Enhanced expression of three monocarboxylate transporter isoforms in the brain of obese mice

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Monocarboxylate transporters (MCTs) are membrane carriers for lactate and ketone bodies. Three isoforms, MCT1, MCT2 and MCT4, have been described in the central nervous system but little information is available about the regulation of their expression in relation to altered metabolic and/or nutritional conditions. We show here that brains of mice fed on a high fat diet (HFD) up to 12 weeks as well as brains of genetically obese (*ob*/*ob*) or diabetic (*db*/*db*) mice exhibit an increase of MCT1, MCT2 and MCT4 expression as compared to brains of control mice fed a standard diet. Enhanced expression of each transporter was visible throughout the brain but most prominently in the cortex and in the hippocampus. Using immunohistochemistry, we observed that neurons (expressing mainly MCT2 but also sometimes low levels of MCT1 under normal conditions) were immunolabelled for all three transporters in HFD mice as well as in *ob*/*ob* and *db*/*db* mice. At the subcellular level, changes were most remarkable in neuronal cell bodies. Western blotting performed on brain structure extracts allowed us to confirm quantitatively the enhancement of MCT1 and MCT2 expression. Our data demonstrate that the expression of cerebral MCT isoforms can be modulated by alterations of peripheral metabolism, suggesting that the adult brain is sensitive and adapts to new metabolic states. This observation could be relevant in the context of obesity development and its consequences for brain function.

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Some nutritional habits can lead to metabolic alterations that certainly have a critical influence not only on health in general but more specifically on brain function. This notion is supported by recent observations implying that the typical diet of Western countries nowadays, rich in sugar and saturated fat, and the associated metabolic consequences for the organism may contribute to cognitive decline in ageing and accelerate the course of dementia in Alzheimer's disease (Kalmijn, 2000). Interestingly, cognitive impairments associated with age and neurodegenerative diseases are correlated with specific reductions in brain metabolism that often precede the first symptoms (De Leon *et al.* 2001; Mosconi, 2005; Montoya *et al.* 2006). As a consequence, studying the physiological impact of altered metabolic states (e.g. obesity) caused notably by specific diet compositions on brain processes is becoming of prime interest.

Glucose is classically considered the main energy substrate of the brain (Sokoloff, 1989). In recent years, however, evidence has been provided for the notion that the brain can make use of alternative energy substrates under specific circumstances. For example, fatty acids provided by the diet readily cross the blood–brain barrier (BBB) and can contribute significantly to fulfil brain energy needs (Ebert *et al.* 2003). In addition to their direct utilization by the brain, fatty acids are converted in the liver to ketone bodies, i.e. β-hydroxybutyrate and acetoacetate, that can be also subsequently oxidized by brain cells. Under normal circumstances, the concentration of ketone bodies in the circulation is low and their utilization by the adult brain is considered of little physiological significance. But it can be reactivated during prolonged ketosis, diabetes and fasting (Cremer, 1982; Hawkins *et al.* 1986). Ketone bodies may thus become an important source of energy for the brain, depending of their blood concentration (for review, see Morris, 2005). Lactate is an additional energy substrate known to be used preferentially by neurons (Bouzier-Sore *et al.* 2003; Itoh *et al.* 2003). In addition to its formation from glucose within the brain parenchyma, notably by astrocytes (Pellerin, 2003), it was shown that elevation of blood lactate, as occurs during moderate exercise, is not only sufficient to promote its use by the brain (Dalsgaard *et al.* 2004) but it also reduces in parallel cerebral glucose consumption (Smith *et al.* 2003). Although the brain has

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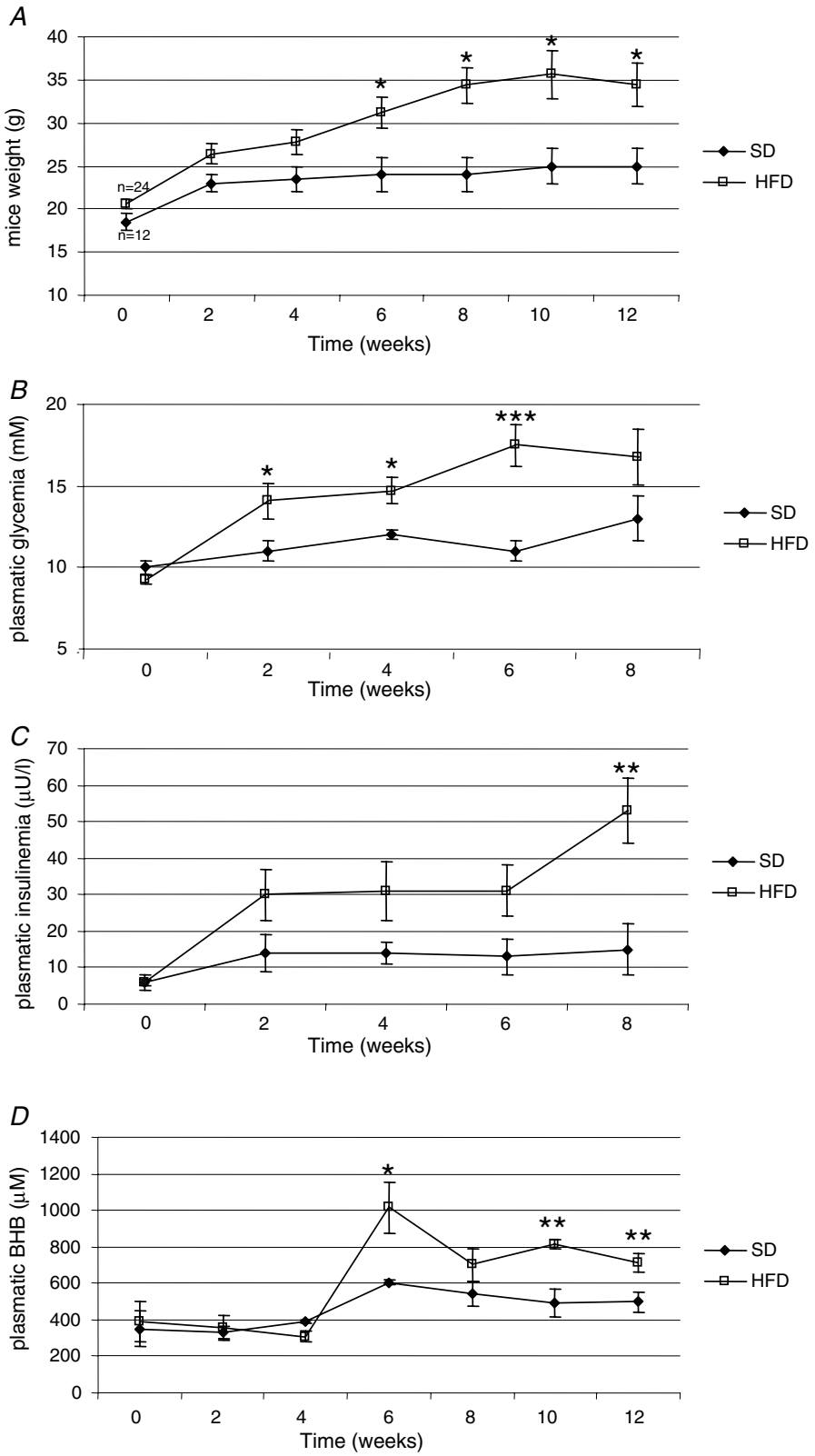


Figure 1. Weight gain as well as plasmatic glucose, insulin and β-hydroxybutyrate levels in mice fed with either a standard diet (SD) or a high fat diet (HFD)

A, mice of each group ($n = 12$ for the SD group and $n = 24$ for the HFD group) were weighed every 2 weeks. Two and four mice of the SD and HFD group, respectively, were killed every 2 weeks from 2 to 12 weeks of standard or high fat diet. Statistically significant differences in the weight of the HFD group relative to baseline values of the

the capacity to use alternative energy substrates in addition to glucose under particular circumstances, it is unknown whether an altered metabolic state (e.g. obesity) can lead to long-lasting brain adaptations that would pertain to both neuroenergetics and brain function.

Monocarboxylate transporters (MCTs) form a large family of proton-linked carriers that have the ability to transport lactate, pyruvate and the ketone bodies (Halestrap & Meredith, 2004; Pierre & Pellerin, 2005). In the central nervous system, three MCTs have been identified and their distribution determined at the cellular level. MCT1 is mostly expressed by endothelial cells and astrocytic processes in rodents (Gerhart *et al.* 1997; Pellerin *et al.* 1998; Leino *et al.* 1999; Hanu *et al.* 2000; Pierre *et al.* 2000; Baud *et al.* 2003) and in humans (Froberg *et al.* 2001; Chiry *et al.* 2006). MCT2 is the major neuronal transporter in the rodent brain (Pierre *et al.* 2002) while MCT4 is exclusively expressed in astrocytes (Bergersen *et al.* 2001; Rafiki *et al.* 2003; Pellerin *et al.* 2005). So far, there are few data reporting possible regulations of MCT expression in the adult brain under specific nutritional manipulations and none in relation to obesity.

In this study, we investigated the effect of a high fat diet (HFD), given up to 12 weeks, on levels of MCT1, MCT2 and MCT4 protein expression in the adult mouse brain. Moreover, we performed a parallel investigation using brains of mutant mice presenting a phenotype exhibiting common features (hyperinsulinaemia, hyperglycaemia, obesity) with mice fed a HFD. Our data reveal an up-regulation of all three monocarboxylate transporter isoforms in neurons from distinct brain areas compared to control mice fed with a standard diet. Such results suggest that the adult brain responds to peripheral metabolic changes with long-lasting adaptations.

