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Chapter III

Selected Fast and Slow Methionine Sulfoximine-Inbred Mice: MSO-Dependent Seizures, Behavior, Brain Glycoprofiles, Neurotransmitters and Glycogen

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Abstract

Methionine sulfoximine (MSO) is a byproduct due to oxidation of methionine during bleaching of wheat used for animal nutrition. The compound was reported to alter animal behavior. Among these alterations, MSO has been described as epileptogenic for a variety of animals. In addition, MSO was glycogenic affecting especially the brain. In order to study the putative relationships between these two effects, we 1) analyzed the concomitant effect of MSO related to seizure induction and brain glycogen content in various strains of mice, and 2) developed strains of mice based upon the latency towards MSO-dependent generalized convulsions. Mice with short latency period were designated as MSO-Fast strain whereas those exhibiting a long latency period were designated as MSO-Slow strain. These two strains which had been obtained after an extensive selection process including inbreeding (giving either iMSO-Fast or iMSO-Slow mice) were used for the study of MSO induced responses in terms of alteration in seizure latency, neurotransmitter concentration, glycogen content, behavior, and in terms of the cerebral glycol-profile in cortical astrocyte cell membrane proteins.

In terms of sensitivity, MSO-Fast mice exhibited a very short latency toward MSO-dependent seizures when challenged with 75 mg/kg MSO (MSO-Fast n=20, 277.6 ± 64.5 min) as compared to MSO-Slow ones which did not responded to that dose during the first 10 hours after application. The EEG confirmed these data. Kainic acid was the only other convulsant that demonstrated a significant difference between the two strains of mice, MSO-Fast being significantly more reactive than MSO-Slow ones (MSO-Fast: 42 ± 30 min; MSO-Slow: 75 ± 52 min, $p<0.05$, n=20). In MSO-Fast mice MSO-induced seizures were significantly antagonized by MK-801. Therefore, we proposed that the glutamatergic pathway is involved in MSO-dependent seizures in our mouse model.

Behavioral open field testing revealed that iMSO-Fast mice were more anxious than iMSO-Slow strain. These observed differences might be related to the differences detected for the cerebral serotonin (5-HT) contents among both strains since brain of iMSO-Fast mice contained 1.48 ± 0.65 pmole/mg protein while that of iMSO-Slow ones contained 0.59 ± 0.31 pmole/mg protein, $p<0.01$, (n=8) as revealed by reversed-phase HPLC coupled to electro-chemical detection,

As observed for 5-HT the basal glycogen content was significantly higher in iMSO-Fast mice (8.4 ± 1.5 nmoles glucosyl/mg protein) compared to iMSO-Slow ones (3.2 ± 1.0 nmoles glucosyl/mg protein, $p<0.001$, n=10). The time course during which glycogen increased after MSO administration was significantly elevated in MSO-Fast mice than in MSO-Slow ones, while no significant variations were detected in both strains during control kinetics. These differences are supported by our *in vitro* studies addressing glycosylation by glycoprofiling with lectins on astrocytes.

Our results showed that the developed model of iMSO-Fast and iMSO-Slow inbred mice enabled us to study the mechanisms related to the appearance of MSO-dependent seizures allowing us also to address the question how seizure sensitivity and brain glycogen content are related to each other. Our current hypothesis suggests that brain glycogen metabolism may constitute a protection against epileptic attack, as glycogen may be degraded down to glucose-6-phosphate that can be used to either postpone the epileptic seizures or to provide neurons with energy when they need it. The ability of regulating this metabolism by variation of brain neurotransmitter contents, in particular the 5-HT neurotransmission, may constitute a defense against MSO-dependent epilepsy.

Keywords: Methionine sulfoximine, epilepsy, seizure, neurotransmitter, glycogen, mouse, glycosylation

I. Introduction

Epilepsies express as a variety of symptoms, such as absences, partial and generalized seizures. The World Health Organization (WHO) recognizes at least 40 forms of epilepsies (Dreifuss, 1987, (CCTILAE), 1989, Everitt and Sander, 1999, Wieser et al.., 2001). The symptoms correspond to a sudden and temporary synchronization of neuronal activity, whose origins are not well understood (Johnston, 1996, Dudek et al.., 1999, McCormick and Contreras, 2001, Rampp and Stefan, 2006, Uhlhaas and Singer, 2006, Scharfman, 2007). Medical imagery of epilepsies is based upon various mechanisms; the uptake of glucose analogs representing one of them (Engel et al.., 1982, Engel et al.., 1983, Sitoh and Tien, 1998, Lai et al.., 2010). This allowed the demonstration that epileptic foci are hyper-metabolic during the ictal period and hypometabolic during the inter-ictal period (Engel et al.., 1982, Engel et al.., 1983, Lai et al.., 2010). Such a hypometabolism of epileptic foci

