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Cell-specific modulation of monocarboxylate transporter expression contributes to the metabolic reprogramming taking place following cerebral ischemia

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ABSTRACT

Monocarboxylate transporters (MCTs) are involved in lactate trafficking and utilization by brain cells. As lactate is not only overproduced during ischemia but its utilization was shown to be essential upon recovery, we analyzed the expression of the main cerebral MCTs at 1 and 24 hours after an ischemic insult induced by a transient occlusion of the left middle cerebral artery (MCAO) in CD1 mice (n = 5, 7 and 10 for control, 1 and 24 hours groups, respectively). After 1 hour of reperfusion, an upregulation of the three MCTs was observed in the striatum (MCT1 ipsilateral 2.73 ± 0.2 and contralateral 2.01 ± 0.4; MCT2 ipsilateral 2.1 ± 0.1; MCT4 ipsilateral 1.65 ± 0.1) and in the surrounding cortex of both the ipsilateral (MCT1 2.4 ± 0.4; MCT2 1.62 ± 0.2; MCT4 1.31 ± 0.1) and contralateral (MCT1 2.78 ± 0.4; MCT2 1.76 ± 0.2) hemispheres, compared to the corresponding sham hemispheres. An increase of MCT1 (ipsilateral 2.1 ± 0.2) and MCT2 (contralateral 1.9 ± 0.1) expression was also observed in the hippocampus, while no effect was observed for MCT4. At 24 hours of reperfusion, total MCT2 and MCT4 expressions were decreased in the striatum (MCT2 ipsilateral 0.32 ± 0.1 and contralateral 0.63 ± 0.1; MCT4 ipsilateral 0.59 ± 0.1) and the surrounding cortex (MCT4 ipsilateral 0.67 ± 0.1), compared to the sham. At the cellular level, neurons which usually express only MCT2 strongly expressed MCT1 at both time points. Surprisingly, staining for MCT4 appeared on neurons and was strong at 24 hours post-insult, in the striatum and the cortex of both hemispheres. A similar expression pattern was observed also in the ipsilateral hemisphere of the sham operated animals at 24 hours. Overall, our study indicates that cell-specific changes in MCT expression induced by an ischemic insult may participate to the metabolic adaptations taking place in the brain after a transient ischemic episode.

Stroke is the second leading cause of death worldwide (World Health Organization 2014; http://www.who.int/mediacentre/factsheets/fs310/en) and the commonest cause of neurological disability in the developed world (Murray and Lopez 1996; MacDonald et al. 2000). Ischemic stroke, resulting from an artery occlusion, is the most common subtype of stroke. In this case, oxygen and glucose supply from the blood is disrupted, leading to energy failure in the concerned area and causing damage to the brain tissue. Brain metabolism therefore plays a critical role in the pathophysiological mechanisms underlying neuronal damage in stroke and recovery. Glucose is the primary energy substrate of the brain under physiological conditions, but the brain is also able to oxidize other intermediate metabolites, notably lactate (Boumezbeur et al. 2010; Wyss et al. 2011). Lactate seems to occupy a central position under ischemic conditions. Indeed, during cerebral ischemia, lactate accumulates in the extracellular space due to the anaerobic metabolism of glucose. Levels can rise from 1.5–3.5 mM in the normal brain to 15–25 mM in the ischemic brain (Rehncrona et al. 1981; Folbergrova et al. 1992; Wagner et al. 1992). Despite a long history of negatively perceived role in ischemia, lactate has been shown to have neuroprotective properties in hypoxic conditions *in vitro* (Schurr et al. 1997; Schurr et al. 1997; Berthet et al. 2009), but also in models of ischemia *in vivo* (Schurr et al. 2001; Berthet et al. 2009; Berthet et al. 2012). Indeed, Berthet et al. (2009, 2012) demonstrated that administration of lactate after reperfusion, either intracerebroventricularly or intravenously, decreased significantly the lesion size in mouse brain and improved the neurological outcome upon recovery from a transient middle cerebral artery occlusion (tMCAO).

Lactate trafficking between cells is facilitated by a family of proton-dependent carriers named monocarboxylate transporters (MCTs). Three of them have been clearly characterized as lactate transporters in the central nervous system (Pierre and Pellerin 2005). MCT1 is predominantly found on endothelial cells forming cerebral blood vessels as well as on glial cells including astrocytes (Gerhart et al. 1997; Hanu et al. 2000; Pierre et al. 2000), oligodendrocytes (Lee et al. 2012; Rinholm et al. 2011) and tanycytes (Cortes-Campo et al. 2011). MCT2 is considered the main neuronal transporter (Pierre et al. 2002; Debernardi et al. 2003). Finally, MCT4 is exclusively expressed by astrocytes but its precise role is not well understood up to now (Rafiki et al. 2003; Pellerin et al. 2005; Rosafio and Pellerin 2014). Interestingly, Schurr et al. (2001) showed that inhibition of lactate transport through the MCTs increases neuronal damage after cardiac arrest-induced transient global cerebral ischemia in a rat model, thus suggesting a critical role for these transporters in the recovery

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from ischemia. In this study, we investigated the changes in cerebral MCT expression pattern occurring in a MCAO ischemic mouse model.

EXPERIMENTAL PROCEDURES

All experiments were conducted in accordance with the Swiss guidelines for animal experimentation and approved by the veterinary authority.

Transient middle cerebral artery occlusion in the mouse

Male CD1 mice (body weight 26-35 g, 5-7 weeks of age; Charles River, L'arbresle, France) were anesthetized with isoflurane (1.5 - 2% in nitrous oxide/oxygen 70%/30%) using a face mask. Body temperature was maintained at $37.0 \pm 0.5°C$ throughout surgery using a heating pad (FHC Inc., Bowdoinham, ME, USA). Regional cerebral blood flow (rCBF) was measured and continuously recorded throughout the operation in all animals by laser-Doppler flowmetry (Perimed, Craponne, France) with a flexible probe fixed on the skull (1 mm posterior and 6 mm lateral from bregma). Transient (30 minutes) focal cerebral ischemia was induced by occlusion of the left MCA with an intra-arterial suture as described previously (Longa et al. 1989; Berthet et al. 2009). Briefly, the left common carotid artery and the left external carotid artery were exposed and ligated following a ventral midline neck incision. Ischemia was induced by inserting a silicon-coated nylon monofilament (0.17 mm diameter) through the left common carotid artery into the internal carotid artery until mild resistance was felt and a drop to less than 20% of initial rCBF was registered. rCBF was monitored and maintained below 20% of the baseline level during ischemia. Reperfusion was considered successful if the rCBF rose above 50% of baseline. Sham mice underwent exclusively the common carotid artery ligation during 30 minutes.

At the beginning of the operation, mice were administered 0.025 mg/kg of buprenorphine subcutaneously for post-surgery analgesia. Once the animals were awake, they were housed overnight in an incubator at 28°C.

Western blotting

For protein expression experiments, young adult male mice were subjected to 30 minutes MCAO as described above and sacrificed either 1 hour or 24 hours after reperfusion. Striatum (caudate and putamen), primary motor and somatosensory cortex and hippocampus were collected using a rodent brain matrix (adult mouse, coronal. ASI Instruments, MI, USA). Total proteins were extracted from brain tissues by cellular lysis in ice-cold RIPA buffer