Methods

Animals

Six-week-old C57Bl6-Rj male mice were obtained from the Janvier Breeding Centre (Le Genest-Saint-Isle, France) and received 1 week prior to the experiments to acclimatize them to the animal facility. They were housed in a temperature-controlled environment with a 12 : 12 h light–dark cycle and given free access to food and water. Animals were then placed randomly upon either a standard diet (SD, Provimi Kliba, Penthalaz, Switzerland. diet no. 34360.13, proteins 28%, carbohydrates 60% and fat 12%) or a fat-enriched diet (HFD, Harlan Teckland, Oxon, UK. Diet no. TD 93075, proteins 21%, carbohydrates 24%, fat 55%) during 2–12 weeks. Mice were weighed every 2 weeks and groups were killed after 2, 4, 6, 8, 10 and 12 weeks on either SD or HFD. Twelve hours prior to kill, animals were fasted. Five-week-old C57BL/KSJ@Rj-db (*db*/*db*) and C57BL/6J@Rj-ob (*ob*/*ob*) mice were obtained from Janvier Breeding Centre and housed 1 week with free access to food (standard diet) and water before being killed. At time of kill, the weight of *db*/*db* and *ob*/*ob* mice was 37.3 ± 1.6 g ($n = 9$) and 35.9 ± 1 g ($n = 9$), respectively. All experimental protocols were approved by the Cantonal Veterinary Office (Vaud, Switzerland).

Blood samples and biochemical assays

Prior to the dietary treatment, and then every 14 days, approximatively 500 μl of blood was taken by retro-orbitary puncture on 12 h-fasted animals and collected in heparinized tubes for glucose, insulin and β-hydroxybutyrate (BHB) plasmatic measurements. The last blood sample was collected by cardiac puncture, just prior to the animal kill. After centrifugation (2300g, 15 min), plasmas were frozen and stored at -80°C. Blood glucose was measured using the glucose oxidase method on a glucose analyser (Beckman Instruments). Plasmatic insulin was measured by the mouse insulin ELISA enzyme immunoassay (Mercodia, Uppsala, Sweden) and BHB levels were determined using D-3-hydroxybutyric acid colorimetric method (Boehringer Mannheim, Roche, Germany).

Immunohistochemistry

Under urethane anaesthesia (1.5 g kg^{-1} I.P.), animals were perfused intracardially with a heparinized solution of saline (25 IU ml^{-1} in 0.9% NaCl, during 2 min) followed by a freshly prepared solution of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4, for 15 min). Brains were removed and postfixed in 4% paraformaldehyde for 2 h, then cut on a vibratome (Leica VT1000M) to obtain coronal slices (40 μm thick), which were collected in phosphate buffer saline (PBS). Sections were rinsed several times in PBS and incubated with casein (0.5% in PBS) for 1 h to block non-specific sites. They were then incubated in rabbit antisera raised against either MCT1, or MCT2 (characterized in Pierre *et al.* 2000) or MCT4 (characterized in Wilson *et al.* 1998) diluted 1/500 in PBS-BSA (PBS containing 0.25% bovine serum albumin) overnight at 4°C in a humidified

SD group were evident after 6 weeks of diet. *B–D*, glucose, insulin and β-hydroxybutyrate (BHB) plasmatic levels were measured every 2 weeks on blood taken by retro-orbital puncture. HFD increases each of these plasmatic parameters when compared to SD. Error bars represent standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t* test.

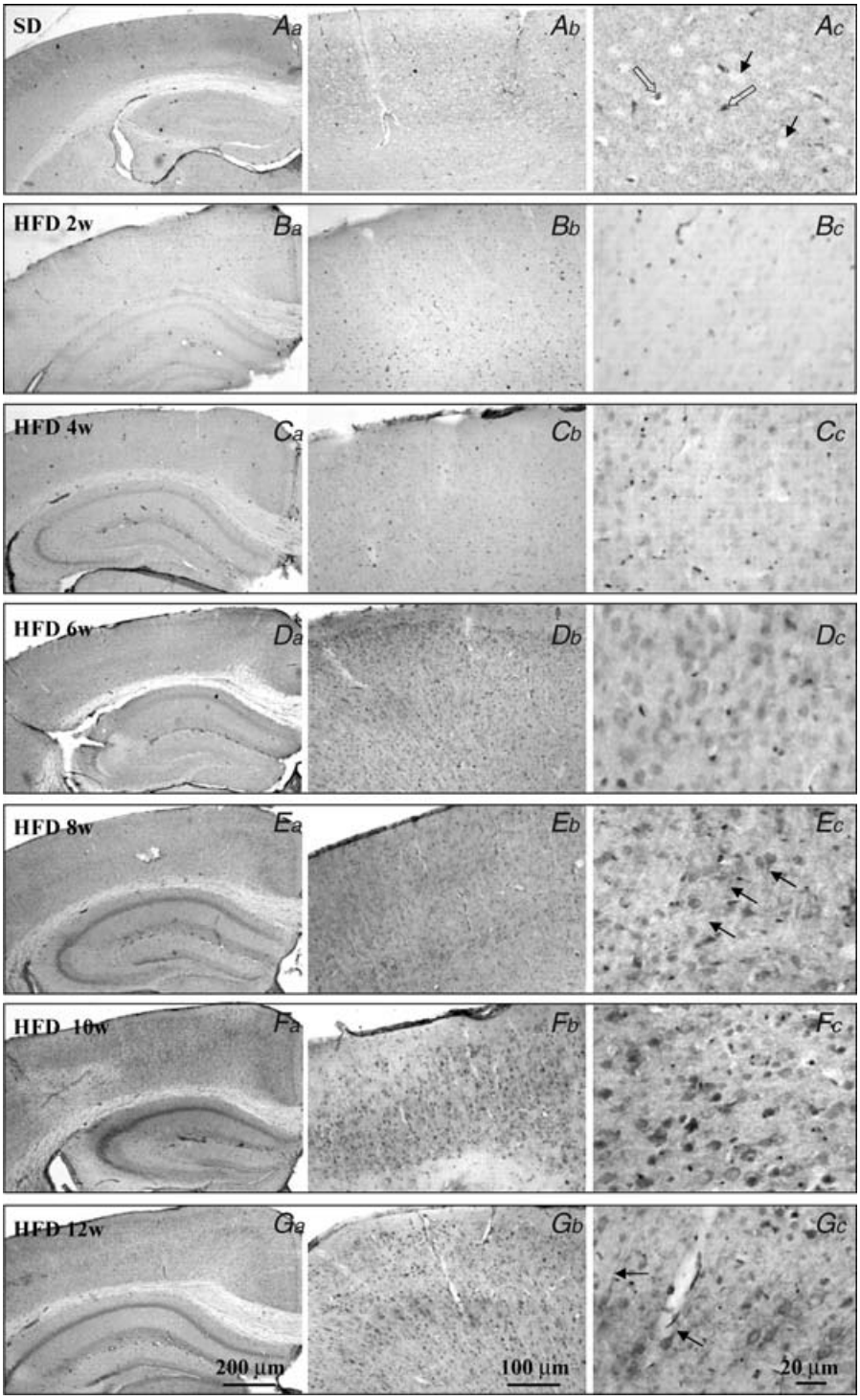


Figure 2. MCT1 expression and distribution in retrosplenial and somatosensory cortex of mice fed with standard diet (SD, *A*) or mice fed with high fat diet from 2 to 12 weeks (HFD 2w–12w, *B–G*)
When mice were fed with SD, MCT1 immunoreactivity occurred in the neuropile (where it was previously shown to be associated with astrocytic processes (Pierre *et al.* 2000), and in endothelial cells forming capillaries (*Aa* and *b*, and *c* at higher magnification, open arrows). Note in *Ac* that neuronal cell bodies remain immunonegative (arrows).This pattern of distribution is similar in cortex of mice fed with SD from 2 to 12 weeks (not shown). By contrast, cortex of mice fed with HFD exhibits, with time of diet, a gradual increase of MCT1 immunolabelling, occurring in neuronal cell bodies and to a lesser extent in their processes (*Ba–Gc*, arrows on processes in *Ec* and *Gc*). Note that MCT1 immunoreaction is also enhanced by the high fat diet in neuronal layers of the hippocampus, visible on pictures at low magnification (*Ba–Ga*). Immunoperoxidase labelling viewed with light microscopy.