might be due to an intrinsic increase in the capacity for brain energy production as a consequence of various alterations in the cell such as glycolysis, gluconeogenesis or glycogenesis, the latter resulting in an accumulation of glycogen. That increase in glycogen content is difficult to demonstrate in humans. Nevertheless, brain glycogen content could now be considered as an index of brain activity, and is regarded as a diagnostic and therapeutic tool. In the future this could be useful when the inherent difficulties of measuring brain glycogen content using non-invasive methodologies, both in humans and animals are overcome (Gruetter, 2002, Gruetter et al.., 2003, Oz et al.., 2003, Brown, 2004, Brown et al.., 2005, Oz et al.., 2007, Cloix et al.., 2008, van Heeswijk et al.., 2010). However, it has been reported that brain biopsies obtained from the hippocampus of epileptic patients contained high glycogen content compared to grey and white matter from the same individuals (Dalsgaard et al.., 2007). During seizures, when neurons need a lot of energy, since they are starved by their high activities, this energy could come from different sources: blood supply, neuronal glycolysis and/or from a glial source especially from astrocytes. When the first two are outpaced, as astrocytes are the major cells capable of glycogen synthesis and accumulation, the astrocytic glycogen could be mobilized, and astrocyte glycolysis of the G-6-P derived from glycogen could then generate lactate, which is transferred to neurons. Neurons are therefore provided with a metabolite that can be used directly by the mitochondria for ATP production without further processing (Cloix et al.., 2008, Cloix and Hevor, 2009).

Among the various epileptic or seizure-induced models, the MSO-model is one which is chemically induced and in which both associated seizures and a specific increase in brain glycogen content is observed (Folbergrova et al.., 1969, Folbergrova, 1973, Hevor and Delorme, 1991, Hevor, 1994, Bernard-Hélary et al.., 2000, Bernard-Hélary et al.., 2002, Cloix and Hevor, 2009, Cloix et al.., 2010a). This model corresponds to seizures induced by methionine sulfoximine (MSO).

I.1. Historical Background

During the 50's, white food was used for feeding animals instead of crude food. The process used for producing the so called white food was a process designated as "bleaching" or "agenizing" which referred to the oxidation of grain products by the use of nitrogen trichloride. Such a process leads to the degradation of proteins up to the release of amino-acids; among them methionine. The latter of which became oxidized to MSO. Later MSO had been identified as the chemical responsible for the behavior abnormalities observed in animals fed with white food. Among abnormalities seizures were reported and described as the most relevant pathology (Gastaut et al.., 1958, Wolfe and Elliot, 1962, Wada et al.., 1967).

I.2. MSO-Induced Seizures

Animals, especially rats and mice, developed seizures after intraperitoneal administration of a single convulsive dose of MSO, and such seizures resembled the human "grand mal", the most striking and debilitating form of human epilepsy. In treated animals epileptiform

seizures developed during a period of 6-8 hours after MSO dosing (pre-convulsive period), followed by recurrent seizures during 24-48 hours (convulsive or ictal period or *status epilepticus*) and then they recovered normal behavior (post-convulsive period) (Hevor, 1994, Bernard-Hélary et al.., 2000, Bernard-Hélary et al.., 2002, Cloix et al.., 2010b). These time periods of MSO-dependent episodes of epilepsy vary from one animal to another, and according to the inbred mouse strains used. Highest augmentation of glycogen leading to high levels of glycogen accumulation takes place as soon as the MSO-induced pre-convulsive period starts which are specifically confined to astrocytes of the cerebral cortex and cerebellum (Hevor et al.., 1985, Hevor et al.., 1986, Hevor, 1994, Bernard-Hélary et al.., 2000, Bernard-Hélary et al.., 2002). In addition, the action of MSO on recurrent alterations in metabolism is also observed *in vitro* in cultured rat and mouse astrocytes. These data show that such metabolic effects occur independent from seizures and are pharmacologically induced well before seizure onset (Hevor and Delorme, 1991, Hevor, 1994, Bernard-Hélary et al.., 2000, Bernard-Hélary et al.., 2002). Alteration of glycogen content is also observed in other models of induced seizures, such as those depending upon homocysteic acid (Folbergrova et al.., 2008), where the accumulation of glycogen was essentially due to astrocytic metabolism. That is also the case in progressive myoclonus epilepsy where inclusion bodies, resembling abnormal glycogen, accumulate during the disease (Wang et al.., 2007). With one exception (Vilchez et al.., 2007) who described that murine neurons posses also the enzymatic machinery to synthesize glycogen, astrocytes are considered to be the only cell types capable for glycogen synthesis (Magistretti and Pellerin, 1996, Pellegri et al.., 1996, Magistretti et al.., 1999, Magistretti, 2006, Cloix et al.., 2008, Cloix and Hevor, 2009).

Some actions of MSO on glutamate receptors, and inhibition of glutamine synthetase resulting in putative accumulation of glutamate, have been reported as being responsible for MSO-dependent seizures (Peters and Tower, 1959, Griffith and Meister, 1978, Eid et al.., 2004, Eid et al.., 2008) whereas others describe a decrease in the brain glutamate content (Fonnum, 1984, Engelsen and Fonnum, 1985, Fonnum and Paulsen, 1990), or no variation (Somers and Beckstead, 1990), after administration of a convulsive dose of MSO in rats, rather than the expected increase. Furthermore, chronic inhibition of brain glutamine synthetase by MSO did not induce seizures in mice (Engelsen and Fonnum, 1985, Blin et al.., 2002), although a unilateral deficiency of hippocampal glutamine synthetase, induced by MSO, causes seizures in a new "pharmacological kindling" model of epileptic rats (Eid et al.., 2008). The precise mechanism responsible for MSO-dependent seizures is therefore not well defined, and further investigations are needed.