(#9806, Cell Signaling, Beverly, MA, USA) supplemented with a mixture of protease inhibitors (Complete 11257000; Roche, Basel, Switzerland). Protein quantification was performed with the Pierce BCA Protein Assay kit (#23227, Thermo Fisher Scientific, Pierce, Lausanne, Switzerland) and about 20 µg of proteins were denatured (95°C) during 5 minutes in SDS-PAGE sample buffer (60 mM Tris-HCl pH 6.8, 5 % SDS, 6.6 % glycerol, 5 mM EDTA, 5 % β-mercaptoethanol and 0.1 % bromophenol blue). Samples were separated on a 10 % acrylamide running gel and a 4 % acrylamide stacking gel using an Electrophoresis Unit (Bio-Rad, Cressier, Switzerland). Proteins were then electroblotted onto nitrocellulose membranes (0.45 µm; #162-0115, Bio-Rad, Cressier, Switzerland) using an Electrophoresis Unit. Non-specific binding sites were blocked for 1 hour at room temperature with a solution of Tris-Buffered-Saline (TBS; 50 mM Tris-HCl pH 7.5, 150mM NaCl) supplemented with 0.1 % Tween-20 and containing 10 % (wt/vol) of skimmed milk. Blots were then incubated overnight at 4°C with specific primary antibodies in TBS-T 0.1 % containing 1 % skimmed milk: polyclonal rabbit anti-mouse MCT1 (1:1000 dilution; (Pierre et al. 2000)), polyclonal rabbit anti-mouse MCT2 (1:1000 dilution; (Pierre et al. 2000)) or polyclonal rabbit anti-mouse MCT4 (1:200 dilution; #sc-50329; SantaCruz, Heidelberg, Germany). Blots were washed three times in TBS-T 0.1% and subsequently incubated 2 hours at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit IgG (#NA9340V, 1:10.000 dilution; GE Healthcare, Glattbrugg, Switzerland). After being washed three times in TBS-T 0.1%, blots were processed using Immun-StarTMWesternCTM Chemiluminescent Kit (#170-5070, Bio-Rad, Cressier, Switzerland). Chemiluminescence detection was performed with the ChemiDoc™ XRS System (#170-8070, Bio-Rad, Cressier, Switzerland). Total protein content assay was performed with the Pierce Reversible Protein Stain kit (#24580, Thermo Fisher Scientific, Pierce, Lausanne, Switzerland) and revealed with the ChemiDoc™ XRS System (#170-8070, Bio-Rad, Cressier, Switzerland). Both types of labeling were quantified with the ImageLab 3.0 software (Bio-Rad, Cressier, Switzerland) and MCT protein expression was normalized to the total protein content. All data were normalized to the corresponding ipsilateral area of the sham.

Immunohistochemistry

Mice were injected intraperitoneally with a lethal dose of pentobarbital (10mL/kg, Sigma, Buchs, Switzerland) and then perfused with 150mL of 4% paraformaldehyde (Sigma-Aldrich, USA) dissolved in 1 x PBS at pH 7.4. Brains were dissected, postfixed overnight at 4°C, cryoprotected 24 hours in 30% sucrose solution (Sigma-Aldrich, USA) and rapidly frozen.

Twenty μm thick coronal microtome-cryostat (leica MC 3050S) sections were stored in cryoprotectant (30% ethylene glycol and 25% glycerin in 1 x PBS) at -20°C. For immunostaining, sections were washed three times in 1 x PBS and blocking of non-specific binding sites was achieved by incubating in 1 x PBS containing 0.25% bovine serum albumin, 0.3% Triton X-100 and 10% normal goat serum during 1 hour. Double immunostainings were carried out overnight at 4°C in PBS containing 0.25% bovine serum albumin, 0.3% Triton X-100: polyclonal anti-MCT1 and anti-MCT2 (1:500 dilution; (Pierre et al. 2000)), polyclonal anti-MCT4 (1:250 dilution; #sc-50329; SantaCruz, Heidelberg, Germany) in combination with either a monoclonal mouse anti-neuronal nuclear antigen (NeuN) antibody (1:500 dilution, Sigma, Buchs, Switzerland), a monoclonal mouse anti-microtubule associated protein (MAP2) antibody (1:250 dilution, Sigma, Buchs, Switzerland) or a monoclonal anti-S100β (1:500 dilution; Sigma, Buchs, Switzerland). After washing three times with PBS, sections were incubated in a PBS solution containing a donkey Cy3-conjugated anti-rabbit antibody (1:500 dilution; #711-165-152, Jackson Immunoresearch, Baltimore, MD, USA) and a donkey FITC-conjugated anti-mouse antibody (1:500 dilution; #715-095-150, Jackson Immunoresearch, Baltimore, MD, USA). After washing twice in PBS, sections were incubated during 5 minutes with Hoechst staining solution (#94403, Sigma, Buchs, Switzerland) dissolved in PBS (1/10'000), rinsed again twice 10 minutes in PBS and then mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Preparations were then maintained at 4°C until observation with a Zeiss LSM 710 Quasar Confocal Microscope (Zeiss, Feldbach, Switzerland).

Statistical analysis

Statistical analyses were performed using either a one-way ANOVA followed by Dunnett's multiple comparison test or a Student t-test. Statistical calculations were carried out using GraphPad-Prism 6.0 (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± s.e.m. $P < 0.05$ was considered statistically significant.

RESULTS

Quantitative changes of MCT expression in striatum, cortex and hippocampus at 1 hour and 24 hours post-ischemia

The protein expression levels of MCT1, MCT2 and MCT4 were evaluated by Western blot in three distinct brain areas both from the ipsilateral and contralateral hemisphere at an

early (1 hour) and a later (24 hours) time point following ischemia and reperfusion. At 1 hour post-ischemia, MCT1 expression was increased in the ischemic mouse brain in all structures analyzed (Figure 1A). Thus, a significant increase was reported both in the ipsilateral (2.73 ± 0.2) and contralateral striatum (2.01 ± 0.4) when compared to sham. Similarly, enhanced levels were observed also in the ipsilateral (2.41 ± 0.4) and contralateral (2.78 ± 0.4) cortex. Interestingly, an increase in MCT1 expression was also observed in more remote regions like the hippocampus, reaching significance for the ipsilateral hemisphere (2.1 ± 0.2). In contrast, significant differences in MCT1 expression were no longer observed at 24 hours in any region examined (Figure 1B). At 1 hour post-ischemia, MCT2 expression was increased in the striatum (2.1 ± 0.1) and the cortex (1.62 ± 0.2) of the ischemic hemisphere compared to the sham. In addition, a significant increase was observed in the cortex (1.76 ± 0.2) and in the hippocampus (1.9 ± 0.1) of the contralateral hemisphere (Figure 1C). On the contrary, at 24 hours post-ischemia, MCT2 expression was reduced in all structures analyzed in both the ipsi- and contralateral hemisphere, reaching significance in the striatum, both in the ipsilateral (0.32 ± 0.1) and contralateral (0.63 ± 0.1) hemispheres, and in the ipsilateral hippocampus (0.63 ± 0.1) (Figure 1D). Finally, MCT4 expression was increased in the ipsilateral hemisphere for both the striatum (1.65 ± 0.1) and the cortex (1.31 ± 0.1) at 1 hour post-ischemia (Figure 1E). In contrast, at 24 hours post-ischemia, the opposite effect was observed for MCT4 expression with a decrease in the ischemic striatum and cortex in comparison to sham (respectively 0.59 ± 0.1 and 0.67 ± 0.1) (Figure 1F).

Cellular changes in MCT expression at 1 hour and 24 hours post-ischemia.

In order to obtain more information about the cellular elements which might exhibit changes in MCT expression, an immunofluorescence characterization was performed. Under basal conditions, MCT1 immunoreactivity had been observed essentially in the neuropil as well as on endothelial cells of capillaries as described previously (Pierre et al. 2000). Here we report the appearance of a strong MCT1 staining on neuronal cell bodies in the striatum (Figure 2B-C), and an even more intense staining in the cortex (Figure 2E-F) of ischemic animals at 1 hour post-ischemia, as compared to the sham hemispheres (Figure 2A and 2D). Blood vessels appear to be more intensely labelled as well in ischemic animals compared to the sham in both structures. Moreover, the overall neuropil staining was clearly enhanced in both areas for both hemispheres. This observation most likely explains the significant increase of MCT1 expression detected by Western blot (Figure 1A). At 24 hours post-ischemia in the striatum, the intensity of the neuropil labelling and the number of neurons labelled decreased

compared to 1 hour post-ischemia (Figure 2H-I). In the cortex, MCT1 expression in the neuropil was reduced compared to 1 hour post-ischemia, especially in the ischemic hemisphere (Figure 2K-L). Interestingly, at 24 hours after surgery, neuronal cell bodies became positive for MCT1 in the sham hemispheres, in the striatum (Figure 2G) and even more in the cortex (Figure 2J) with a small enhancement of the neuropil labelling. Such an induction in the sham might explain the absence of difference observed between the sham and the ischemic animals by western blotting 24 hours post-ischemia (Figure 1B). This observation reveals an effect of the sham condition *per se* (a transient reduction in blood flow due to 30 min CCA ligation) on brain metabolism.

