor regions and also in the CA3 region of the hippocampus. Although GLUT1 has been used previously to estimate blood vessel density in the context of angiogenesis (Van der Borght, et al., 2009), the present results cannot completely dissociate angiogenesis from an increase in the number of GLUT1 on existing blood vessels. For example, an increase in GLUT1 density on existing blood vessel branches could lead to an increase in fluorescence of smaller blood vessels or of blood

vessels situated under the immediate brain slice surface and lead to increased number of visible blood vessels. However, this hypothesis is not supported by the observation of significant increase in blood vessel area (as estimated) in all motor regions and the increase in blood vessel area in the CA3 that is not matched by an increase in overall estimate of GLUT1 density in other brain regions such as the CA1 of the hippocampus. From this perspective, 48 hours of running wheel access would induce angiogenesis and an increase in the number of GLUT1 in most motor regions while neuronal plastic changes are only observed in the cerebellum. Because of the limitations associated with using GLUT1 immunoreactivity to measure both angiogenesis and GLUT1 proper, the experiment will have to be replicated using other markers specific to the vascular epithelial cells.

Because the access to the running wheel was limited to 48 hours, we hypothesized that this would induce neuronal plasticity but not the more non-specific changes associated with long-term exercise (Black, et al., 1990; Ding, et al., 2006; Isaacs, et al., 1992; Kleim, Cooper, et al., 2002; Swain, et al., 2003). One such factor that has been invoked is brain-derived neurotrophic factor (BDNF) that has been shown to be involved in many of the functional effects of exercise (Vaynman, Ying, & Gomez-Pinilla, 2004).

Interestingly, a recent study performed by Van der Borght and colleagues (2009) compared the effects of short-term and long-term running on blood vessel density in the hippocampus. Mice had access to a running wheel for 1, 3, or 10 days followed by GLUT1 immunostaining as a measure of blood vessel density. They found that GLUT1 immunostaining was somewhat greater for mice with running wheels in comparison to sedentary mice, however, the difference was not statistically significant in the CA1 which

is consistent with the present results. However, there was a significant increase in the area of GLUT1-positive vessels in the dentate gyrus after 3 days that disappeared 1 day after the cessation of exercise (Van der Borght, et al., 2009). The last observation indicates that angiogenesis is a very labile process that spans hours rather than days.

In the dentate gyrus, the researchers did not find any significant changes in the optical density of the GLUT1-positive structures suggesting that there was not a significant alteration in GLUT1 expression in pre-existing blood vessels (Van der Borght, et al., 2009). The authors suggest that the increase in the surface area of the GLUT1-positive vessels they observed in the dentate gyrus was a measure of the change in vascular density rather than an increase in staining intensity. The same limitations noted above also apply to this study since GLUT1 was also used to detect blood vessels. In light of previous observations that show that GLUT1 increases associated with neuronal activity are transients (Choeiri, et al., 2005), it appears that short-term GLUT1 expression plasticity might be more likely than angiogenesis at this juncture. This dissociation between angiogenesis and the number of GLUT1 will need to be addressed in future projects by measuring blood vessel density with other markers.

The results of the Van Der Borght study (2009) suggest that the effects of running on cerebral vasculature appear to be region-specific in the hippocampus. This may be due to a link between angiogenesis and neurogenesis that occurs primarily in the dentate gyrus (Van der Borght, et al., 2009). We did not measure GLUT1 in the dentate gyrus in the present experiment but there were no apparent changes in GLUT1 immunoreactivity in that region in our sample. Furthermore, the study involving the operant learning task

(Choeiri, et al., 2005) did not find significant differences in GLUT1 expression in the dentate gyrus of learning animals.

The small but non-significant increase in the area occupied by GLUT1-containing vessels in the stationary wheel group compared to the no wheel group suggests the possibility that the novelty of the wheel has a small stimulatory effect on angiogenesis. However, this effect was absent in measures of GLUT1 density. This may be an avenue for future research measuring angiogenesis independent of GLUT1.

Some studies have shown a transitory increase in learning-induced local cerebral glucose uptake (Sif, et al., 1991) and a prominent change in the hippocampus, particularly in the CA1 pyramidal cells as a result of operant conditioning (Messier, Durkin, Mrabet, & Destrade, 1990; Stein, Xue, & Belluzzi, 1994). In that experiment, control groups that were forced to exercise in a treadmill for the same time they were placed in the operant chamber failed to show any increase in glucose uptake. Interestingly, the same operant bar-pressing task produced an increase in GLUT1 expression in the hippocampus and the sensorimotor cortex (Choeiri, et al., 2005) suggesting a link between increased glucose uptake and increased GLUT1 expression when new behaviors lead to neuronal activation.