The purpose of the present study is to compare two inbred strains of mice, C57BL/6J and CBA/J, and two inbred lines of mice, iMSO-Fast and iMSO-Slow, selected according to their latency toward MSO-dependent seizures (Cloix et al.., 2010b). Indeed, on account of the high resemblance of MSO models to human epilepsy, and because of the recent metabolic data on the associated conditions, we are interested in addressing the basic mechanisms that generated epileptiform activities in our MSO model which may contribute to a better understanding of epileptogenesis. Our approach was based upon selecting mice that responded differently to the administration of MSO, and upon comparing the metabolism and the behavior of selected inbred lines in order to find abnormalities, which can trigger seizures. In preliminary investigations, based upon the first few stages of inbreeding, we observed that MSO basically separated mice into two types, one that developed seizures minutes after MSO injection (MSO-Fast) and secondly those that developed seizures much later after administration

(MSO-Slow) (Cloix and Hevor, 2009, Cloix et al.., 2010a). The comparison of these various strains of mice revealed different sensitivities toward MSO in terms of latency, brain glycogen and neurotransmitter contents. In addition for the MSO-Fast and MSO-Slow inbred lines we investigated effects of various other convulsants and anticonvulsants, as well as the MSO-dependent changes in brain neurotransmitter and glycogen contents as well as altered behavioral characteristics such as anxiety and the capacity for spatial learning. These latter approaches addressed specifically the involvement of glutamatergic and GABAergic pathways in relation to glutamine synthetase activity, neurotransmitter and glycogen content and their putative interrelationships with behavior.

II. Materials and Methods

II.1. Animals

Ten week-old male CBA/J and C57BL/6J mice were obtained from the Transgenèse et Archivage d'Animaux Modèles (TAAM, CNRS UPS44, Orléans, France). MSO-Slow and MSO-Fast mice were selected according to a previously described procedure using latencies toward a 75 mg/kg dose of MSO (Cloix et al.., 2010b, Boissonnet et al.., 2012). Thereafter, 4 additional MSO-challenges were performed in order to increase the selection pressure: MSO-Slow mice were challenged with 100 mg/kg MSO, while MSO-Fast ones were challenged using 50 mg/kg MSO. This was followed by 10-12 inbreeding generations of brother-sister crosses to generate mice (iMSO-Fast, iMSO-Slow) that were 10 week-old at the time of the experiments. Doses of MSO to induce generalized seizures were chosen according to previously described methods, and data describing the different responses toward various MSO-doses (Cloix et al.., 2010b). All protocols were approved by the local ethical committee with the agreement number CREEA CL2007-023, and were in accordance with the European Community Council Directive of 24 November 1986 (86/609/ECC).

Mice were sacrificed by decapitation, and the heads were immediately frozen in liquid nitrogen and thereafter conserved at -80 °C. Dorsal cortices were dissected from the brain at -20 °C and pulverized with a liquid nitrogen frozen homogenizer, and then kept at -80 °C until used. Brain cortices were homogenized in 5 volumes of the appropriate solution (0.2 M $HClO_4$ for glycogen and neurotransmitter measurements; 100 mM imidazol buffer for glutamine synthetase activity). Protein content was determined using the Lowry procedure (Lowry et al.., 1951) with bovine serum albumin as a standard.

II.2. Chemicals and Seizure Latency

Methionine sulfoximine (MSO), pentylenetetrazole (PTZ, an GABA antagonist), kainic acid (KA, an glutamatergic agonist), valproic acid (VPA, an GABA agonist), pilocarpine (PC, an acetylcholine agonist) and MK-801 (an NMDA antagonist) were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). The chemicals were dissolved in 0.9% NaCl and the pH was adjusted to 7.4 with 1 N NaOH for KA and VPA only. They were administered intraperitoneally (i.p.), and the latencies to seizures were determined as previously described

(Cloix et al.., 2010b). Maximal times of observation were as follows: MSO, 600 min; KA and PC, 120 min; PTZ 180 sec. Mice that did not seize during the observation period were given a score of 600 for MSO, 120 for KA, and 180 for PTZ, and measured latencies included the values corresponding to the time when mice were not convulsing. Doses of various convulsants and anticonvulsants were as follows: MSO, various doses as indicated hereafter and ranging from 0 to 200 mg/kg for MSO-Fast and 0 to 400 mg/kg for MSO-Slow; PTZ, 75 mg/kg; PC, 300 mg/kg; KA, 25 mg/kg; VPA, 250 mg/kg; MK-801, 1 mg/kg. Doses of MSO given to each strain of mice were determined as previously described, and with reference to the sensitivity of each strain for MSO-dependent seizures (Bernard-Hélary et al.., 2000, Bernard-Hélary et al.., 2002, Cloix et al.., 2010b). Such doses corresponded to sub-convulsant doses of MSO. The ED_{50} for MSO were calculated using GraphPad Prism software with a non-linear fitting of data, normalized response and variable slope. In this analysis, only the generalized convulsions were considered. Ten CBA/J and 15 C57BL/6J mice (age 10 weeks) were used. For the experiments with MSO 20 ten-week old mice (10 females and 10 males for each strain) were used.