Under basal conditions, MCT2 expression was reported to be abundantly expressed in the neuropil associated with neuronal elements, mainly on processes and spines (Pierre et al. 2002; Bergersen et al. 2005). At 1 hour post-ischemia, while most neuronal cell bodies were barely stained for MCT2 in the sham (Figure 3A and 3D), in the ischemic animals neuronal cell bodies were intensely stained in the striatum and even more in the cortex of both hemispheres (Figure 3B-C and 3E-F, respectively). Moreover, the neuropil staining was also more intense. Interestingly, endothelial cells forming blood vessels exhibited a slight MCT2 expression in ischemic animals (Figure 3B-C and 3E-F), which was not present in brain areas from sham animals (Figure 3A and 3D). These data are consistent with the quantitative measurements obtained by Western blot (Figure 1C). At 24 hours post-ischemia, a strong reduction of the overall MCT2 expression was observed in the ipsilateral hemispheres of ischemic animals (Figure 3I and 3L) in accordance with the Western blot data (Figure 1D). In parallel, MCT2 expression associated with blood vessels was strongly increased in all cases (Figure 3G-L), including in brain areas from sham animals (Figure 3G and 3J). MCT2 expression on microvessel elements was previously reported but was scarce under non-ischemic conditions (Pierre et al. 2000).

MCT4 is normally expressed exclusively on astrocytes (Rafiki et al. 2003). Here we confirmed that MCT4 colocalizes with the astrocytic marker S100β in both brain areas of the sham (Figure 4A and 4D). At 1 hour post-ischemia, the overall MCT4 staining was strongly enhanced in the striatum and cortex of both ipsilateral and contralateral hemispheres (Figure 4B-C and 4E-F) compared to the sham. It was also noticed that some cells which are negative for the S100β staining exhibit MCT4 labelling. A few of them were found in brain areas from sham animals (Figure 4A and 4D), and much more in both the striatum (Figure 4C) and the cortex (Figure 4F) of ischemic hemispheres. At 24 hours, similarly to MCT1 expression, some cells resembling neurons were clearly stained for MCT4 in the striatum of the ischemic

hemisphere (Figure 4I), and in the cortex of both hemispheres, even though the labeling was less intense in the ipsilateral hemisphere (Figure 4K-L). Interestingly, both structures in the sham presented also neuronal-like cell associated MCT4 staining (Figure 4G and 4J), highlighting the likely effect of the sham treatment on brain metabolism. In order to confirm the presence of MCT4 on neurons, double labeling experiments with the neuronal marker NeuN were performed. Indeed, many cells stained for the neuronal marker NeuN in the striatum at 1 hour (Figure 5A-C) and 24 hours (Figure 5G-I) post-ischemia as well as in the cortex at 1 hour (Figure 5D-E) and 24 hours (Figure 5J-L) post-ischemia exhibited MCT4 staining. Results obtained by immunofluorescence suggest that the increased MCT4 expression observed by western blot at 1 hour compared to the sham (Figure 1E) might be attributable to enhanced astrocytic expression, while at 24 hours the lack of difference observed in western blot might be due to the increase in neuronal MCT4 expression in the sham (Figure 1F).

Energetic shortage caused by interruption of oxygen and glucose supply during ischemic stroke leads to metabolic adaptations promoting a switch between oxidative and glycolytic metabolism (Arnberg et al. 2015). Such phenotypic changes lead first to the production and the accumulation in the extracellular space of large amounts of lactate (Bruhn et al. 1989; Jones et al. 2000; Thoren et al. 2006). In this context, lactate transport through monocarboxylate transporters might play an important role in the new metabolic processes that take place during and following ischemia. Indeed, it has been shown that inhibition of lactate transport by acting on MCTs increases neuronal damage in different rat models of cerebral ischemia (Schurr et al. 2001; Wang et al. 2011). Thus, an analysis of cerebral MCT expression pattern following ischemia is essential to understand the metabolic reprogramming occurring in the brain upon reperfusion, and also to explain the reported protective role of lactate under such a condition (Berthet et al. 2009; 2012). So far, only few studies have investigated the cell-specific changes in the expression of MCTs occurring in different rat models of ischemia (Tseng et al. 2003; Zhang et al. 2005; Moreira et al. 2009; Geng et al. 2015). However, none of those studies thoroughly examined the cell-specific evolution in the expression of the three MCT isoforms at the protein level after ischemia. In our study, not only global changes of expression for all three MCT isoforms were observed, but the cell-

specific pattern of expression was found to be modified following ischemia. The cell-specific changes observed have been summarized in a schematic form in Figure 6.

Modification of neuronal MCT expression following transient MCAO and reperfusion

Under normal conditions, neurons strongly express the high affinity transporter MCT2 (Figure 6A; Pierre et al. 2000; Pierre et al. 2002). Pharmacological inhibition of MCT2 was found to be deleterious in neuronal cultures exposed to OGD (An et al. 2014), but also in MCAO in rat, while MCT2 overexpression led to a reduced infarct size (Wang et al. 2011). Although the authors showed the importance of MCT2 activity for protection from ischemia, they did not assess in that study the impact of MCAO itself on MCT2 expression. Our data show that MCT2 expression is increased at 1 hour post-ischemia in neurons of all brain areas analyzed, in the ipsilateral and contralateral hemispheres. Such an early enhancement in MCT2 expression on neurons is consistent with the fact that MCT2 expression is regulated primarily at the translational level in neurons, allowing for a rapid protein synthesis following stimulation (Chenal and Pellerin 2007; Chenal et al. 2009; Robinet and Pellerin 2010). This response might offer a first mechanism to preserve neurons from the consequences of an ischemic insult such as excitotoxicity (Lai et al. 2014). Indeed, it was shown that overexpression of MCT2 in neurons together with lactate availability protects them from an excitotoxic insult *in vitro* (Bliss et al. 2004). Interestingly, in addition to enhanced MCT2 expression, appearance of MCT1 on neurons was observed at this time point (Figure 6B). Neuronal MCT1 immunoreactivity is not detected under normal physiological conditions in the mouse brain (Pierre et al. 2000; Pierre et al. 2002), except in some rare neuronal populations (Lee et al. 2012). However following ischemia, neuronal MCT1 expression was reported in the rat hippocampus (Tseng et al. 2003) and in the hypertensive rat cortex (Zhang et al. 2005). If we consider that postischemic glucose utilization is depressed between 1 and 48 hours in the neocortex and the striatum (Pulsinelli et al. 1982; Thoren et al. 2006), and that despite this fact, the ATP/ADP ratio and phosphocreatine content are essentially preserved during reperfusion (Thoren et al. 2006), it seems likely that neurons modify their metabolism by increasing MCT2 expression and by inducing MCT1 expression in order to favor lactate utilization while sparing glucose during reperfusion, at least in the first hours post-ischemia. Since glucose utilization is depressed, the increase in MCT1 and MCT2 expression observed in our study is unlikely to be related to a hypothetical increased glycolysis, but rather that the most likely purpose of their induction would be to allow neuronal lactate uptake and oxidation with reperfusion rather than release. Since the affinity of MCT1 for lactate is lower than

MCT2, it would make it ideal for the transport of lactate inside neurons when extracellular lactate concentrations are elevated like following ischemia. Moreover, several studies are supporting this idea. In fact, *in vitro* experiments showed that ischemic neurons enhance their ability to utilize lactate as an energy substrate and that lactate, but not glucose, is necessary for the recovery of synaptic function from hypoxia (Schurr et al. 1997a; Schurr et al. 1997b; Kitano et al. 2002). Thus, both increased MCT1 and MCT2 expression observed at 1 hour post-ischemia may reflect a first metabolic reprogramming to the post-ischemic environment (i.e. with reperfusion), in which neurons might oxidize more lactate to sustain their energy needs instead of glucose.

At 24 hours post-ischemia, while neurons were still strongly expressing MCT1, we observed a general decrease in MCT2 expression, in both the ipsilateral and contralateral hemispheres, as highlighted by the difference in band intensities at 24 hours compared to 1 hour post-ischemia. Such a phenomenon was also recently observed in another model of hypoxic ischemia in young mice (Zovein et al. 2004). Although neuronal cell death might contribute to this reduced expression in the ischemic hemisphere, it would not explain the decrease in the contralateral hemisphere, thus arguing for a specific decrease in expression (Figure 6C). Interestingly, at this later time point, MCT4 expression was observed on the somata of dying neurons in the lesion (striatum), but also on healthy neurons of the surrounding and more distant structures (cortex and hippocampus). While only a few of them were expressing MCT4 at 1 hour post-ischemia, at 24 hours post-ischemia most of them displayed such expression (Figure 6C). Up to now, MCT4 was considered as an exclusive astrocytic transporter under physiological conditions (Bergersen et al. 2002; Rafiki et al. 2003; Pellerin et al. 2005). But similarly to MCT1 expression, appearance of MCT4 on neurons was reported previously in the context of obesity, in ob/ob mice and in high fat diet-induced obese mice, as well as in db/db diabetic mice (Pierre et al. 2007). It seems likely that MCT1, and later on MCT4, might play an important role for neurons to achieve a new metabolic state following ischemia. Since MCT4 expression was shown to be under the control of the transcriptional factor HIF-1α (Ullah et al. 2006), it can be suggested that neuronal MCT4 expression could be part of a neuroprotective mechanism activated by HIF-1α, as previously demonstrated through a pharmacological stabilization of HIF-1α in both rat and mouse ischemic stroke models, for which the authors showed a reduction in the infarct volume and improved behavior (Nagel et al. 2011; Reischl et al. 2014). Strenghtening this point, loss of HIF-1α function in neurons was shown to increase their susceptibility to hypoxia-induced death both

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in vitro and *in vivo* (Baranova et al. 2007; Vangeison et al. 2008). Of note, an induction of MCT1 and MCT4 expression in neurons was observed also in the sham hemisphere at 24 hours post-ischemia. If we consider that due to the common carotid ligation, cerebral blood flow (CBF) is reduced in the sham condition between 80 to 50% of the initial measurement and that in our experimental model we consider true ischemia when it drops below 20%, it can be suggested that a moderate and rather short duration reduction in oxygen and metabolite supply is sufficient to influence some metabolic characteristics of neurons.