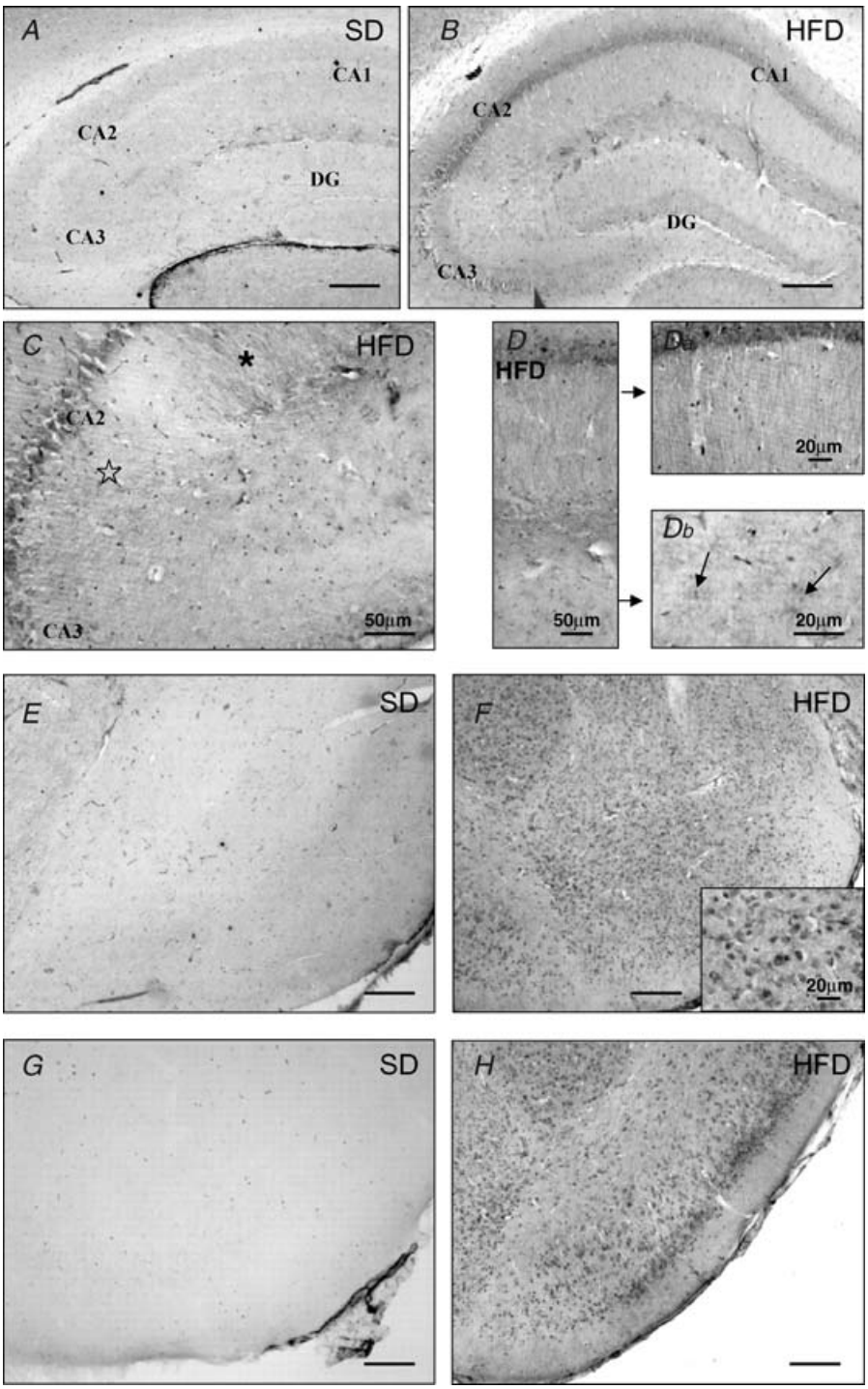
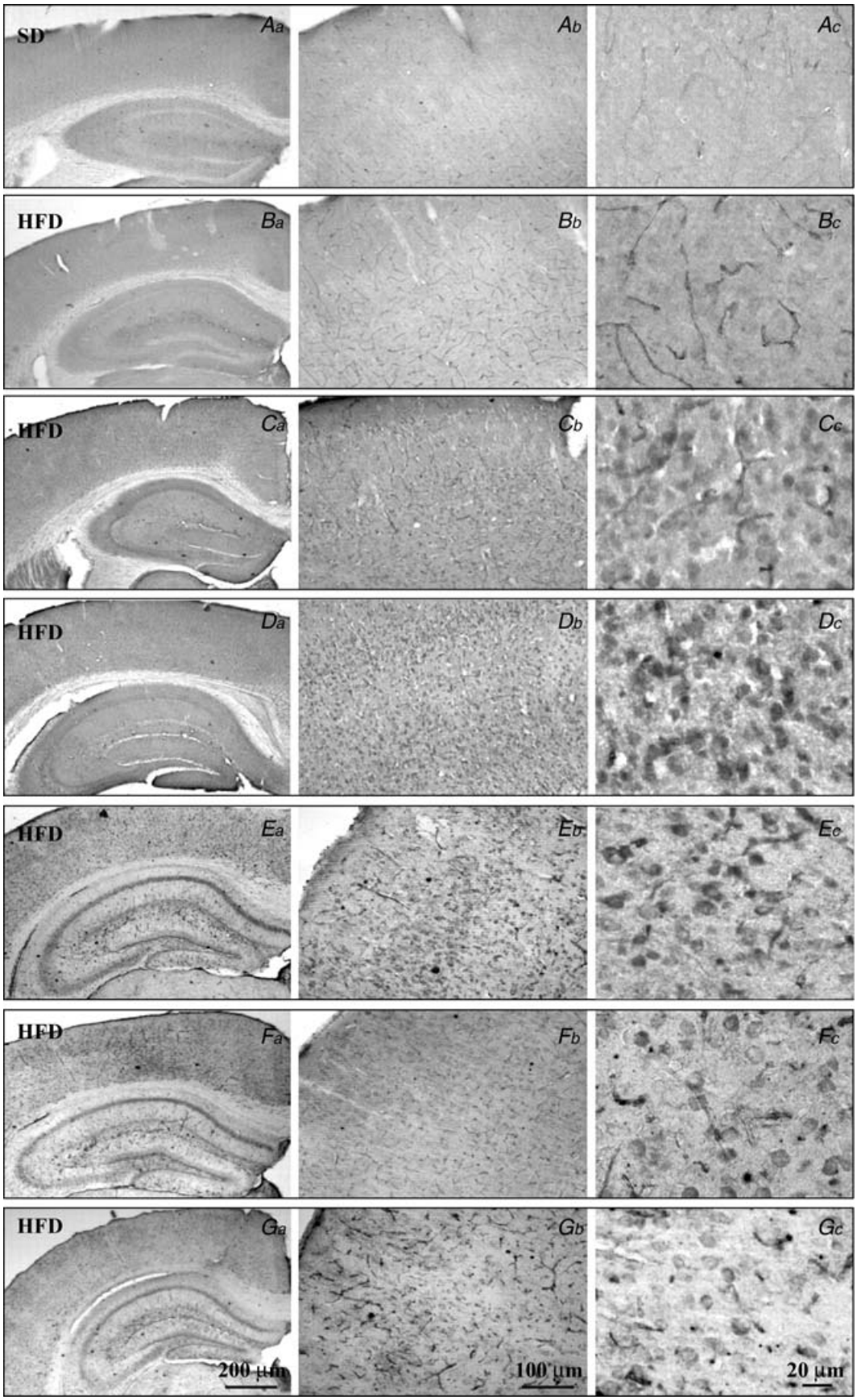


Figure 3. High fat diet alters cellular expression of MCT1 in different cortical and subcortical structures
A–D, while there is little MCT1 immunoreactivity in the hippocampus of mice fed with standard diet (SD, *A*), a strong immunolabelling is visible in hippocampal neuronal layers of mice fed with high fat diet (HFD, *B–D*). The pyramidal layer of the hippocampus is more strongly immunolabelled for MCT1 in CA1 and CA2 areas than in CA3 (*B*, *C*, *D* and *Da*). Moreover, MCT1-immunopositive neuronal fibres are visible in CA1 and CA2 (*C*, asterisk, *Da*) but not in CA3 (*C*, star). The granular layer of the dentate gyrus is not strongly immunolabelled (DG, *B*). Moreover, MCT1-immunopositive astrocytes are visible in the molecular layer (*Db*, arrows). *E–H*, comparison of MCT1 immunoreactivity in amygdala and piriform cortex of mouse fed with a standard diet (SD; *E*: amygdala; *G*: pirifom cortex) and of mouse fed with HFD (HFD; *F*: amygdala, *H*: piriform cortex). Note that HFD induces a dramatic increase of MCT1 immunolabelling in many neurons of these structures. Immunoreactivity revealed using peroxidase. Brightfield optics. Calibration bar represents 100 μm unless indicated otherwise.



chamber. Sheep antirabbit immunoglobulins (Igs, 1/200, Jackson Immunoresearch Laboratories) followed by rabbit peroxidase–antiperoxidase complexes (1/500, Sigma) were used as immunolabels. The peroxidase reaction product was revealed with glucose oxidase-nickel-3,3′-DAB (Shu *et al.* 1988). For double labelling experiments, sections were incubated in a mixture of primary antibodies raised against one MCT (see above) and either against the neuronal marker NeuN (1/500, mouse monoclonal antibody, Chemicon International) or the glial fibrillary acidic protein (GFAP) (1/500, mouse monoclonal antibody, Sigma) overnight at 4°C. Immunofluorescence was revealed by incubation in a solution of second fluorescent antibodies (anti-rabbit Cy-3 and anti-mouse fluorescein isothiocyanate (FITC)-conjugated Igs, diluted 1/200, from Jackson Immunoresearch). Controls included omission of the primary serum or its replacement by non-immune rabbit serum. No specific staining was visible on such preparations. Sections were examined with a Zeiss axioplan2 microscope (Zeiss, Germany), using brightfield optics or epifluorescence. Pictures were acquired using a CCD camera (Axiocam, Zeiss, Germany) together with the 2.05 Axiovision software (Zeiss). They were also examined under a Leica SP5 AOBS confocal microscope using the Ar 488 nm and He/Ne 543 nm laser illumination. Frame size of the images was 512 × 512 pixels with 8-bit colour depth and stacks were made of images taken with 0.5–0.8 μm step size.

Western blotting

For Western blotting, we used extracts from whole brain or isolated cortex, hippocampus and cerebellum that were homogenized in a buffer of 0.32 M sucrose containing Hepes 1 mM, $MgCl_2$ 1 mM, $NaHCO_3$ 1 mM, phenyl-methyl-sulphonyl fluoride 0.1 mM, pH 7.4, in the presence of a complete set of protease inhibitors (Complete, Roche, Switzerland). Protein concentrations were determined by the method of Bradford (1976). Ten micrograms of proteins was heated at 95°C in SDS-PAGE sample buffer (62.5 mM Tris-HCl, 50 mM DTT, 2% SDS, 10% glycerol and 0.1% bromophenol blue) and loaded onto 10% polyacrylamide gels. After electrophoresis, samples were transferred to nitrocellulose membranes (Biorad Laboratories). Membranes were blocked in a blocking solution of Tris-buffered saline (TBS) containing 0.1% Tween, 10% non-fat milk and 1% BSA for 1 h. Membranes were incubated overnight at 4°C with a mixture containing either anti-MCT1 or anti-MCT2 antibody, diluted 1/1000 in TBS, 0.1% Tween, 1% milk (TBSTM) and anti-β-actin antibody (diluted 1/10000; Sigma). After several washes in TBSTM, they were incubated in a solution of IR-dye 800 anti-mouse IgG (diluted 1/5000, LI-COR Biosciences, Rockland) and Alexa Fluor 680 anti-rabbit IgG (Molecular Probes, Invitrogen detection technologies). Bound antibody signal quantification was done by infrared detection and analysis (Odyssey infrared detection system, LI-COR Biosciences). Statistics were performed using Student's *t*-test.