II.3. Glycogen Measurements

Tissue homogenates were centrifuged at 20,000 × g for 1 h at 4°C, and 100 µl of the supernatants and were buffered at pH 4.8 using 0.2 M sodium acetate buffer. Glycogen in tissue homogenates was cleaved to glucose by α-amyloglucosidase; and the glucose released was measured using a standard glucose oxidase/peroxidase method to obtain total glucose (i.e. free glucose plus glucose from glycogen hydrolysis). Glucose levels were also determined without α-amyloglucosidase hydrolysis to obtain free glucose; glycogen content was calculated by subtraction of total and free glucose levels, as described (Verge et al.., 1996, Cloix et al.., 2010a).

II.4. Measurement of Monoamine Concentrations

Tissue homogenates were centrifuged at 20,000 × g for 1 h at 4°C, and 100 µl of the supernatants and were injected, at least twice, directly into a high-performance liquid chromatograph (HPLC) coupled to an electrochemical detector. Monoamine separations were performed using a Beckman Coulter pump (Gold system 118, Fullerton, California, USA) connected to a Zorbax octadecylsilane column of 4.6 × 250 mm size (Agilent, Palo Alto, California, USA). The electrochemical detector was an amperometric detector (Intro, Antec Leyden, Zoeterwoude, The Netherlands) with a glassy carbon electrode. The mobile phase was run using a Beckman Coulter Pump Gold 118, and was constituted of 100 mM sodium acetate, 20 mM citric acid, 0.4 mM sodium octylsulfate, 0.15 mM disodium EDTA, 8% methanol, pH 5.0. The HPLC was developed using a flow rate gradient summarized in Table 1. The various monoamines were determined, and the retention times were as follows. Tyr (tyrosine), RT=7.28min; DOPAC (3,4-dihydroxyphenylacetic acid), RT=14.12min; NE (norepinephrine), RT=17.68min; MHPG (3-methoxy-4-hydroxyphenylglycol), RT=19.17min; 5-HTP (5-hydroxytryptophan), RT= 23.52min; E (epinephrine), RT=28.63min; 5-HIAA (5-hydroxyindoleacetic acid), RT=29.20min; HVA (homovanillic acid), RT=32.23min; NME

(normetepinephrine), RT=32.65min; Trp (tryptophan), RT=36.50min; DA (dopamine), RT=40.97min; ME (metepinephrine), RT=43.23min; and 5-HT (5-hydroxytrypamine or serotonin), RT=57.97min. Concentrations of the different authentic neurotransmitters were injected at least 3 times, and a standard curve was determined for each neurotransmitter, corresponding to the response of the detector as a function of injected contents. Such standard curves were used to determine the amounts of neurotransmitters in the supernatant from brain cortices, which were expressed as pmol/mg protein. For some neurotransmitters, in order to be sure that the obtained peak corresponded to the standard neurotransmitter, the brain samples were over-loaded with known amount of standard neurotransmitter and injected to the system.

Table 1. Chart of gradient flow rates used in the determination of monoamines using HPLC.

Flow (ml/min)	Duration (min)	Starts at (min)	Ends at (min)
0.5	4.0	0.0	4.0
0.1	0.3	4.0	4.3
0.1	19.7	4.3	24
0.4	0.5	24.0	24.5
0.6	9.5	24.5	34.0
0.6	1.0	34.0	25.0
0.4	0.5	35.0	35.5
0.4	10.5	35.5	46.0
2.5	2.0	46.0	48.0
2.5	9.0	48.0	57.0
0.5	2.0	57.0	59.0
0.5	1.0	59.0	60.0

II.5. Glutamine Synthetase Activity

Ten animals (5 females and 5 males) were used to determine kinetic properties of glutamine synthetase (GS) from both inbred strains of MSO-Fast and MSO-Slow mice. Their cortices were pulverized and the powder was processed, as previously described (Blin et al.., 2002), except for homogenization that was performed in 100 mM imidazol buffer. Protein content was determined using the Lowry procedure (Lowry et al.., 1951). Maximal enzyme activity and substrate affinity were determined by increasing amount of substrate, i.e. glutamate from 0.2 to 50 mM; and the inhibition constant was calculated for MSO varying from 1.5 µM to 25 mM, at a glutamate concentration of 50 mM.

II.6. Behavior and EEG

We tested 40 animals, 20 females and 20 males. The radial-maze test, to determine spatial memory learning capacities, was performed using a previously described method (Blin et al.., 2002). Open-field analysis, to determine anxiety, was performed according to a slight modification of previously described protocols (Belzung and Griebel, 2001, Crusio, 2001,

Einat et al.., 2003). In brief, the open-field design corresponded to a dark plastic homemade open-field with 9 square-cases of 15 cm each, separated by a 2 cm wide blue line. A dark plastic wall of 30 cm height surrounded the 9 cases. Mice were positioned in the centre square and observed for 5 minutes. Before each mouse entered the open-field it was wiped with a paper cloth soaked with 70% ethanol and with water thereafter. The following values were recorded by the same experimenter: time before the first movement, corresponding to the time needed to pass to the next square; number of visited squares, which corresponded to the line crossed by at least the animal's shoulders; the number of total rearing; and the number of defecation pellets. These values were considered as an index of the animal's anxiety (Belzung and Griebel, 2001, Crusio, 2001, Einat et al.., 2003). Electroencephalogram (EEG) recording was performed as follows. Under isoflurane anesthesia (1.5%), mice were implanted with three monopolar surface electrodes placed in the cranial bone. Two electrodes were set bilaterally over the parietal cortex and a ground electrode was placed over the frontal cortex. Electrodes were made of a tungsten wire (diameter 250 µm) soldered to a male connector (Wire-pro, Farnell, Villefranche sur Saône, France). They were inserted in the skull so that only the tip (0.5 mm) protruded onto tissue. The electrodes were glued to the skull with cyanoacrylate and dental cement. EEG was recorded on freely moving mice, after a recovery period of 7-10 days. EEG was monitored for a several hours lasting period (8 h) using a Powerlab 26T model ML856 ADInstruments with a LabChart v7 sofware (Oxford, UK). The behavior of the mice was observed during the same period of time.