The particular pattern of MCT isoform expression in neurons reported here seems to reflect a complicated metabolic reprogramming that should allow them to face the metabolic challenges imposed on them following ischemia. However, such important processes need to be further investigated. Indeed, it is unknown if MCT1, and especially later on, MCT4 expression, contribute to enhanced lactate uptake and utilization by these cells, or rather if it would be associated with an hyperglycolytic neuronal metabolism at this stage of reperfusion after MCAO. Indeed, oxidative metabolism was generally decreased following stroke (Sims and Muyderman 2010) and in humans, glucose metabolism was found to be increased in the contralateral hemisphere (Mountz et al. 2003). Thus, it could be that a certain proportion of neurons progressively switch to a hyperglycolytic metabolism when glucose supply is restored, which could explain the large accumulation of lactate observed up to several days following cerebral ischemia (Rehncrona et al. 1981; Folbergrova et al. 1992; Wagner et al. 1992)

Modification of astrocytic MCT expression following transient MCAO and reperfusion

Under normal conditions, astrocytes express MCT1 (Gerhart et al. 1997; Hanu et al. 2000; Pierre et al. 2000) and MCT4 (Rafiki et al. 2003; Pierre and Pellerin 2005; Rosafio and Pellerin 2014) (Figure 6A). Recently, it was demonstrated that oxygen levels regulate MCT4 expression in primary cultures of mouse cortical astrocytes (Rosafio and Pellerin 2014). Our observation that MCT4 expression on astrocytes in the ipsilateral hemisphere increased at 1 hour post-ischemia and then comes back to pre-ischemic levels at 24 hours post-ischemia would be consistent with an oxygen-dependent regulation of MCT4 transcription. Indeed, at this later time point, re-oxygenation would display its effect and might downregulate MCT4 expression in astrocytes by inhibiting the HIF-1α-dependent pathway. It is of interest to note that the neuroprotective effect produced by pharmacological stabilization of HIF-1α observed in ischemic stroke models of both rat and mouse (Nagel et al. 2011; Reischl et al. 2014) could be related also to the initial induction of astrocytic MCT4 expression. Thus, metabolic

reprogramming in astrocytes is essentially taking place in the first hours following ischemia, and might contribute to protect neurons by allowing prominent lactate release that can be used by neurons as an alternative energy fuel upon reperfusion.

Modification of microvessel-associated MCT expression following transient MCAO and reperfusion

As a key element of the "tripartite synapse" (Perez-Alvarez and Araque 2013), microvessels play also an important role in brain metabolism. Due to the blood-brain barrier disruption occurring with stroke, cerebral blood vessels are strongly affected in such a condition (Moskowitz et al. 2010). In a paradigm of ischemia and reperfusion, it seems reasonable to assume that the metabolism of cells composing the microvasculature will be altered as well. In this study, it was found that microvessels undergo a strong enhancement of their MCT1 expression in mice at 1 hour and at 24 hours post-ischemia. A similar observation was previously reported in a rat model of global ischemia (Zhang et al. 2005). In addition, at 24 hours post-ischemia, a strong MCT2 expression appeared on microvessels, an observation that was never reported in ischemia up to now, although such a peculiar expression was previously reported on several microvessel-associated elements under physiological conditions (Pierre et al. 2000). The specific cellular localization of MCT2 expression associated with microvessels will need to be further confirmed in order to eventually understand its precise role. Nevertheless, in view of these observations, it can be concluded that microvessel-associated elements seem to participate also to the metabolic reprogramming taking place in response to ischemia.

Metabolic reprogramming on the contralateral side of the lesion

It is inferred that modifications in MCT expression occurring after an ischemic event are part of a large metabolic reprogramming that affect not only all cell types within the lesion, but also around it and in more remote regions. Indeed, modified MCT expression was also observed in the contralateral hemisphere, suggesting a metabolic reprogramming even in the unlesioned side of the brain. Previously, effects on MCT expression in the contralateral side were observed in a model of traumatic brain injury (Moreira et al. 2009), while increased glucose metabolism was observed in the contralateral cortical area after stroke in humans (Mountz et al. 2003). Moreover, we observed that expression of MCTs was modified also in more remote structures such as the hippocampus. Effects on remote areas caused by a focal lesion have been well documented in several ischemia models and can affect both mRNA and

protein expression (Witte et al. 2000). Other factors such as spreading depression and motor compensation during the recovery period might also contribute to the remote alterations observed both in the ipsilateral and contralateral hemispheres.

CONCLUSIONS

Significant changes in the expression of MCTs take place in the ischemic brain at different time points of recovery following the ischemic event (see Figure 6 for the overview), supporting the concept of metabolic reprogramming occurring in various cell types. In particular, the unexpected neuronal expression of the MCT1 and MCT4 isoforms, and the expression of MCT2 on the microvasculature will deserve to be further investigated to understand their exact role. Notwithstanding, these events appear to concur to promote recovery from the ischemic insult and favor adaptation to a new environment. Thus, efforts to promote the expression and efficiency of monocarboxylate transporters might well represent a new therapeutic avenue to try to reduce the adverse outcomes of stroke in patients.

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FIGURE LEGENDS

Figure 1. Quantitative immunoblot analysis of MCT1, MCT2 and MCT4 expression in the ipsi- and contralateral striatum, cortex, and hippocampus following 30 minutes MCAO and reperfusion in mice. (A) MCT1 expression at 1 hour of reperfusion following MCAO in both the ipsilateral and contralateral hemisphere *vs.* sham. (B) MCT1 expression at 24 hours of reperfusion following MCAO in both the ipsilateral and contralateral hemisphere *vs.* sham. (C) MCT2 expression at 1 hour of reperfusion following MCAO in both the ipsilateral and contralateral hemisphere *vs.* sham. (D) MCT2 expression at 24 hours of reperfusion following MCAO in both the ipsilateral and contralateral hemisphere *vs.* sham. (E) MCT4 expression at 1 hour of reperfusion following MCAO in both the ipsilateral and contralateral hemisphere *vs.* sham. (F) MCT4 expression at 24 hours of reperfusion following MCAO in both the ipsilateral and contralateral hemisphere *vs.* sham. Values of MCT expression were normalized to the total protein content. An ANOVA followed by Dunnett's multiple comparison test was performed for statistical analysis. (*P <0.05, **P < 0.01 and ***P < 0.001). For sham n = 3; For MCAO at 1 hour n = 5; For MCAO at 24 hours n = 7.

Figure 2. Immunocytochemical analysis of MCT1 expression in the ipsi- and contralateral striatum and cortex following 30 minutes MCAO and reperfusion in mice. (A-C) MCT1 expression (in red) in the striatum of sham animals and in the striatum of the ipsilateral and contralateral hemispheres of mice at 1 hour of reperfusion following MCAO. *Inset*, Double immunolabeling on the same section with the neuronal marker NeuN (in green). (D-F) MCT1 expression (in red) in the cortex of sham animals and in the cortex of the ipsilateral and contralateral hemispheres of mice at 1 hour of reperfusion following MCAO. *Inset*, Double immunolabeling on the same section with the neuronal marker NeuN (in green). (G-I) MCT1 expression (in red) in the striatum of sham animals and in the striatum of the ipsilateral and contralateral hemispheres of mice at 24 hours of reperfusion following MCAO. *Inset*, Double immunolabeling on the same section with the neuronal marker NeuN (in green). (J-L) MCT1 expression (in red) in the cortex of sham animals and in the cortex of the ipsilateral and contralateral hemispheres of mice at 24 hours of reperfusion following MCAO. *Inset*, Double immunolabeling on the same section with the neuronal marker NeuN (in green). Nuclei are labeled with DAPI (in blue). White arrows on image and in inset indicate examples of colocalization between MCT1 (in red) and the neuronal marker NeuN (in green) present in

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neuronal nuclei. White arrowheads (>) indicate blood vessels expressing MCT1 (in red). Scale bar = 40 μM. For sham n = 2; For MCAO at 1 hour n = 2; For MCAO at 24 hours n = 3. For each animal, 5-10 sections were examined for each structure.