It is interesting that learning induces GLUT1 plastic expression in the hippocampus but not in the motor cortex (Choeiri, et al., 2005), while access to a running wheel induces GLUT1 plastic expression in the motor cortex but not in the hippocampus. This suggests a clear link between GLUT1 plastic changes and neuronal activation.

Finally, both BDNF (Donovan, et al., 2000; Gustafsson, Lindvall, & Kokaia, 2003) and vascular endothelial growth factor (VEGF) have been demonstrated to

participate in the angiogenesis (Ding, et al., 2006) and neurogenesis (Fabel, et al., 2003) that is associated with exercise and this may provide a mechanism by which GLUT1 plasticity and angiogenesis occur following training in new tasks (Van der Borght, et al., 2009). It would be interesting to measure the level of these growth factors associated with each structure studied in the present experiment to determine if an association exists. It will also be useful to examine whether the number of mitochondria as well as metabolism also increases in the neurons in the activated brain areas.

The differential expression of astrocytic and endothelial GLUT1 could be addressed in future studies by using the Western blot technique. If the astrocytic 45-kDa GLUT1 protein is identified, this may give support to the astrocyte-neuron lactate shuttle. In addition, it would be of interest to examine the colocalization of GLUT1 and MCTs to see if glucose uptake in neurons and astrocytes occurs in same regions where lactate metabolism may take place.

In summary, the present experiment demonstrated that access to a running wheel for 48 hours induced plastic changes in the expression of GLUT1, Tuj and GLUT1-associated estimate of blood vessel surface in motor regions. The results tend to support the plastic association between mechanisms of energy supply and plastic reorganization of neurons following a new training experience.

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Table 1

GLUT1 and Tuj Immunohistochemical Mean Grey Value (±S.E.) and F Value in Various Brain Regions Following Access or Not to a Running Wheel in Mice

	GLUT1				Tuj			
Brain Region	No Wheel	Wheel/No Running	Runners	F	No Wheel	Wheel/No Running	Runners	F
CA1	23.9±1.8	23.9±1.4	27.6±2.8	1.0	43.5±4.5	37.8±4.9	47.0±4.9	1.0
CA2	25.4±2.1	26.3±1.5	30.0±2.8	1.2	45.4±5.0	39.0±6.0	42.3±5.1	.4
CA3	25.9±2.2	26.0±1.7	29.8±2.7	1.0	48.6±5.8	42.9±8.1	41.9±5.4	.3
Sensorimotor Cortex	29.1±1.9	30.9±2.8	40.7±5.1	3.1	50.0±4.5	59.8±7.9	67.0±9.2	1.3
Striatum	25.3±1.5	23.6±1.0	28.9±2.0	3.0	38.2±2.9	34.5±2.8	43.0±3.6	1.8
Motor Cortex	27.8±1.6	27.5±2.0	42.2±6.5	4.3*	48.8±3.9	49.8±5.9	64.7±8.7	1.9
Cerebellum	35.3±5.3	33.7±5.6	50.7±6.2	2.7	52.8±5.7	53.3±6.7	86.3±7.7	8.1*

* $p \leq .05$

Table 2

*The Percent Differences in Mean Optical Density (OD) of the Running Group and the Control Groups in Tuj and GLUT1 Staining (Mean OD of Running Group-Mean OD of Control/Mean OD of Control*100)*

	Running vs. no wheel			Running vs. Wheel/No running		
Brain Region	Tuj (%) (A)	GLUT1 (%) (B)	A-B (%)	Tuj (%) (C)	GLUT1 (%) (D)	C-D (%)
CA1	102	113	11	117	112	-5
CA2	87	114	27	102	110	8
CA3	81	112	31	91	111	20
Sensorimotor Cortex	127	136	9	106	129	23
Striatum	109	113	4	121	121	0
Motor Cortex	124	199	75	121	201	80
Cerebellum	165	150	-15	164	158	-6

Note: A percentage of 164% represents a 64% greater mean OD of running group compared to control group.