II.7. Cell Culture of Astrocytes and Cell Surface Glycoprofilling

II.7.1. Astrocyte Culture

Astrocytes were cultured according to a previously described method (Bernard-Hélary et al.., 2002). The MSO dose of 10 mM and the duration of incubation had been previously reported (Bernard-Hélary et al.., 2002). For the detection of the astrocytes that were recognized by lectins, these astrocytes were harvested by treatment for 5 min with PBS containing trypsin (250 µg/ml) and EDTA (200 µg/ml). After harvesting, cells were washed in PBS and collected by centrifugation. The resulting pellet was resuspended at 2 x 10^6 cells/ml and stained with 20 µM diacetylated N-succinimidyl-carboxyfluoresceine (CFSE) for 15 min at 37 °C. After washing with PBS, cells were resuspended at 2 x 10^6 cells/ml in PBS supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$.

II.7.2. Preparation of Lectin Arrays

Lectins are presented as function of their inhibitive saccharide properties. *N*-acetyl-D-galactosamine: BPA (*Bauhinia purpurea* agglutinin) and WFA (*Wisteria floribunda* agglutinin); galactose: MPA (*Maclura pomifera* agglutinin), AIA (*Artocarpus integrifolia* agglutinin) and RCA-I (*Ricinus communis* agglutinin-I); *N*-acetyl-D-glucosamine: GSL-II (*Griffonia simplicifolia* lectin-II); sialic acid: MAA (*Maackia amurensis* agglutinin); complex structures: PHA-E (*Phaseolus vulgaris* agglutinin-E). Lectins were directly printed in triplicates on Biomat (Rovereto, Italy) fluorescent black 96 wells plates according to GLYcoDiag technology (Landemarre et al.., 2013, Landemarre and Duverger, 2013).

II.7.3. Application to Lectin Array and Data Processing

One hundred µl (about 2 x 10^5 cells) were incubated for 2h at 37 °C under gentle agitation (25 rpm). After washing, fluorescence intensity was measured using Alpha Fusion universal microplate analyzer (Packard).

II.8. Statistical Analyses

Data are expressed as mean ± standard error of the mean (SEM) for the numbers of animals indicated. The results of the latency toward MSO, various convulsants and anticonvulsants, and glutamine synthetase activity were analyzed using Student *t*-test. The results for glycogen and neurotransmitter variations and for radial-maze were evaluated by repeated measures of ANalysis Of VAriance (ANOVA), with STRAIN (MSO-Slow *vs* MSO-Fast) and SEX as between-subjects factors, and DAYS as within-subject factor. Strain and sex comparisons for individual days and convulsion were made by means of two-way ANOVAs. As some animals submitted to radial-maze did not always take the food reward the first time they entered an arm on days 1 and 2, only the data from days 3-5 were used for the statistical evaluation. The results of open-field trials were evaluated by repeated measures of ANOVA, with STRAIN (MSO-Slow *vs* MSO-Fast) and SEX as between-subject factors. Data was considered significant when the *p* value was less than 0.05%.

III. Results

III.1. MSO Induced Various Latencies in the Four Strains of Mice

When MSO was i.p. administered to various mouse strains as a unique dose latencies toward MSO-dependent generalized seizures varied, as exemplified in Figure 1. Although the convulsant doses were not the same, a significant difference in latencies was observed between CBA/J and C57BL/6J mice, and between MSO-Fast and MSO-Slow mice. The latency period towards MSO-dependent seizures was significantly increased in C57BL/6J than that in CBA/J, and in MSO-Slow when compared to MSO-Fast. Moreover, latencies obtained after MSO administration were significantly different from each other, as determined using ANOVA analysis (Figure 1A). The percentage of convulsing mice was differed among the four stains of mice. The order of the decreasing latencies of convulsing mice after MSO administration was MSO-Slow, C57BL/6J, CBA/J, and MSO-Fast, the latter being the fastest responder toward MSO (Figure 1B). These data were in agreement with the ED_{50} of MSO that induced seizures in 50% of mice (Figure 1C). The most resistant strain was MSO-Slow as the ED_{50} of MSO was not computable, since only 25-30% of mice seized when given large MSO-doses such as 300-500 mg/kg. The ED_{50} of MSO was significantly different between CBA/J and C57BL/6J, and between MSO-Fast and MSO-Slow. An increasing ED_{50} of MSO was observed in the following order: MSO-Fast ≈ CBA/J < C57BL/6J < MSO-Slow. In addition, ED_{50} values obtained after MSO administration were significantly different from each other, as determined by ANOVA analysis (Figure 1C). In addition, it should be mentioned that no seizure was observed during the test duration of 10 hours after administration of 75 mg/kg MSO to MSO-Slow inbred mice. These data strongly suggested

that MSO-Slow were really resistant to MSO-dependent seizures whereas MSO-Fast ones were highly sensitive.