Results

To evaluate the impact of feeding animals with a high fat diet (HFD) on cerebral MCT expression, mice were treated for 2–12 weeks with a diet containing 55 kcal% fat in comparison to a standard rodent chow (standard diet, SD), containing 12 kcal% fat. Maximal weight gain was more pronounced for mice fed with HFD ($+13.8 \pm 2.48$ g) than with SD ($+6.5 \pm 2$ g) ($P < 0.05$, see Fig. 1*A*). Glycaemia and insulinaemia were measured every 2 weeks during 8 weeks of diet on blood samples obtained by retro-orbital puncture. Plasmatic glucose levels increased significantly from 2 to 6 weeks of diet when compared to SD (~1.5-fold after 6 weeks, $P < 0.001$, Fig. 1*B*). Insulinaemia increased already after 2 weeks of diet in plasma of mice fed with HFD but enhancement was highly significant only after 8 weeks of HFD (~3.5-fold, $P < 0.01$, Fig. 1*C*). Moreover, β-hydroxybutyrate (BHB) levels were significantly greater in plasma of mice fed with HFD than in SD-fed mice after 6 weeks of HFD and remained elevated up to 12 weeks (~1.5-fold, $P < 0.01$, Fig. 1*D*), except at 8 weeks where the difference was not significant.

Enhanced MCT expression in brains of mice fed with a high fat diet

Immunohistochemistry performed on brain sections from mice fed up to 12 weeks with a standard chow (SD) showed, in agreement with previous data (Pierre *et al.* 2000), that MCT1 immunoreactivity was very low throughout the brain. It occurred in the neuropile around

Figure 4. MCT2 expression and distribution in cortex of mice fed with either a standard diet (SD, *A*) or a high fat diet up to 12 weeks (HFD 2–12 weeks, *B–G*)
MCT2 immunoreactivity occurs in the neuropile around immunonegative neuronal somata in brain of mice fed with a standard diet (*Aa–c*), independently of time on diet and age of the mouse. It is also associated with endothelial cells forming capillaries (*Aa* and *b*, and *c* at higher magnification). This distribution was modified by food composition since cortex of mice fed with HFD exhibited with time a gradual but strong increase of MCT2 immunostaining, occurring in neuronal somata (*Ba–Gc*) and reaching a maximum of intensity after 6 weeks of diet. Neuronal layers of the hippocampus became also strongly immunolabelled for MCT2 after 6–8 weeks of HFD (*Da–Ga*). Peroxidase immunostaining viewed with light microscopy.

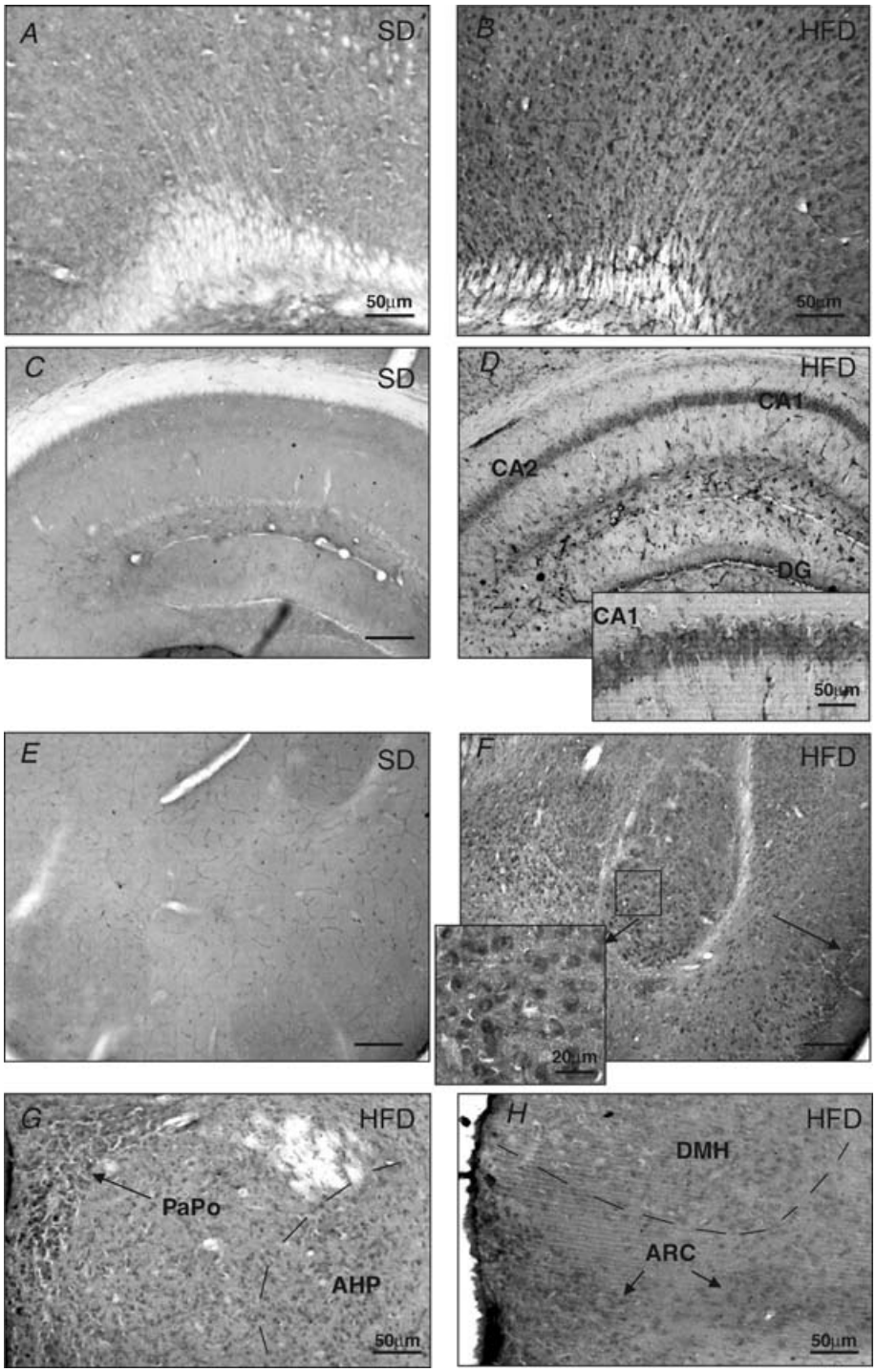


Figure 5. HFD alters cellular MCT2 expression in various cortical and subcortical structures
A, *C* and *E*, MCT2 immunoreactivity in retrosplenial cortex, hippocampus and amygdala of mouse fed with a standard diet. *B*, *D* and *F*, neuronal MCT2 expression is strongly enhanced by high fat diet in retrosplenial cortex (*B*), hippocampus (*D*) and amygdala (*F*, and at higher magnification in the inset) as well as piriform cortex (*F*, arrow). In the hippocampus (*D*), cell bodies and processes of the pyramidal layer are highly immunolabelled for MCT2 in CA1 and CA2 (CA1 at high magnification in the inset). MCT2 immunoreactivity is also increased in the granular layer of the dentate gyrus (DG, *D*) but to a lesser extent than in the pyramidal cell layer. *G* and *H*, HFD induced the appearance of MCT2 immunopositive neurons in hypothalamic nuclei like the posterior paraventricular nucleus (PaPo), the arcuate nucleus (ARC), the anterior hypothalamic area (AHP), and the dorsomedian hypothalamic nucleus (DMH). Calibration bar represents 100 μm unless indicated otherwise. Immunoperoxidase staining viewed with microscopy using brightfield optics.