Figure 3. Immunocytochemical analysis of MCT2 expression in the ipsi- and contralateral striatum and cortex following 30 minutes MCAO and reperfusion in mice. (A-C) MCT2 expression (in red) in the striatum of sham animals and in the striatum of the ipsilateral and contralateral hemispheres of mice at 1 hour of reperfusion following MCAO. *Inset*, Double immunolabeling on the same section with the neuronal marker MAP2 (in green). (D-F) MCT2 expression (in red) in the cortex of sham animals and in the cortex of the ipsilateral and contralateral hemispheres of mice at 1 hour of reperfusion following MCAO. *Inset*, Double immunolabeling on the same section with the neuronal marker MAP2 (in green). (G-I) MCT2 expression (in red) in the striatum of sham animals and in the striatum of the ipsilateral and contralateral hemispheres of mice at 24 hours of reperfusion following MCAO. *Inset*, Double immunolabeling on the same section with the neuronal marker MAP2 (in green). (J-L) MCT2 expression (in red) in the cortex of sham animals and in the cortex of the ipsilateral and contralateral hemispheres of mice at 24 hours of reperfusion following MCAO. *Inset*, Double immunolabeling on the same section with the neuronal marker MAP2 (in green). Nuclei are labeled with DAPI (in blue). White arrows on image and in inset indicate examples of colocalization between the neuronal marker MAP2 (in green) and MCT2 (in red). White arrowheads (>) on image or inset indicate blood vessels, in some cases expressing MCT2 (in red). Scale bar = 40 μM. For sham n = 2; For MCAO at 1 hour n = 2; For MCAO at 24 hours n = 3. For each animal, 5-10 sections were examined for each structure.
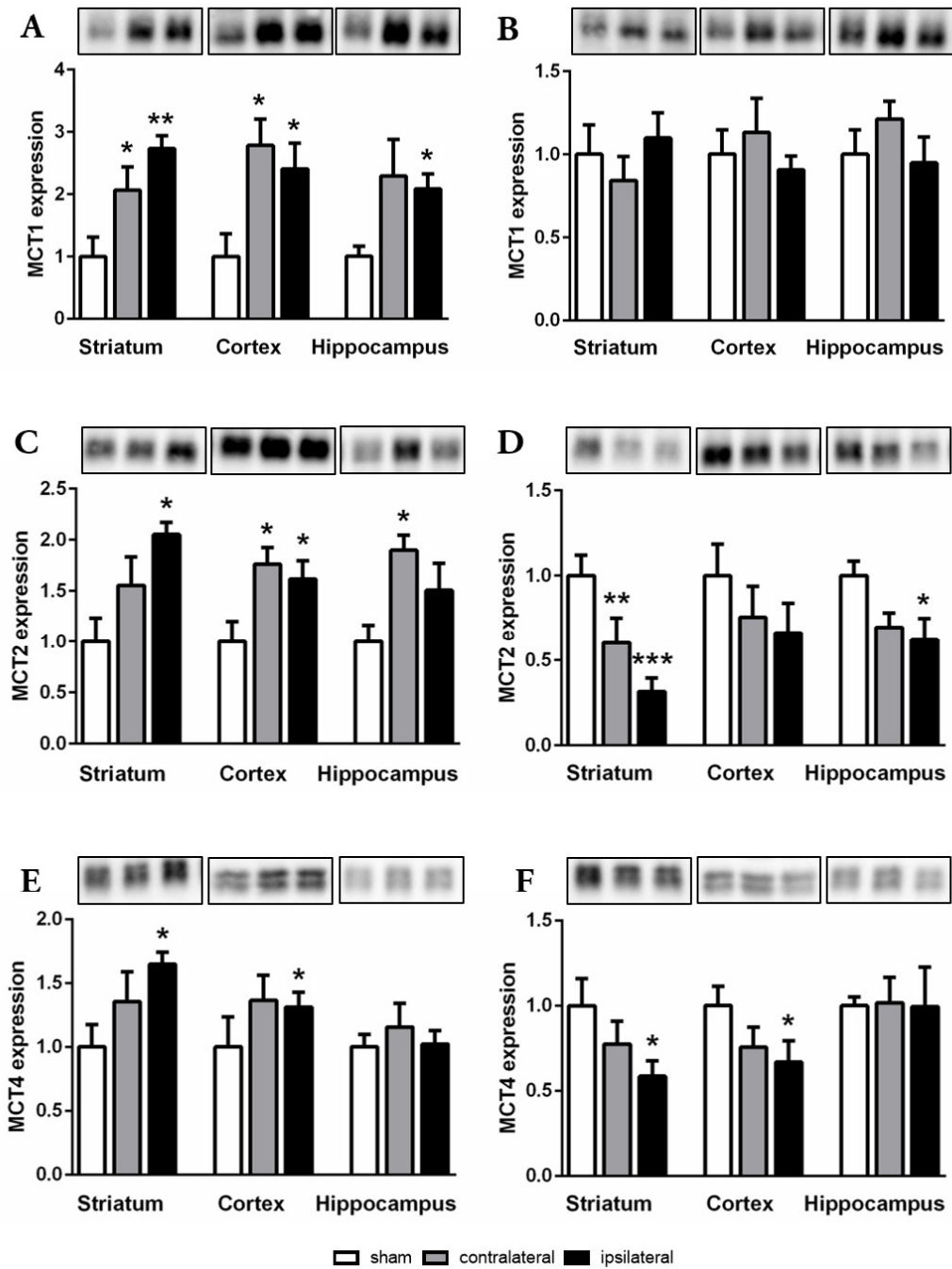
Figure 4. Immunocytochemical analysis of astrocytic MCT4 expression in the ipsi- and contralateral striatum and cortex following 30 minutes MCAO and reperfusion in mice. (A-C) MCT4 expression (in red) in the striatum of sham animals and in the striatum of the ipsilateral and contralateral hemispheres of mice at 1 hour of reperfusion following MCAO. *Inset*, Double