C57BL/6J and CBA/J showed an intermediate responsiveness toward MSO indicating that C57BL/6J the more resistant one of the two. Moreover, the data revealed that the selection of the MSO-Fast and MSO-Slow strains (Cloix et al.., 2010a, Cloix et al.., 2010b) was efficient enough to produce two strains each with a different, extreme responsiveness toward MSO-induced seizures. These data showed that the selection of inbred iMSO-Slow and iMSO-Fast provided experimental animals to address various modes of action of MSO in a more pronounced manner compared to other animal models as intended.

III.2. MSO Induced a Large Increase in Brain Glycogen Content in the Four Strains of Mice

The different time courses observed for the increasing glycogen content in cerebral cortices of mice after an injection of a single dose of MSO are presented in Figure 1D. In all strains, glycogen increased significantly as a function of time. However, the observed increase differed in a strain-specific manner. Glycogen increased to significantly higher levels in C57BL/6J than in CBA/J and it was significantly higher in MSO-Slow than in MSO-Fast mice. Moreover, glycogen variation showed no difference between CBA/J and MSO-Fast mice, the latter two containing significantly lower glycogen levels compared to both MSO-Slow and C57BL/6J.

The presented data suggested that, in terms of cerebral glycogen accumulation, the two strains of mice, C57BL/6J and CBA/J, are intermediate to the two selected strains, MSO-Fast and MSO-Slow. Comparing the obtained data for the glycogen content with the one observed for seizure responsiveness (Figure 1A and 1B), it became obvious that increasing amounts of glycogen correlated with longer latency periods towards MSO-induced seizures, as previously hypothesized (Bernard-Hélary et al.., 2000, Belzung and Griebel, 2001, Crusio, 2001, Bernard-Hélary et al.., 2002, Einat et al.., 2003).

Therefore, as CBA/J and C57BL/6J are in between MSO-Fast and MSO-Slow for the above reported effects of MSO, it might be more than relevant to obtain inbred MSO-Fast and MSO-Slow mice. Thus, we decided to further enhance the diverting responsiveness toward MSO-induced effects of the selected MSO-Fast and MSO-Slow strains by an additional sequence of inbreeding (Fig. 2). The comparison of the responses of these two new "hyperinbred" lines designated as iMSO-Fast and iMSO-Slow with that of their respective parent strains was expected to provide a better understanding of the causes why animals respond differently to MSO- or pharmacologically-induced seizures.

III.3. Selection of MSO-Fast and MSO-Slow Mice

The process of selection of MSO-Fast and MSO-Slow mice was schematized on Figure 2 (Cloix et al.., 2010a, Cloix et al.., 2010b, Boissonnet et al.., 2012). The G0 was generated employing a genetic mixture of 8 different inbred strains of mice. During the 6 first steps in the selection process each mouse was challenged with 75 mg/kg MSO. Mice with the shortest latencies were inter-crossed to lead to a MSO sensitive line, or MSO-Fast mice as shown in

Fig. 2. Conversely, mice with the longest latency period were inter-crossed to give rise to the MSO resistant line, or MSO-Slow mice (Fig. 2). After these first 6 challenges, the selection pressure was increased, and MSO-Fast mice and MSO-Slow mice were challenged using 50 and 100 mg/kg MSO, respectively. After these two steps, during which the crosses between sister and brother were avoided and the response toward MSO was considered as obtained, the process of inbreeding was started for at least 12 crosses between sister and brother only. Afterward, the obtained iMSO-Fast and iMSO-Slow mice were considered as inbred.