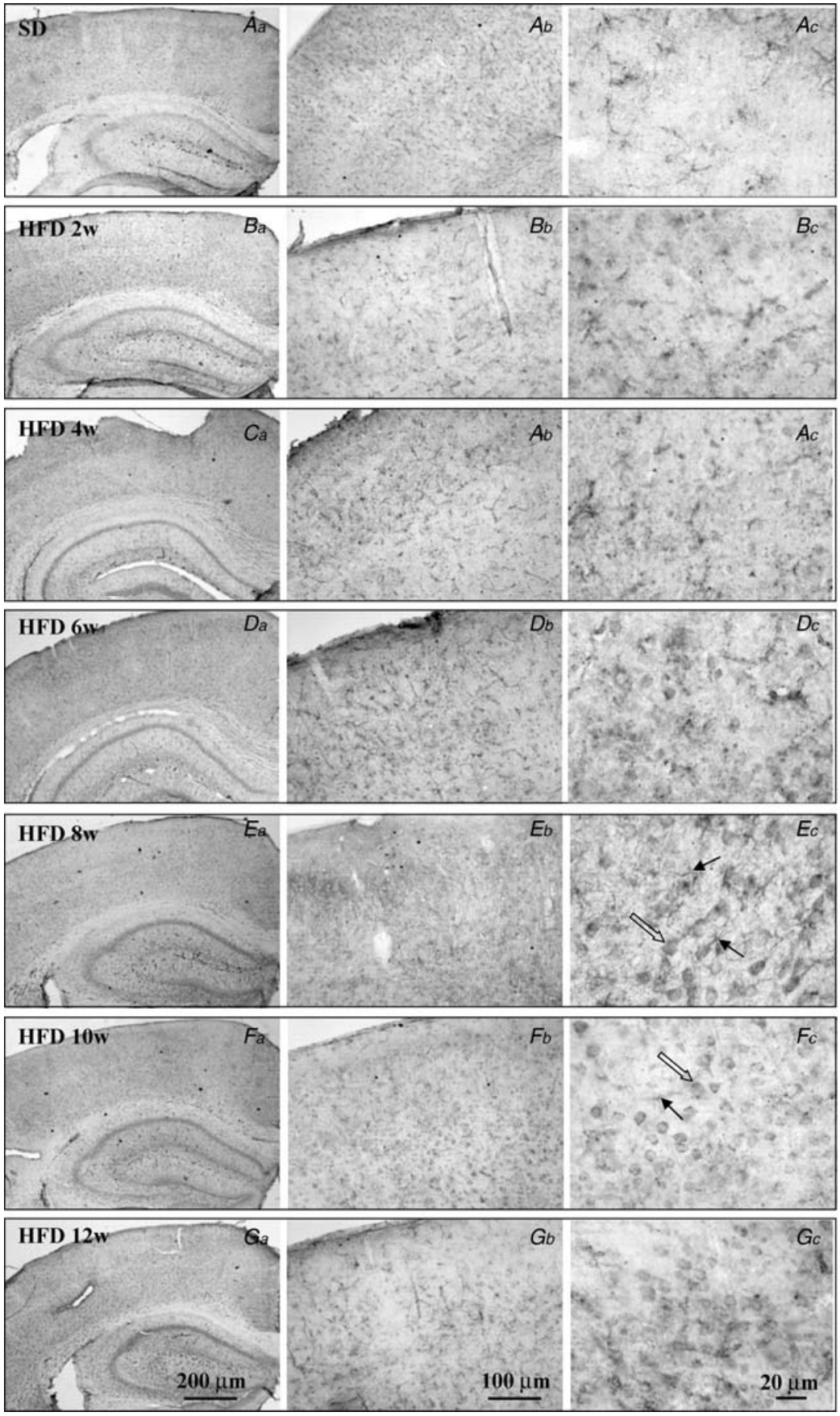
immunonegative neuronal cell bodies and in endothelial cells forming microvessels (Fig. 2*A*). By contrast, feeding mice with HFD for 2 weeks only altered this pattern of expression since a very faint immunoreactivity for MCT1 appeared in neuronal somata throughout the cortex and also in the pyramidal layer of the hippocampus (Fig. 2*B*). Intensity of this neuronal immunolabelling increased with the duration of HFD feeding and reached a maximum between 6 and 10 weeks of HFD feeding (Fig. 2*B–G*). These changes were more pronounced in posterior sections, where the retrosplenial cortex is present but not the cingular cortex (not shown). After 8 weeks of HFD, cortical and hippocampal neurons displayed intense immunoreactivity for MCT1, occurring in their somata as well as in their processes (Figs 2*Ec–Gc* and 3*B–D*). The presence of immunopositive neuronal fibres was evident in cortical neurons as well as in pyramidal cells of CA1 and CA2 in the hippocampus, but not in CA3 (see Fig. 3*C*). Double labelling experiments using MCT1 and either NeuN, a neuronal marker, or GFAP, expressed by glial cells, confirmed that MCT1 immunoreactivity induced by HFD occurred specifically in neurons and not in astrocytes (Fig. 8*Aa–c* and *D*). HFD affected MCT1 distribution in other brain structures, such as the amygdala (Fig. 3*E–F*), as well as the auditory (not shown) and piriform cortices (Fig. 3*G–H*) where MCT1 immunolabelling occurred in cell bodies and proximal processes of many neurons (Fig. 3*F* and *H*). Increased neuronal MCT1 immunoreactivity was also observed in the striatum and the hypothalamus, but enhancement was less pronounced than in the cortex or the hippocampus (not shown).

As previously described (Pierre *et al.* 2002), MCT2 was expressed in the neuropile (associated with neuronal processes) in brain of mice fed with SD, but could not be detected in neuronal somata (Fig. 4*Aa–c*). It was also visible in capillaries (Fig. 4*Aa*). Two weeks of HFD triggered important changes in MCT2 expression since neuronal cell bodies became immunolabelled (Fig. 4*B*), as confirmed by double labelling experiments with NeuN (see Fig. 8*Ba–c* and *E*). Moreover, blood vessels became more intensely immunostained from 2 to 12 weeks of diet (Fig. 4*Bc–Gc*). Intensity of MCT2 labelling in neuronal somata increased gradually with time of diet, with a maximum reached after 6 weeks of HFD feeding, and had a tendency to decrease after 12 weeks of HFD (Fig. 4*B–G*). MCT2-immunopositive neurons were very abundant throughout the retrosplenial, somatosensory, auditory and piriform cortices (Fig. 5*B* and *F*), the hippocampus (Fig. 5*D*) and the amygdala (Fig. 5*F*), and to a lesser extent in the hypothalamus (Fig. 5*G–H*) and the striatum (not shown). Similarly to MCT1, MCT2-positive neuronal processes became visible after 6 weeks of diet.

MCT4 is considered to be a specific astrocytic transporter based on its previously established distribution (Bergersen *et al.* 1999; Bergersen *et al.* 2001; Rafiki *et al.* 2003; Pellerin *et al.* 2005). Consistent with these observations, MCT4 immunoreactivity in brain of mice fed with SD up to 12 weeks was found exclusively in astrocytes throughout the whole brain (Fig. 6*A*). After 2 weeks of HFD, neuron-like cells appeared immunostained for MCT4 in the mouse cortex, in addition to astrocytes (Fig. 6*B*). We performed double labelling experiments with NeuN or GFAP in order to identify the cell type(s) expressing MCT4 in brains of mice fed with HFD. As for both MCT1 and MCT2, MCT4 was expressed by neurons, as shown by superposition of MCT4 and NeuN immunostainings (Fig. 8*Ca–c*), but remained also visible in astrocytes since MCT4 and GFAP immunoreactivities partly colocalized (Fig. 8*F*). Neuronal cell bodies and processes that were immunostained for MCT4 displayed gradually a more intense immunoreaction with time of diet, while labelling of MCT4-positive astrocytes remained unchanged (Fig. 6*C–G*). Neuronal MCT4 expression induced by HFD was found mainly in the same brain structures where enhanced MCT1 and MCT2 expression was observed, i.e. the cortex (Fig. 7*B*), the amygdala (Fig. 7*D*) and the hippocampus (Fig. 7*F*). As compared to the other transporters, the changes in MCT4 expression were less convincing in the hypothalamus and the striatum (not shown).

Quantification of MCT1 and MCT2 expression induced by HFD

Since the increased expression of MCTs due to nutritional manipulations was stronger in rostral (containing retrosplenial, somatosensory and auditory cortex) than in frontal brain coronal sections, we performed brain dissection following the scheme shown in Fig. 9*A*, in order to distinguish what we termed cortex 1 (containing cingulate and motor cortex), cortex 2, including piriform, insular and entorhinal cortex, and cortex 3, composed of the somatosensory, the retrosplenial and the visual cortex. The effect of a 6 weeks of HFD on MCT expression was quantified using Western blots performed on extracts from different brain structures (Fig. 9*B*). We averaged results from two to three Western blots made on extracts obtained from different mice in order to perform statistical analysis. MCT1 protein level was slightly increased in cortex 1 of mice fed with HFD when compared to mice fed with SD ($\sim$1.2-fold, $P < 0.05$, Fig. 9*C*). By contrast, the increase was highly significant in cortex 2 ($\sim$1.4-fold, $P < 0.01$), cortex 3 ($\sim$1.85-fold, $P < 0.01$) and hippocampus ($\sim$1.45-fold, $P < 0.01$), while it was not affected in cerebellum (Fig. 9*C*). MCT2 expression was neither increased in cortex 1 nor in cerebellum of mice fed with HFD compared to mice fed with SD (Fig. 9*C*). By contrast, it was significantly enhanced in cortex 2 ($\sim$1.8-fold, $P < 0.001$), cortex 3 ($\sim$1.5-fold, $P < 0.01$) and hippocampus ($\sim$1.44-fold, $P < 0.01$) (Fig. 9*C*).