Table 3

Mean percent area of GLUT1 intense labeling (±S.E.) and F value in various brain regions following access or not to a running wheel in mice

	Area of GLUT1 intense labeling (%)			
Brain Region	No Wheel	Wheel/No running	Runners	F
CA1	0.9±0.2	1.1±0.2	1.4±0.2	1.9
CA2	0.9±0.2	1.0±0.1	1.4±0.2	2.2
CA3	0.9±0.2	1.1±0.2	1.7±0.2	4.4*
Sensorimotor Cortex	0.9±0.2	1.5±0.3	2.2±0.3	5.1*
Striatum	0.6±0.1	0.8±0.1	1.4±0.3	5.9*
Motor Cortex	1.0±0.5	1.4±0.9	2.8±0.5	7.6*
Cerebellum	0.7±0.2	1.2±0.4	2.0±0.4	4.6*

* $p \leq .05$

Figure 1. Tuj (upper left: control 2, upper right: runner) and GLUT1 (bottom left: control 2, bottom right: runner) immunostaining in the CA1 region.

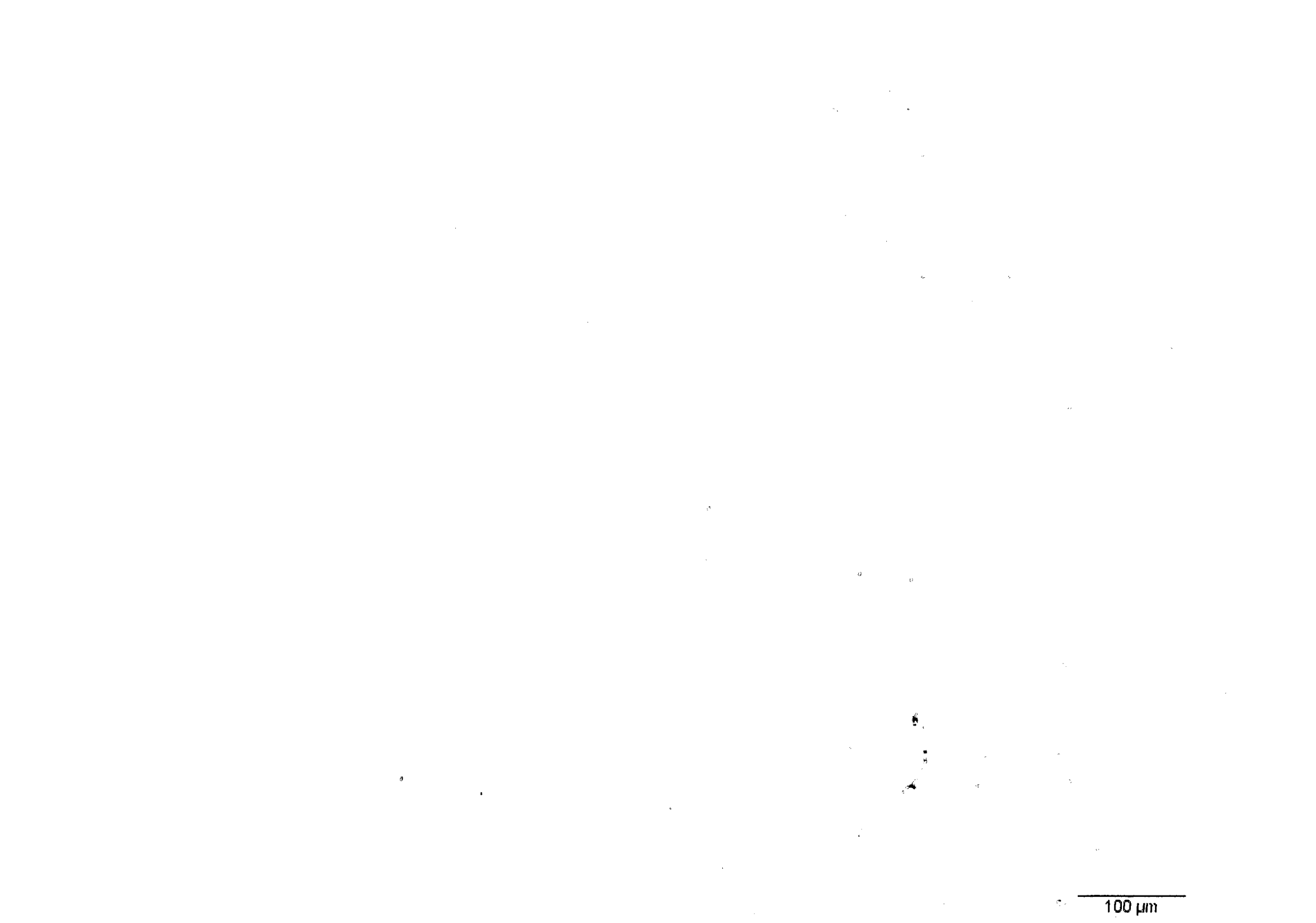

Figure 2. Tuj (upper left: control 2, upper right: runner) and GLUT1 (bottom left: control 2, bottom right: runner) immunostaining in the CA2 region.