immunolabeling on the same section with the astrocytic marker S100β (in green). (D-F) MCT4 expression (in red) in the cortex of sham animals and in the cortex of the ipsilateral and contralateral hemispheres of mice at 1 hour of reperfusion following MCAO. *Inset*, Double immunolabeling on the same section with the astrocytic marker S100β (in green). (G-I) MCT4 expression (in red) in the striatum of sham animals and in the striatum of the
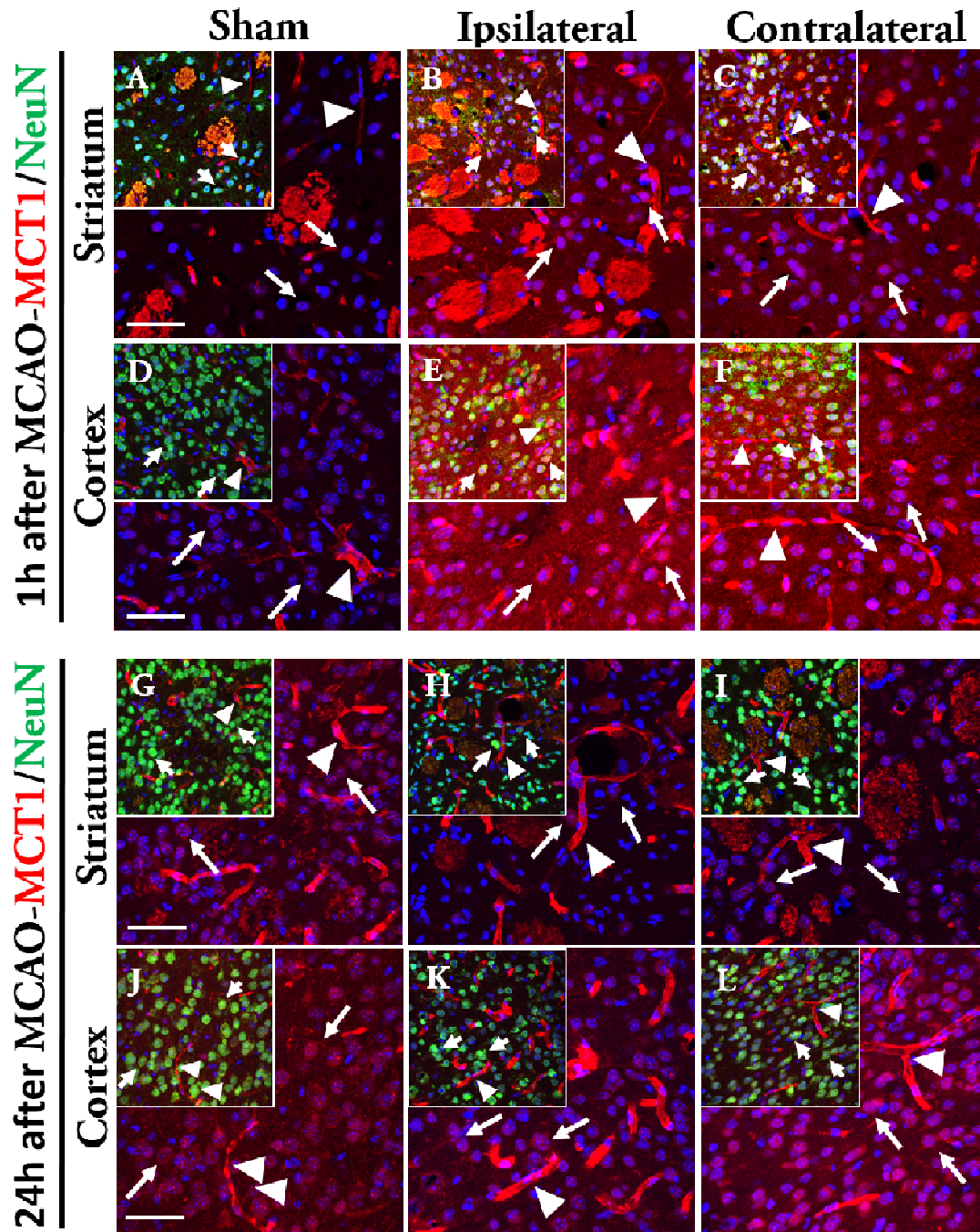
ipsilateral and contralateral hemispheres of mice at 24 hours of reperfusion following MCAO. *Inset*, Double immunolabeling on the same section with the astrocytic marker S100β (in green). (J-L) MCT4 expression (in red) in the cortex of sham animals and in the cortex of the ipsilateral and contralateral hemispheres of mice at 24 hours of reperfusion following MCAO. *Inset*, Double immunolabeling on the same section with the astrocytic marker S100β (in green). Nuclei are labeled with DAPI (in blue). White arrows on image and in inset indicate examples of colocalization between the astrocytic marker S100β (in green) and MCT4 (in red). For sham n = 2; For MCAO at 1 hour n = 2; For MCAO at 24 hours n = 3. For each animal, 5-10 sections were examined for each structure.
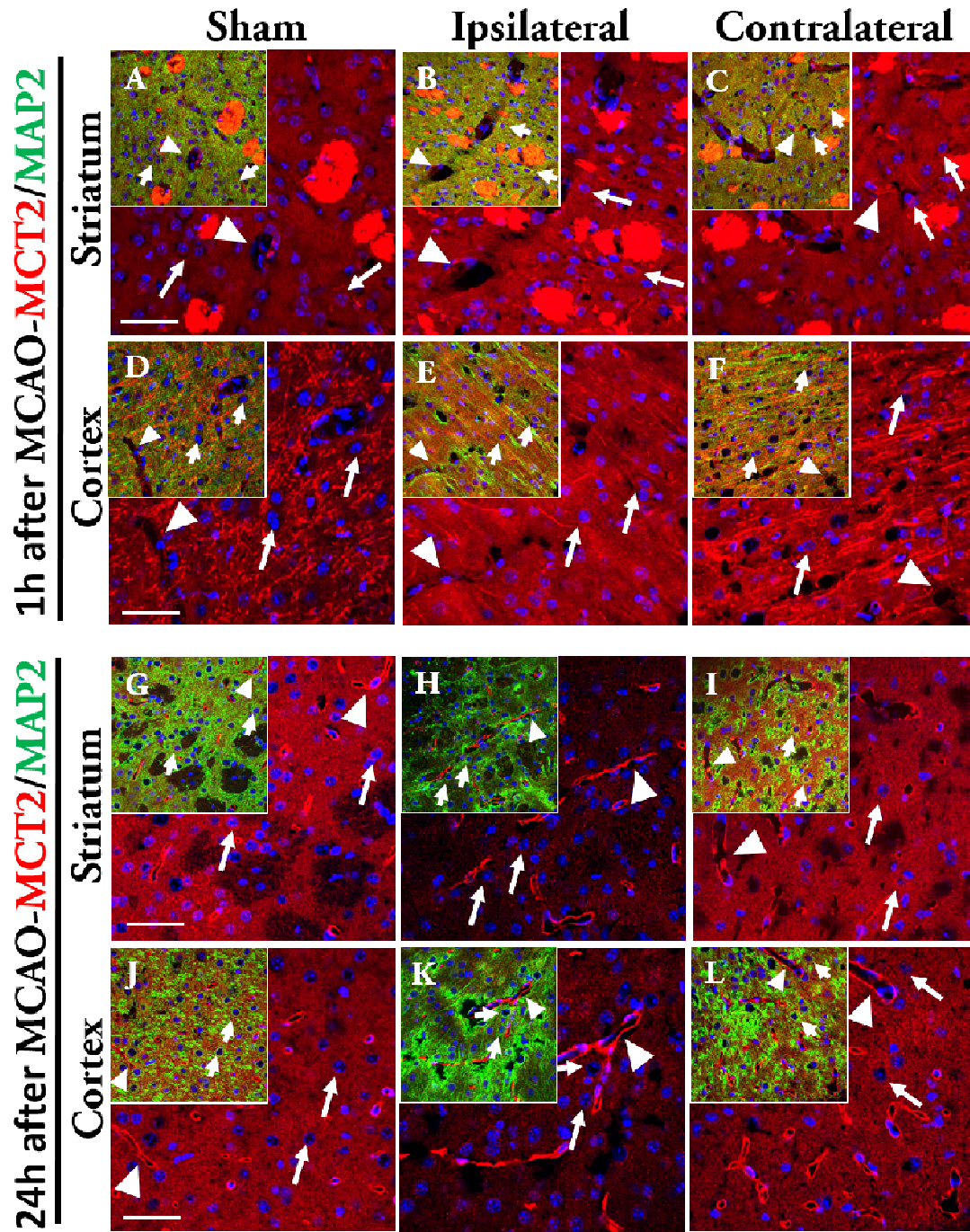
Figure 5. Immunocytochemical analysis of neuronal MCT4 expression in the ipsi- and contralateral striatum and cortex following 30 minutes MCAO and reperfusion in mice. (A-C) MCT4 expression (in red) in the striatum of sham animals and in the striatum of the ipsilateral and contralateral hemispheres of mice at 1 hour of reperfusion following MCAO. *Inset*, Double immunolabeling on the same section with the neuronal marker NeuN (in green). (D-F) MCT4 expression (in red) in the cortex of sham animals and in the cortex of the ipsilateral and contralateral hemispheres of mice at 1 hour of reperfusion following MCAO. *Inset*, Double immunolabeling on the same section with the neuronal marker NeuN (in green). (G-I) MCT4 expression (in red) in the striatum of sham