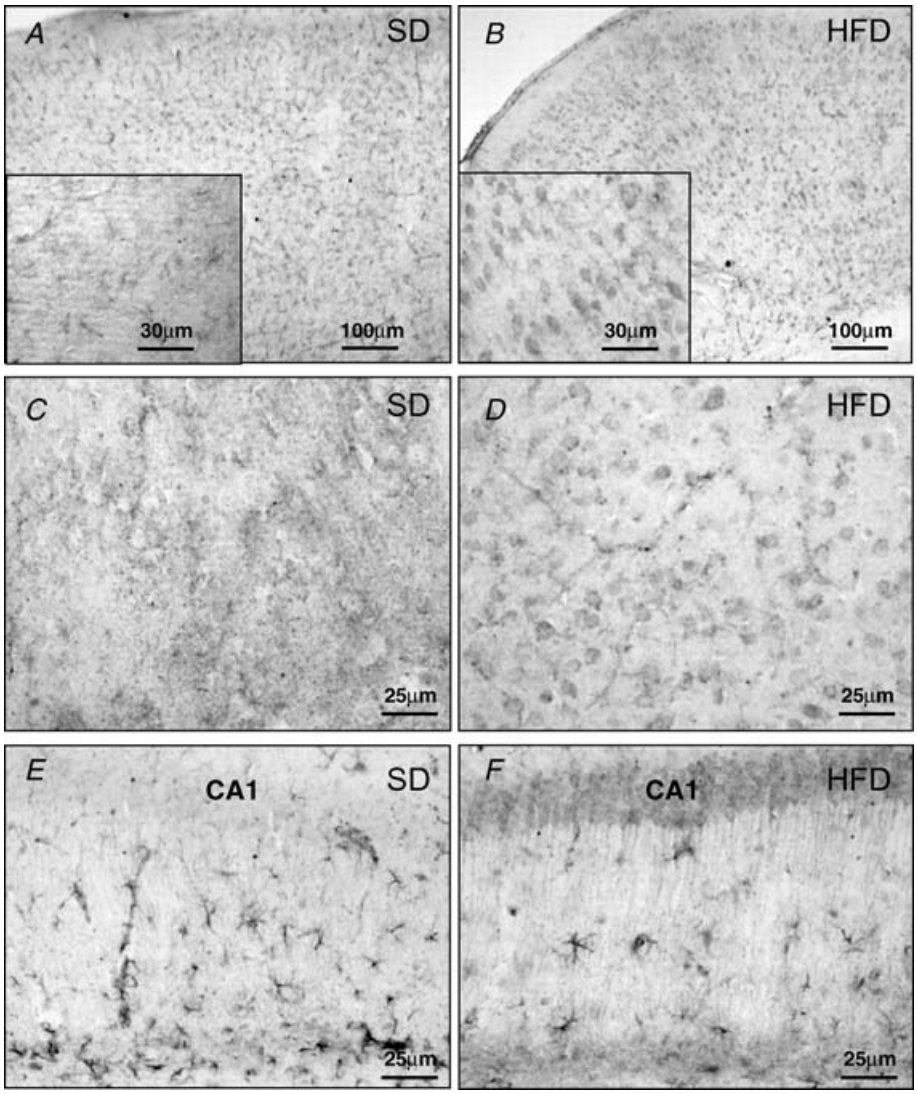


Figure 7. Cellular MCT4 expression is altered by HFD in different brain areas
MCT4 immunoreactivity occurred in astrocytes and their processes in the somatosensory cortex (*A*, clearly visible in inset), the amygdala (*C*) and the CA1 area of the hippocampus (*E*) in mouse fed with a standard diet (SD). In mice fed with a high fat diet during 8 weeks, MCT4 expression was strongly enhanced in these structures (HFD, *B*, *D* and *F*). Intensity of the immunoreactivity did not change in astrocytes, but clearly increased in neurons (*B*, *D* and *F*). In CA1, somata and processes of pyramidal neurons were highly immunolabelled for MCT4 together with astrocytes (*F*). Immunoreaction using peroxidase and viewed using brightfield optics.

Increased brain MCT expression in *ob*/*ob* or *db*/*db* mice fed with a standard diet

In order to distinguish between a direct effect of nutrients (e.g. fatty acids) on brain from other possible consequences of the resulting altered metabolic state induced by a high fat diet, we assessed MCT expression levels in brains of two mouse models presenting some common metabolic features (e.g. obesity) but fed with a standard diet. Six-week-old *ob*/*ob* or *db*/*db* mouse brains were

Figure 6. MCT4 expression and distribution in cortex of mice fed with either a standard diet (SD, *A*) or a high fat diet from 2 to 12 weeks (HFD, *B*–*G*)
In mice fed with SD, MCT4 immunolabelling occurred exclusively in astrocytes throughout the whole brain (*Aa*–*c*, cortical astrocytes clearly visible at high magnification in *Ac*). Following high fat diet, immunopositive neurons for MCT4 appeared and became more numerous as well as more strongly labelled with longer period of diet, in addition to astrocytes that remained immunolabelled (*Ba*–*Gc*, black arrows showing astrocytes, empty open for neurons). A maximal intensity for MCT4 neuronal immunoreactivity was reached after 8 weeks of diet. Immunoperoxidase staining visualized with light microscopy.

investigated for MCT1, MCT2 and MCT4 expression levels. Immunolabelling using immunoperoxidase showed that the three MCTs were expressed in numerous neurons of the brain of both *ob*/*ob* and *db*/*db* mice (Fig. 10). Only cortical immunostaining is illustrated (Fig. 10) but many other brain structures were immunostained for all three MCTs, similarly to what was observed in the brain of mice fed with HFD (not shown). Double fluorescence immunolabelling using the neuronal marker NeuN confirmed neuronal expression of the three transporters (Fig. 10*Ac–Fc*). MCT1 and MCT2 protein levels were quantified in *ob*/*ob* and *db*/*db* *versus* wild-type (WT, C57Bl/6J) mice using Western blots performed on extracts from different brain structures. We averaged results from two Western blots made on extracts obtained from different mice in order to perform statistics (Fig. 11). In both cortex 1 and cerebellum, neither MCT1 nor MCT2 levels were significantly changed in *ob*/*ob* or *db*/*db* mice when compared to WT. By contrast, they increased significantly in cortex 2 of *ob*/*ob* mice (∼2-fold, $P < 0.001$ for MCT1; ∼2.5-fold, $P < 0.001$ for MCT2) and *db*/*db* mice (∼1.5-fold, $P < 0.01$ for MCT1; ∼3.8-fold, $P < 0.001$ for MCT2). A significant increase was also observed in cortex 3 of both *ob*/*ob* (∼1.3-fold, $P < 0.01$ for MCT1; ∼2.3-fold, $P < 0.001$ for MCT2) and *db*/*db* (∼1.3-fold, $P < 0.01$ for MCT1 and ∼2.3-fold, $P < 0.001$ for MCT2) mice as well as in the hippocampus (∼1.8-fold, $P < 0.001$ for MCT1 and ∼1.5-fold, $P < 0.001$ for MCT2 in *ob*/*ob* mice, and ∼1.8-fold, $P < 0.001$ for MCT1 and ∼1.7-fold, $P < 0.001$ for MCT2 in *db*/*db* mice).

Discussion

Little information is available concerning the regulation of MCT expression in the central nervous system, especially in relation to different metabolic states. During development, levels of brain MCT1, 2 and 4 were shown to increase in the preweaning period and to decrease rapidly

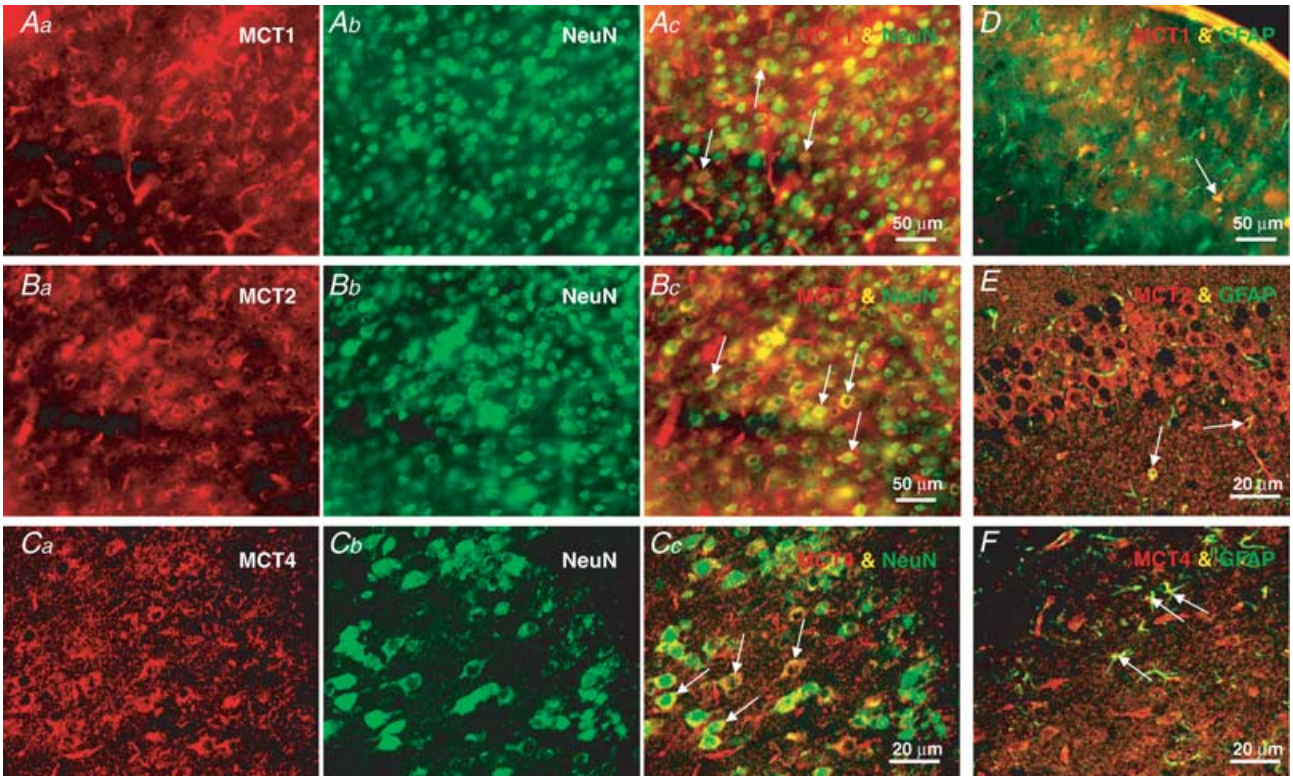


Figure 8. Cellular determination of MCT expression in mice fed with HFD
Ac–Cc, double immunolabelling performed for either MCT1 (red, *Aa*), MCT2 (red, *Ba*) or MCT4 (red, *Ca*) and the neuronal marker NeuN (green, *Ab–Cb*) showed a colocalization between each of the transporters and NeuN (orange or yellow, *Ac–Cc*, arrows) in cortical neurons of mice fed with a HFD during 6 weeks. *D*, *E* and *F*, double immunofluorescence labelling for the astrocytic marker GFAP (green) and MCT1 (red, *D*), MCT2 (red, *E*) or MCT4 (red, *F*). No colocalization between MCT1 or MCT2 immunoreactivities and GFAP immunostaining could be detected except on capillaries (yellow, arrows, *D* and *E*). By contrast, MCT4 immunolabelling (*F*) occurred in neurons (red) as well as in astrocytes, as shown by the yellow coloration of astrocytic processes (arrows) resulting from superimposition of red (MCT4) and green (GFAP) staining. *D*, piriform cortex; *E*, hippocampus; *F*, hypothalamus. *Aa–Bc* and *D*, fluorescence microscopy; *Ca–c*, *E* and *F*, projections of 10–18 sections (0.6 μm thickness) made using confocal microscopy.