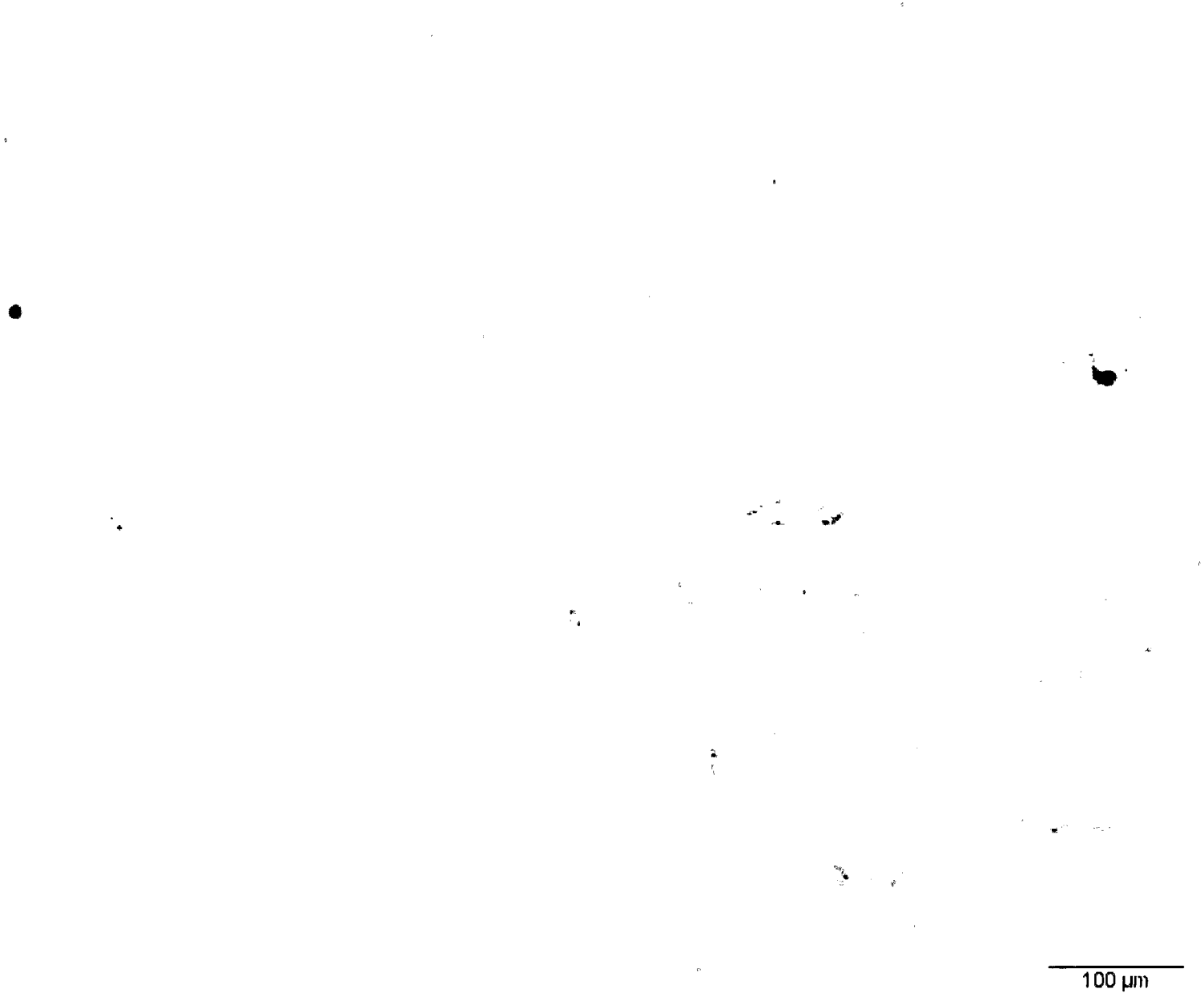

Figure 3. Tuj (upper left: control 2, upper right: runner) and GLUT1 (bottom left: control 2, bottom right: runner) immunostaining in the CA3 region.