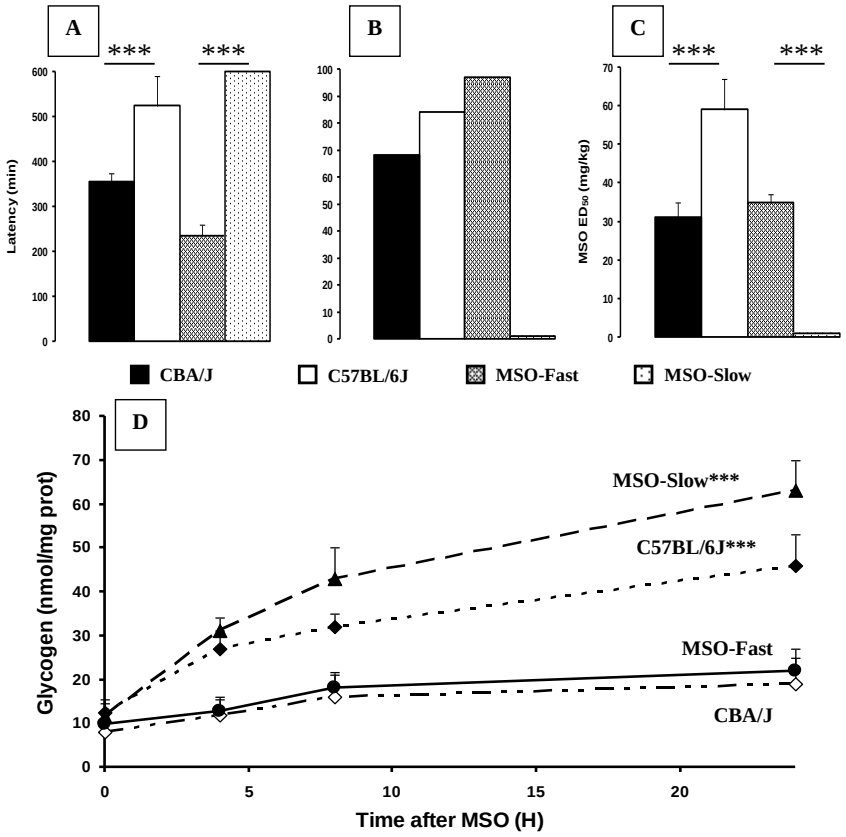

Figure 1. Characteristics of MSO-dependent seizures within two inbred mouse strains, and MSO-Fast and MSO-Slow after G6. MSO was administered using intraperitoneal injection as a unique dose to different inbred strains of mice of both sexes: CBA/J, 40 mg/kg, n=10; C57BL/6J, 100 mg/kg, n=15; MSO-Fast, 75 mg/kg, n=20; MSO-Slow, 250 mg/kg, n=20. A: Latency toward MSO determined as time-duration between MSO injection and the first generalized seizure. When mice did not seize during the 10 hour period of observation a score of 600 was assigned. B: Percentage of mice that seized. C: ED_{50} of MSO was determined as previously described (Bernard-Hélary et al.., 2002). NC: no computable value using GraphPrims software. Values are expressed as mean ± SEM, and significance between two inbred mice, C57BL/6J *vs* CBA/J, MSO-Fast *vs* MSO-Slow, was assumed using Student *t*-test as $p<0.001$: ***. D: Variation of brain glycogen content in the four strains of mice. Mice were given an unique dose of MSO according to the strain: CBA/J, 40 mg/kg, n=10; MSO-Fast, 75 mg/kg, n=20; C57BL/6J, 100 mg/kg, n=15; MSO-Slow, 75 mg/kg, n=20. Mice were sacrificed by decapitation at various times as indicated and glycogen was determined as previously described in materials and methods (Cloix et al.., 2010a). Values are expressed as mean ± SEM, and significance between glycogen variation as a function of time in inbred mice was tested using repeated ANOVA test as $p<0.001$: ***.

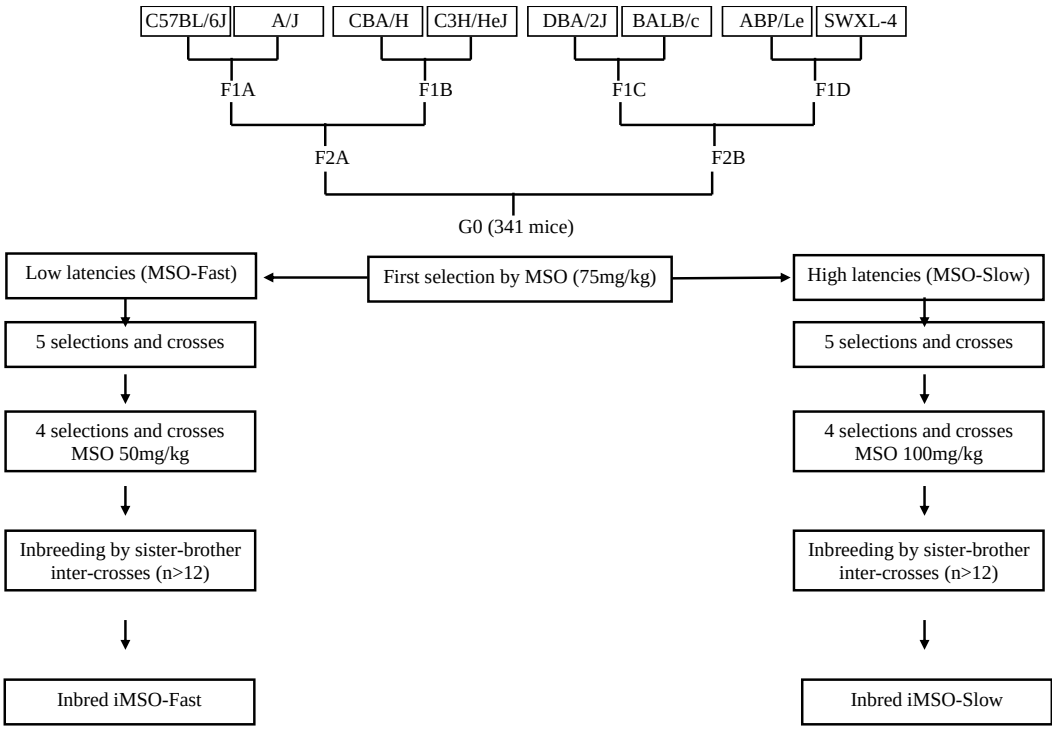

Figure 2. Selection of MSO-Fast and MSO-Slow inbred mice based upon MSO-dependent seizures latencies.

These iMSO-Fast and iMSO-Slow mice after inbreeding were characterized and in part compared to their respective ancestors (MSO-Fast; MSO-Slow, G6 = sixth generation) in terms of certain parameters specified below.

III.4. Response Toward MSO of iMSO-Fast and iMSO-Slow Mice

III.4.1. Latency and Seizures

The MSO-dependent seizure responses were analyzed in both strains, iMSO-Fast and iMSO-Slow during and after the selection process, and are exemplified in Figure 3. In terms of latencies, iMSO-Slow mice were significantly lower responders or no-responders toward MSO than other lines of inbred mice (Figure 3A). These data were similar to those obtained when only mice that seized were considered (Figure 3B). The reported data confirmed the usefulness of the two inbred strains, iMSO-Fast and iMSO-Slow. These inbred mice offered the potential for studying the mechanisms involved in MSO-dependent seizure induction.