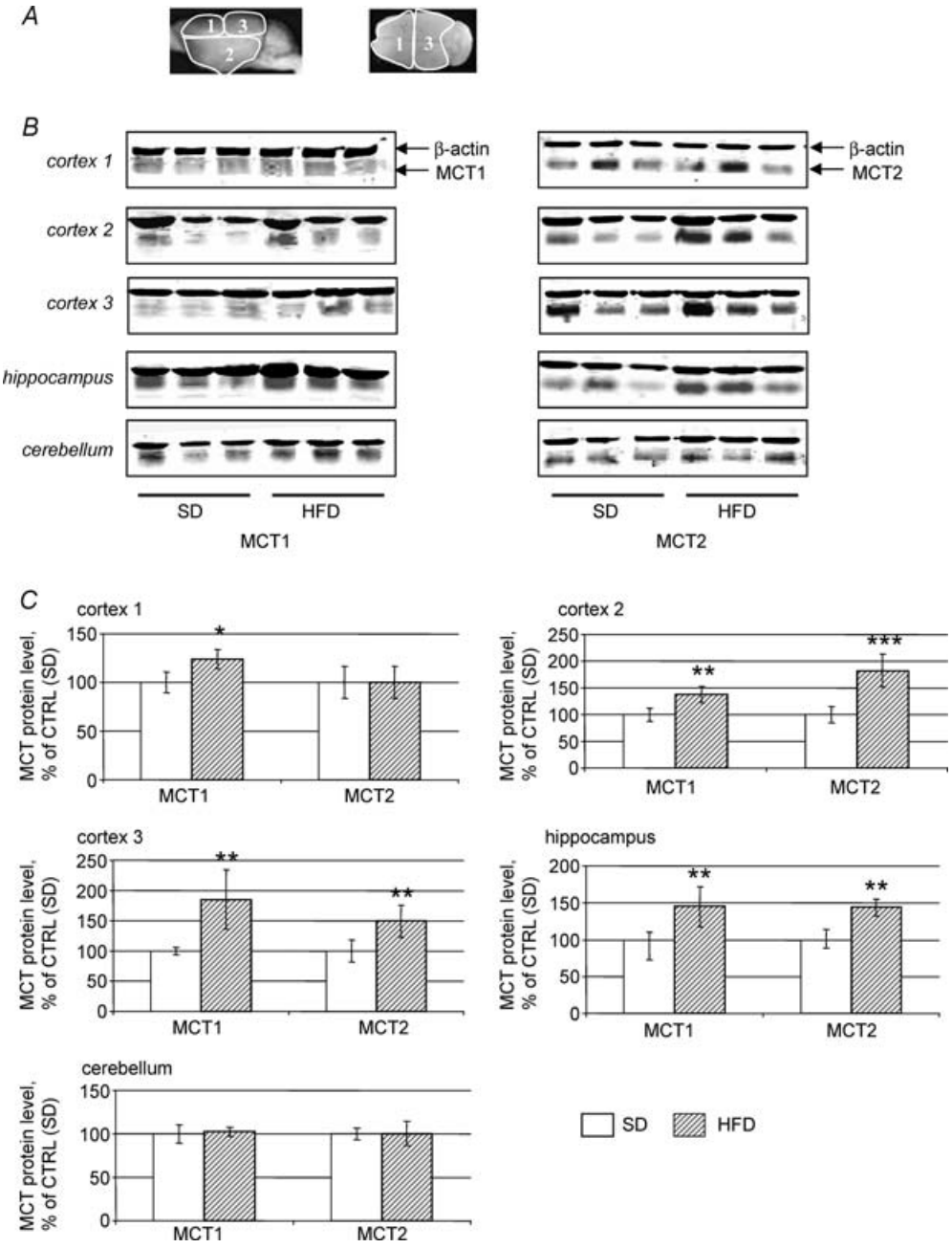
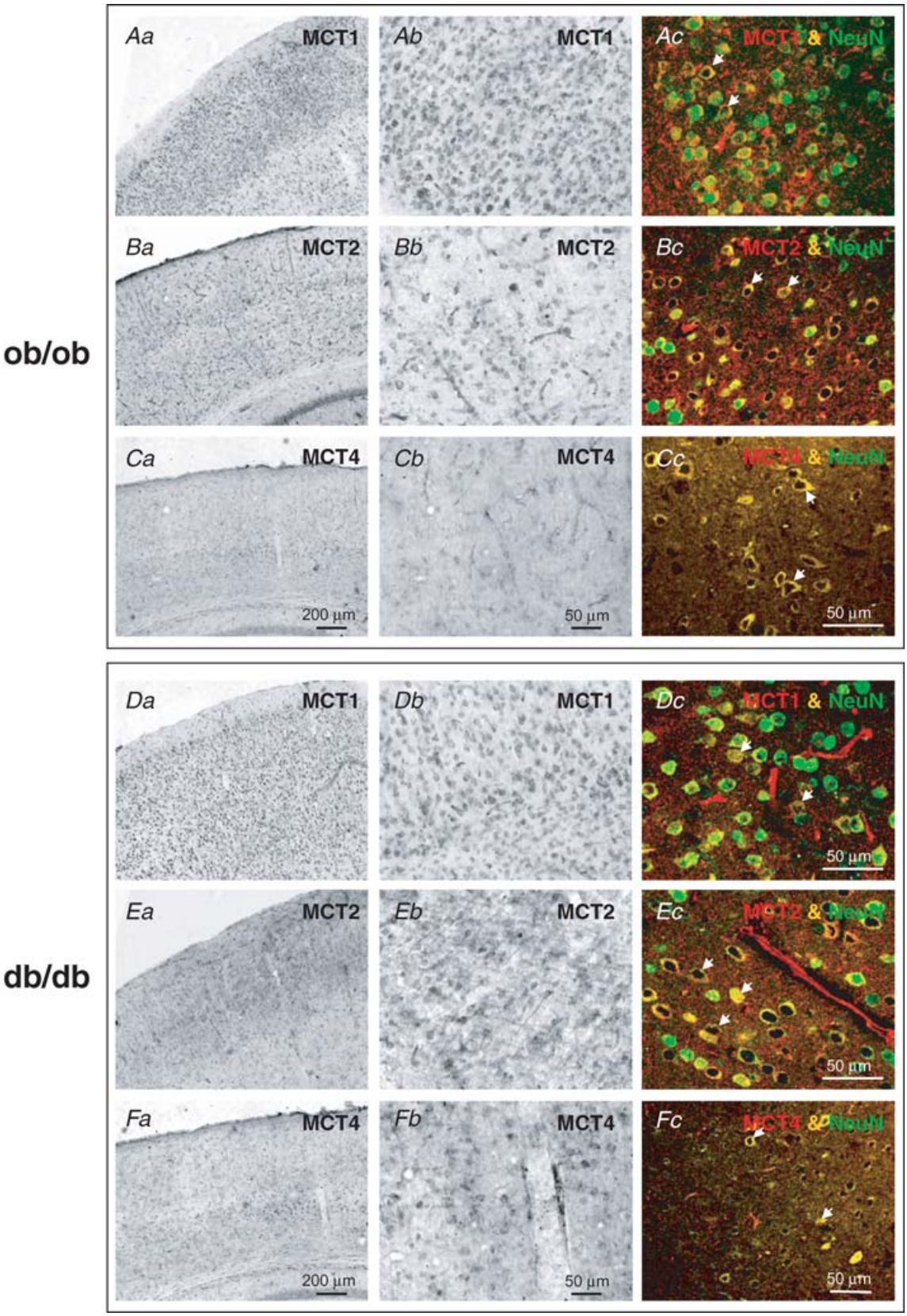


Figure 9. Quantitative determination of HFD-induced changes of MCT1 and MCT2 protein expression in different brain structures

A, identification of the different parts of the mouse brain cortex considered for quantitative purposes. Cortex 1: frontal motor and cingulate cortex; cortex 2: piriform, insular and entorhinal cortex; cortex 3: somatosensory, retrosplenial and visual cortex. *B*, infrared Western blot analysis showing MCT1 (MCT1, band at ∼43 kDa), MCT2 (MCT2, band at ∼40 kDa) and β-actin (β-actin, band at ∼50 kDa) in different brain structures of mice fed with either a standard diet (SD, 3 wells corresponding to the identified brain structure from 3 different mice) or a high fat diet (HFD, 3 wells corresponding to the identified brain structure from 3 different mice). *C*, quantification was done with the Odyssey software (LI-COR, Rockland) on Western blots that were performed on brain structure extracts obtained from at least three animals in each condition (SD or HFD) and subsequently averaged to perform statistics. Results show that MCT1 and MCT2 protein expressions are significantly increased in cortex 2 and 3 as well as in the hippocampus of mice fed with the HFD. MCT1 protein level was also increased in cortex 1 ($P < 0.05$), while MCT2 expression did not change in this structure. In cerebellum, we did not observe any modification of MCT1 or MCT2 protein expression level. Results are expressed as percentage of control after the values had been normalized using β-actin signal as a reference. Error bars represent standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ using Student's *t* test.



after weaning, in parallel with available blood ketones as fuel (Gerhart *et al.* 1997; Pellerin *et al.* 1998; Leino *et al.* 1999; Baud *et al.* 2003). Increased expression was found both on brain capillaries (to facilitate substrate entry) and on parenchymal cells, i.e. neurons and astrocytes. It is purported that such changes are necessary to allow the developing brain to use circulating ketone bodies derived from fatty acids present in maternal milk as energy substrates (Nehlig & Pereira de Vasconcelos, 1993). In adulthood, cerebral MCT1 expression was shown to be up-regulated after 3 weeks of a ketogenic diet (Leino *et al.* 2001). Immunostaining was enhanced both on endothelial cells forming capillaries and on unidentified cellular processes in the neuropile, but not in neuronal somata. As opposed to such an effect of a ketogenic diet, our data are the first to describe gradual and extensive changes in the expression of all three MCT isoforms found in the central nervous system in adult animals exposed to a high fat diet, as well as elevated expression of the same MCT isoforms in brains of genetically obese or diabetic mice. The most striking observations are the prominent enhancement of expression of each MCT specifically in neurons and their appearance in neuronal somata. Indeed, MCT4 has been considered up to now as an exclusive astrocytic transporter, never having been detected in neurons (Bergersen *et al.* 2002; Rafiki *et al.* 2003; Pellerin *et al.* 2005). Similarly, under normal conditions, MCT1 expression occurred mainly in endothelial cells and astrocytic processes (Gerhart *et al.* 1997; Leino *et al.* 1999; Hanu *et al.* 2000; Pierre *et al.* 2000). Although MCT1 expression could be observed in cultured neurons of the rat hypothalamus (Ainscow *et al.* 2002; Kang *et al.* 2004), it remained undetectable using Western blot analysis or as very faint immunoreactivity in mouse cortical neurons in culture (Debernardi *et al.* 2003). *In vivo*, a few scattered MCT1-positive neurons were detected in the rat brain (Leino *et al.* 1999), but immunoreactivity for MCT1 in neurons was never observed in the mouse brain (Pierre *et al.* 2000; Pierre *et al.* 2002). In addition, we observed that modifications of MCT expression do not occur uniformly throughout the brain as it takes place in some areas but not others (e.g. hippocampus or hypothalamus *versus* cerebellum) or predominate in parts of the same structure (e.g. visual cortex *versus* motor cortex). Although the specific reasons for such cellular and regional heterogeneities are unknown, they are likely to be related to differential distribution of effector mechanisms, e.g. receptors and/or transduction mechanisms.