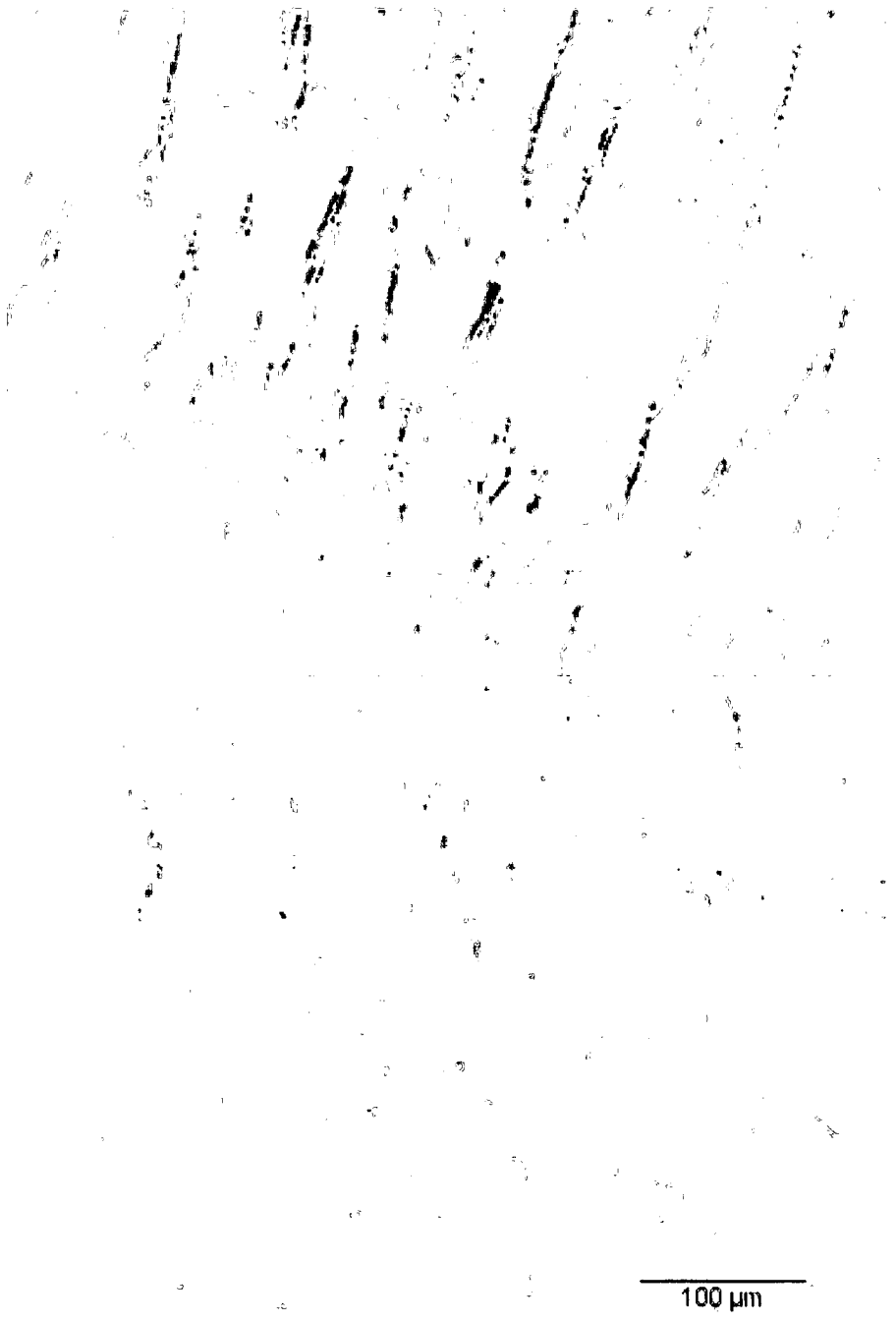

Figure 4. Tuj (upper left: control 2, upper right: runner) and GLUT1 (bottom left: control 2, bottom right: runner) immunostaining in the sensorimotor cortex.

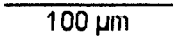

Figure 5. Tuj (upper left: control 2, upper right: runner) and GLUT1 (bottom left: control 2, bottom right: runner) immunostaining in the striatum.