animals and in the striatum of the ipsilateral and contralateral hemispheres of mice at 24 hours of reperfusion following MCAO. *Inset*, Double immunolabeling on the same section with the neuronal marker NeuN (in green). (J-L) MCT4 expression (in red) in the cortex of sham animals and in the cortex of the ipsilateral and contralateral hemispheres of mice at 24 hours of reperfusion following MCAO. *Inset*, Double immunolabeling on the same section with the neuronal marker NeuN (in green). Nuclei are labeled with DAPI (in blue). White full arrows on image and in inset indicate examples of colocalization between MCT4 (in red) and the neuronal marker NeuN (in green) present in neuronal nuclei. White empty arrows indicate NeuN positive neurons (in green) which do not express MCT4 on their cell body. Scale bar = 40 μM. For sham n = 2; For MCAO at 1 hour n = 2; For MCAO at 24 hours n = 3. For each animal, 5-10 sections were examined for each structure.
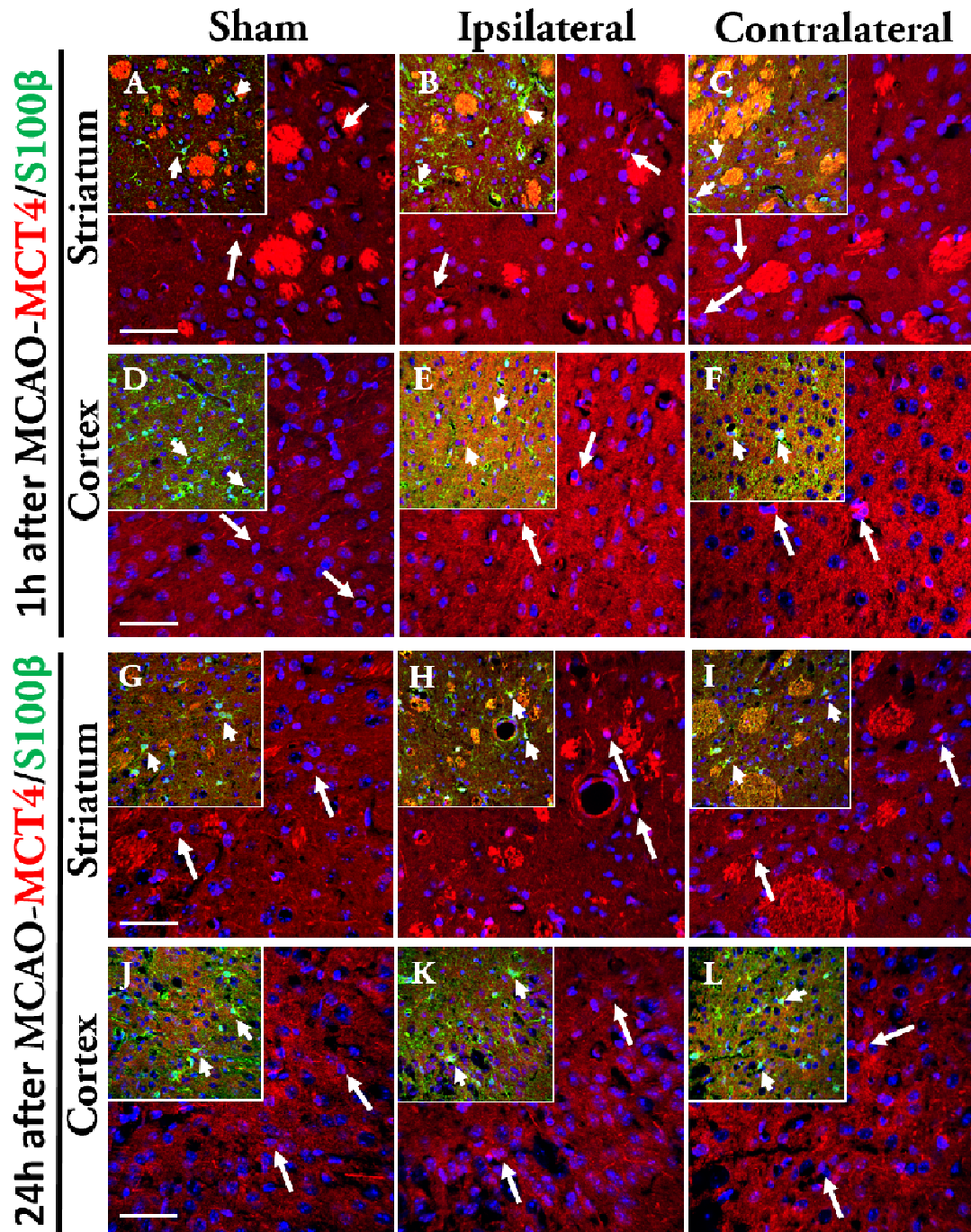
Figure 6. Schematic representations of the major cell-specific changes in MCT expression following ischemia and reperfusion in mice. (A) Under normal conditions, neurons essentially
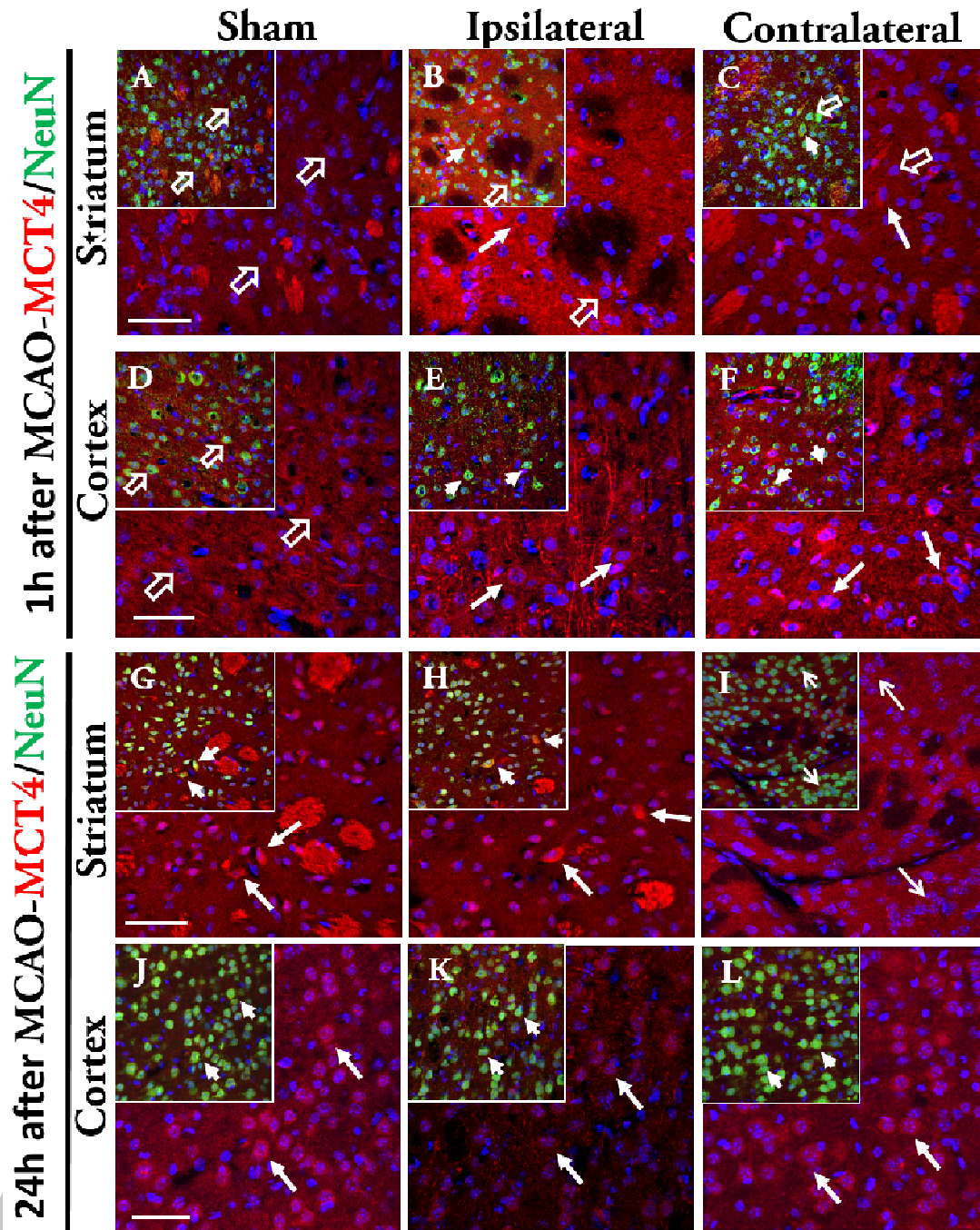
express MCT2, astrocytes exhibit both MCT1 and MCT4 expression while endothelial cells forming blood vessels display MCT1 expression. (B) At 1 hour of reperfusion following the ischemic episode, a strong enhancement of MCT1 expression by blood vessels and of MCT4 expression by astrocytes is observed. In parallel, neurons not only display enhanced MCT2 expression, but they also start to exhibit MCT1 expression. (C) At 24 hours of reperfusion following the ischemic episode, MCT4 expression on astrocytes returns to basal levels. On blood vessels, MCT1 expression remains elevated but MCT2 expression become visible. Finally, while MCT1 and MCT4 expression on neurons is still enhanced, neuronal MCT2 expression decreases below basal levels.
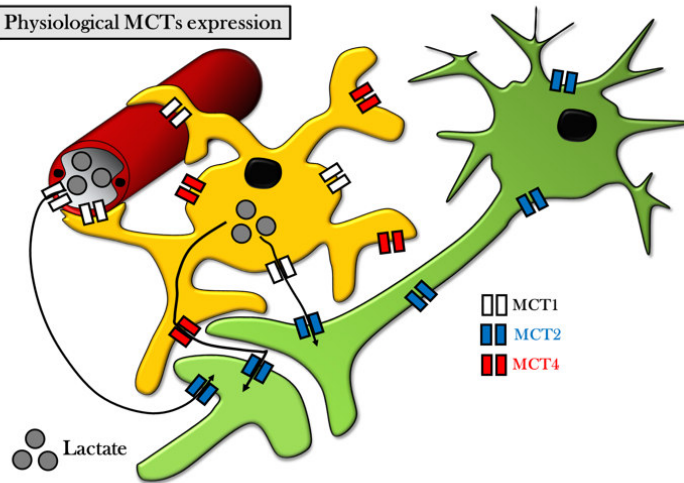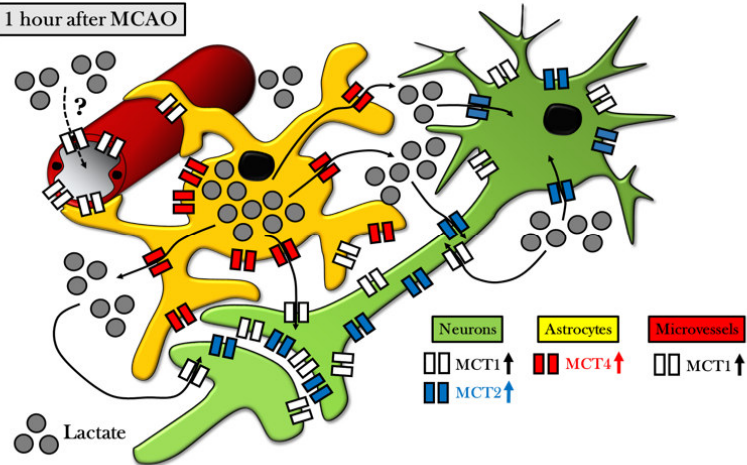
Physiological MCTs expression