The precise mechanism by which exposure to a high fat diet leads to enhanced MCT expression in brain remains uncertain for the moment. However, our observations that genetically induced obese mice (*ob*/*ob*) as well as genetically induced diabetic mice (*db*/*db*) fed on a standard diet exhibit a similar enhancement of MCT expression argue against a direct role of fatty acids. As an alternative possibility, hormonal alterations occurring as a consequence of exposure to a high fat diet and development of obesity could participate in the up-regulation of MCT expression in the central nervous system. Increased fat mass induced by a high fat diet, as occurs in obesity, leads to enhanced production and circulating levels of adiposity signals such as leptin and insulin (Benoit *et al.* 2004; Woods *et al.* 2004). Leptin was shown to activate several signal transduction mechanisms and induce gene expression in peripheral organs as well as in the central nervous system (Fruhbeck, 2006). However, in both the *ob*/*ob* mouse, which carries a mutation of the gene coding for leptin, and the *db*/*db* mouse, which has a mutated leptin receptor, the same alterations in MCT expression were present despite the disruption of the putative leptin signal. Thus, it is unlikely that leptin is the main signal causing enhanced cerebral MCT expression in mice fed with a high fat diet. Another feature commonly observed in obese animals is an elevation of circulating levels of insulin (Woods *et al.* 2004). Hyperinsulinaemia was not only observed in our mice fed with a high fat diet but it is also a characteristic of both *ob*/*ob* and *db*/*db* mice. Moreover, it is well known that insulin enters into the brain and modulates a number of cerebral functions, from energy and glucose homeostasis to learning and memory (Gerozissis, 2003; Plum *et al.* 2005). Interestingly, we have recently observed that insulin can enhance the expression of MCT2 in cultured cortical neurons (Chenal & Pellerin, 2006). Based on these observations, we believe that insulin represents a good candidate for the putative signal to mediate the changes of neuronal MCT expression in each obese mouse model we have investigated.

Figure 10. MCT expression in *ob*/*ob* (*Aa–Cc*) and *db*/*db* (*Da–Fc*) mouse brain
Immunoperoxidase staining showed that MCT1 (*Aa* and *b*; *Da* and *b*), MCT2 (*Ba* and *b*; *Ea* and *b*) and MCT4 (*Ca* and *b*; *Fa* and *b*) immunoreactivities occurred in neuronal-like structures and on capillaries in the cortex of a *ob*/*ob* (*Aa–Cb*) or *db*/*db* (*Da–Fb*) mouse. *Ab–Fb* pictures show higher magnifications of, respectively, *Aa–Fa* views. Neuronal expression of MCT1, MCT2 and MCT4 in the cortex of both *ob*/*ob* and db/db mice was confirmed by double labelling for MCT1, 2 or 4 (red) and the neuronal marker NeuN (green) resulting in an orange coloration in the cytoplasm of neuronal cell bodies (arrows, *Ac–Fc*). *Aa–Fb*, immunoperoxidase viewed with brightfield optics. *Ac–Fc*, immunofluorescence visualized using a confocal microscope with *Ac* and *Dc* pictures corresponding to a projection of 18 sections of 0.5 μm and *Bc*, *Cc*, *Ec* and *Fc* pictures representing single sections (central z planes) of 0.5–0.8 μm thickness.

Monocarboxylate transporters have been implicated in the intercellular transfer of lactate in different organs including the brain (Brooks, 1986; Pellerin & Magistretti, 1994; Gladden, 2004). Moreover, as mentioned previously, they are involved in the import and the use of energy substrates such as lactate and the ketone bodies in particular metabolic situations. Exposure to a high fat diet sets a new metabolic environment for the brain that most likely leads to specific metabolic adaptations. Similarly, mice developing features of the metabolic syndrome following specific genetic mutations are likely to undergo cerebral modifications in response to such altered metabolic conditions. Although insulin can exert a certain influence on brain glucose metabolism, it might also have a significant impact on other aspects of neuroenergetics, including the use of alternative energy substrates such as monocarboxylates. In parallel with hyperinsulinaemia and hyperglycaemia, one common feature encountered with the development of obesity is the appearance of insulin resistance (Kahn *et al.* 2006). Such resistance to the action of insulin is well described for tissues like muscle or liver, but it was recently proposed that it might also affect the brain (Isganaitis & Lustig, 2005). Specific metabolic consequences of altered insulin sensitivity in the brain have not been explored but overexpression of monocarboxylate transporters by neurons as observed here could be a response to overcome brain insulin resistance and its impact on the utilization of energy substrates by brain cells. Such an observation might be of interest in relation to the suggestion that a relation exists between

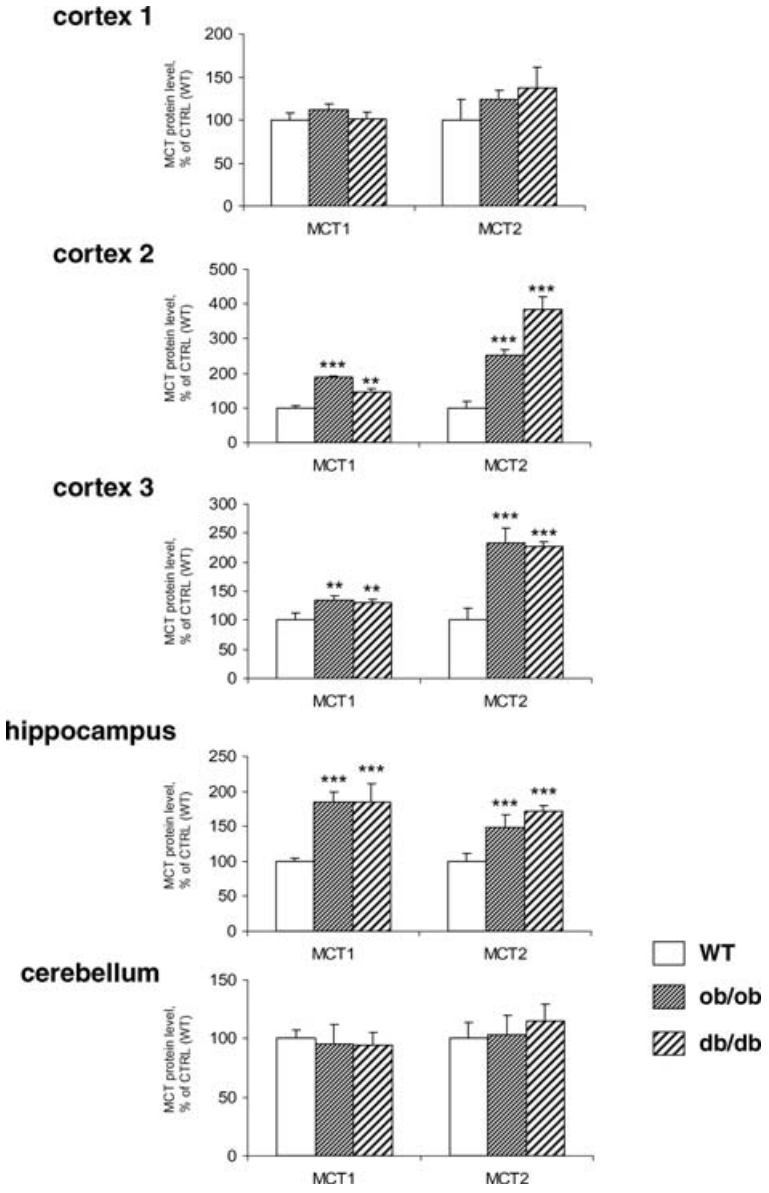


Figure 11. Quantitative determination of MCT1 and MCT2 protein expression in different brain structures of *ob*/*ob* and *db*/*db* mice
Quantification was done with the Odyssey software (LI-COR, Rockland) on Western blots that were performed on brain structure extracts obtained from at least three animals in each condition (wild-type (WT), *ob*/*ob* or *db*/*db*) and subsequently averaged to perform statistics. Results showed that MCT1 and MCT2 protein expression was significantly increased in cortex 2 and 3 as well as in the hippocampus of both *ob*/*ob* and *db*/*db* mice. In cortex 1 or cerebellum, we did not observe any significant modification of MCT1 or MCT2 protein expression level. Results are expressed as percentage of control (WT) after the values had been normalized using β-actin signal as a reference. Error bars represent standard deviation. $^{**}P < 0.01$, $^{***}P < 0.001$ using Student's *t* test.

insulin resistance, metabolic deficits and the development of cognitive decline as observed in ageing and neurodegenerative diseases (Watson & Craft, 2006).

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