Figure 6. Tuj (upper left: control 2, upper right: runner) and GLUT1 (bottom left: control 2, bottom right: runner) immunostaining in the motor cortex.

Figure 7. Tuj (upper left: control 2, upper right: runner) and GLUT1 (bottom left: control 2, bottom right: runner) immunostaining in the cerebellum.

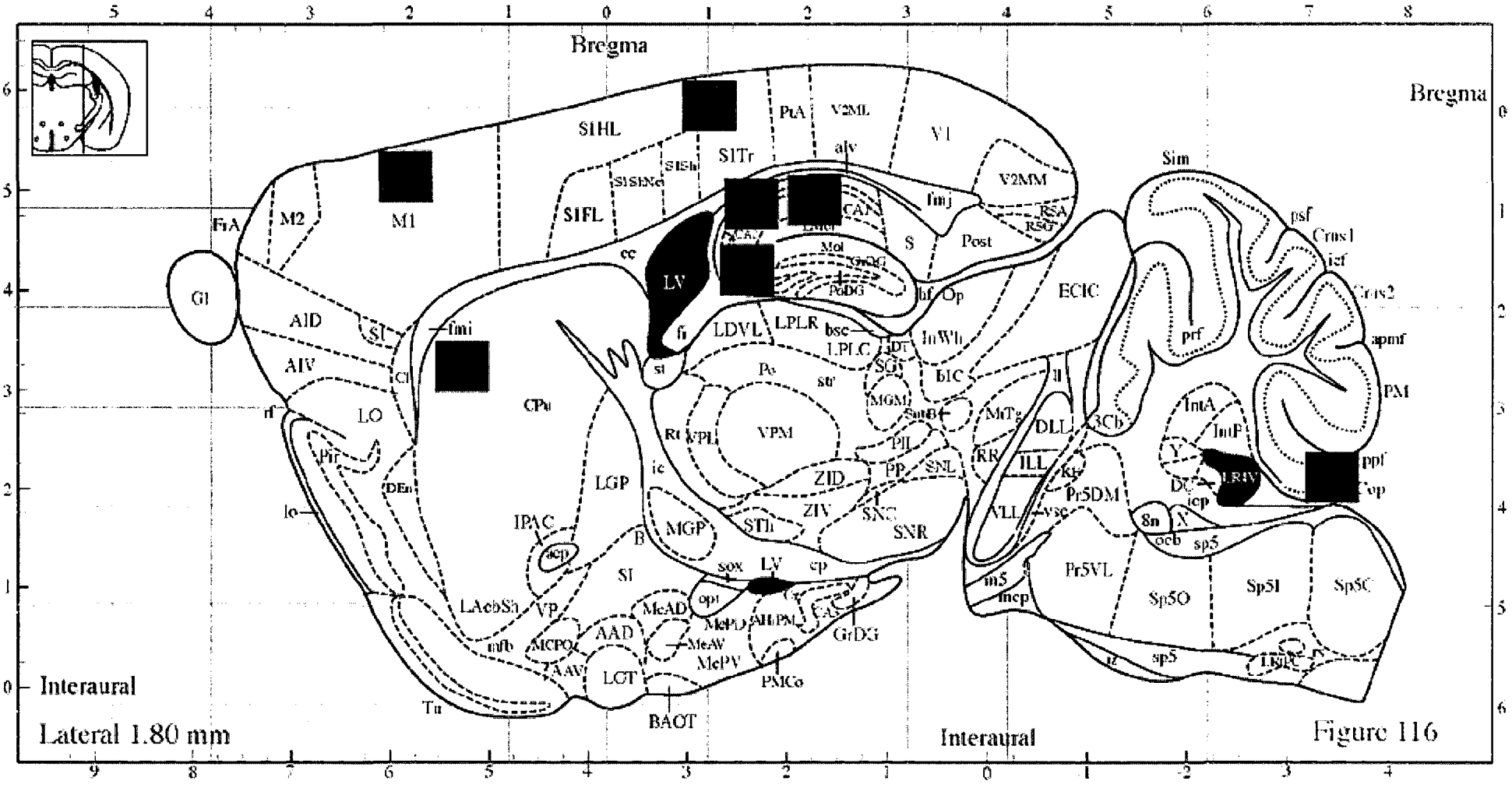

Figure 8. Approximate regional localization of GLUT1 and Tuj optical density measurements (black boxes).