▯▯ MCT1
▮▮ MCT2
▮▮ MCT4

Lactate

1 hour after MCAO

Neurons	Astrocytes	Microvessels
▯▯ MCT1↑	▮▮ MCT4↑	▯▯ MCT1↑
▮▮ MCT2↑		

Lactate

24 hours after MCAO

Neurons	Microvessels
▯▯ MCT1↑	▯▯ MCT1↑
▮▮ MCT2↓	▮▮ MCT2↑
▮▮ MCT4↑	

Lactate

Physiological MCTs expression

□□ MCT1
■■ MCT2
■■ MCT4

Lactate

1 hour after MCAO

Neurons	Astrocytes	Microvessels
□□ MCT1 ↑	■■ MCT4 ↑	□□ MCT1 ↑
■■ MCT2 ↑		

Lactate

24 hours after MCAO

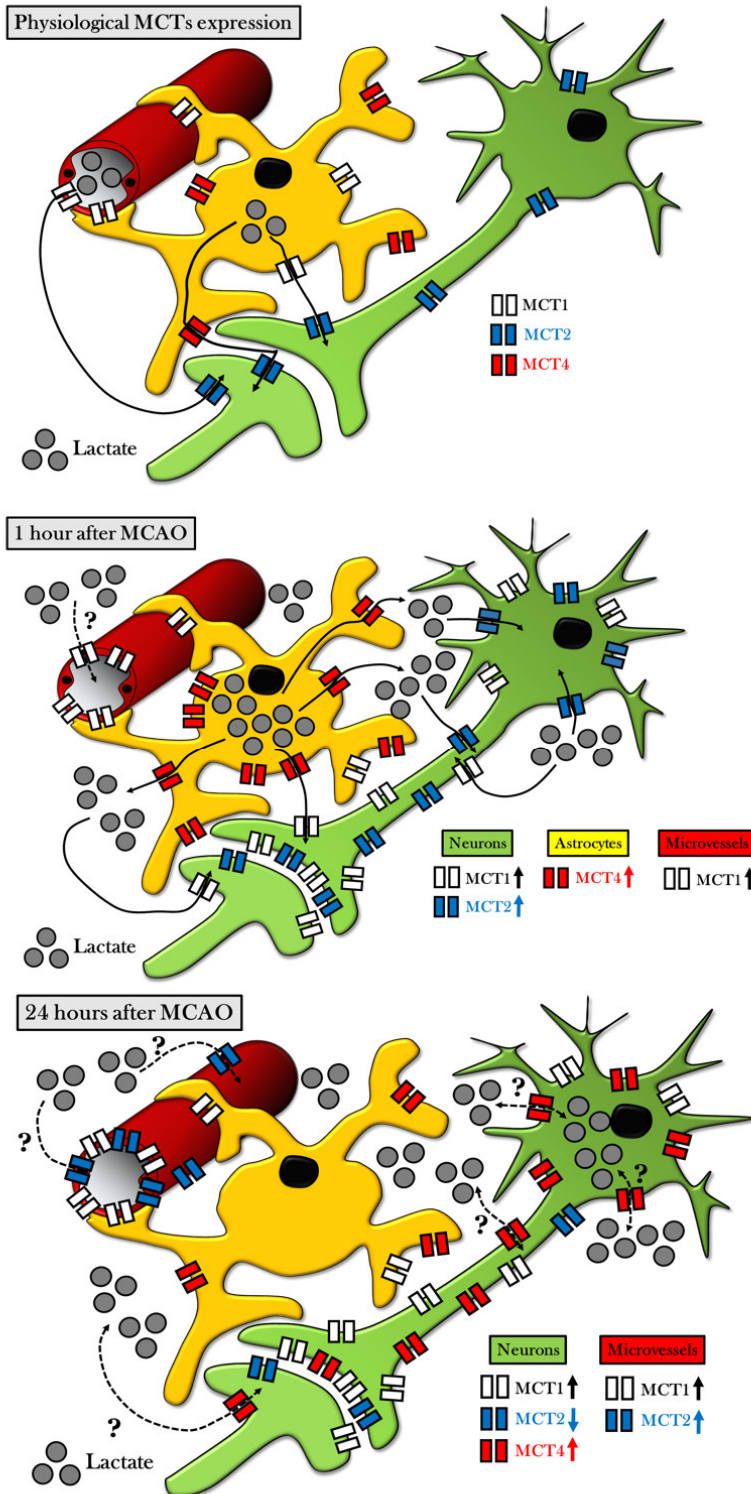| Neurons	Microvessels
□□ MCT1 ↑	□□ MCT1 ↑
■■ MCT2 ↓	■■ MCT2 ↑
■■ MCT4 ↑	

Lactate
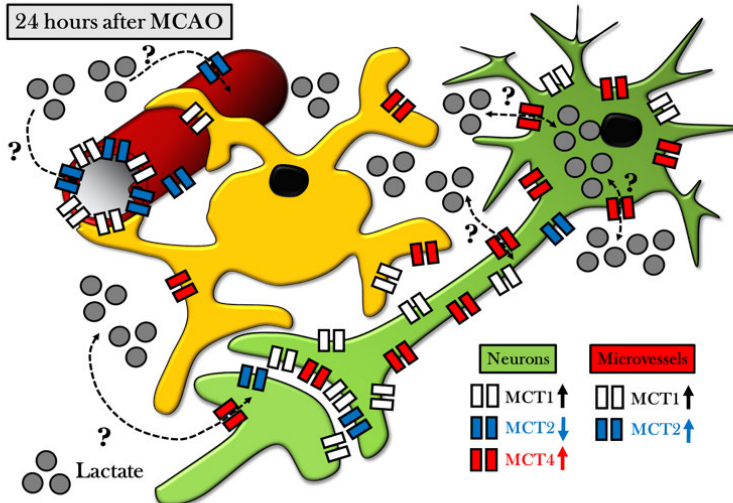
Highlights

- After 1 hour, MCT1, MCT2 and MCT4 expressions were upregulated in the ipsilateral and contralateral striatum and cortex

- Neuronal MCT2 but also MCT1 expression, astrocytic MCT4 expression and microvessel MCT1 expression all increased at 1 hour

- After 24 hours, overall MCT2 and MCT4 expressions decreased in the ipsilateral and contralateral striatum and cortex

- Neuronal MCT2 expression was reduced but MCT4 expression appeared while microvessels exhibited MCT2 expression at 24 hours