These data were corroborated by the EEG measured in inbred MSO-Fast and MSO-Slow mice (Figure 4) receiving 75 mg/kg MSO to MSO-Fast and 350 mg/kg MSO to MSO-Slow. The MSO-Fast indeed responded few alterations in EEG 2 hours after MSO administration which were higher than those observed at the same time point in MSO-Slow. Moreover, 4½ hours after MSO injection EEG was deeply altered in MSO-Fast mice whereas a more moderate modification could be observed in inbred MSO-Slow mice.

Altogether, these data demonstrated that the selection process indeed produced two different inbred lines of mice, which differed in terms of their seizure responses toward MSO

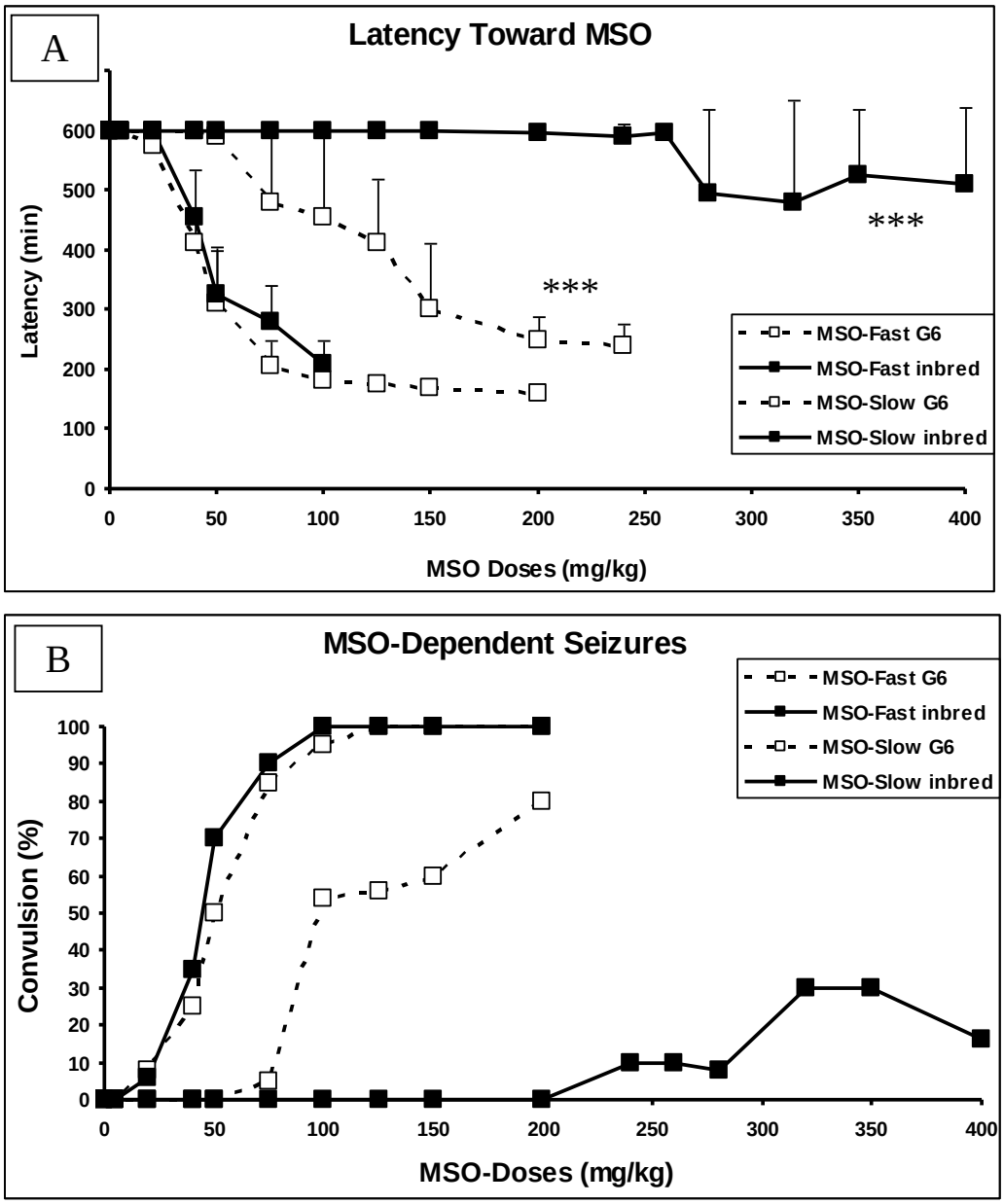

Figure 3. Characteristics of MSO-dependent seizures of MSO-Fast and MSO-Slow of both sexes (20 mice, 10♂, 10♀). Used mice corresponded to either G6 mice or inbred mice, i.e., iMSO-fast and iMSO-Slow mice. A: Latencies toward various doses of MSO administered i.p. Latencies were determined as time period between MSO injection and the first observed generalized seizure. Mice which did not seize were arbitrarily given a score of 600. ***: $p<0.001$ using ANOVA analysis. B: Seizures were expressed as percentage of seizing mice.

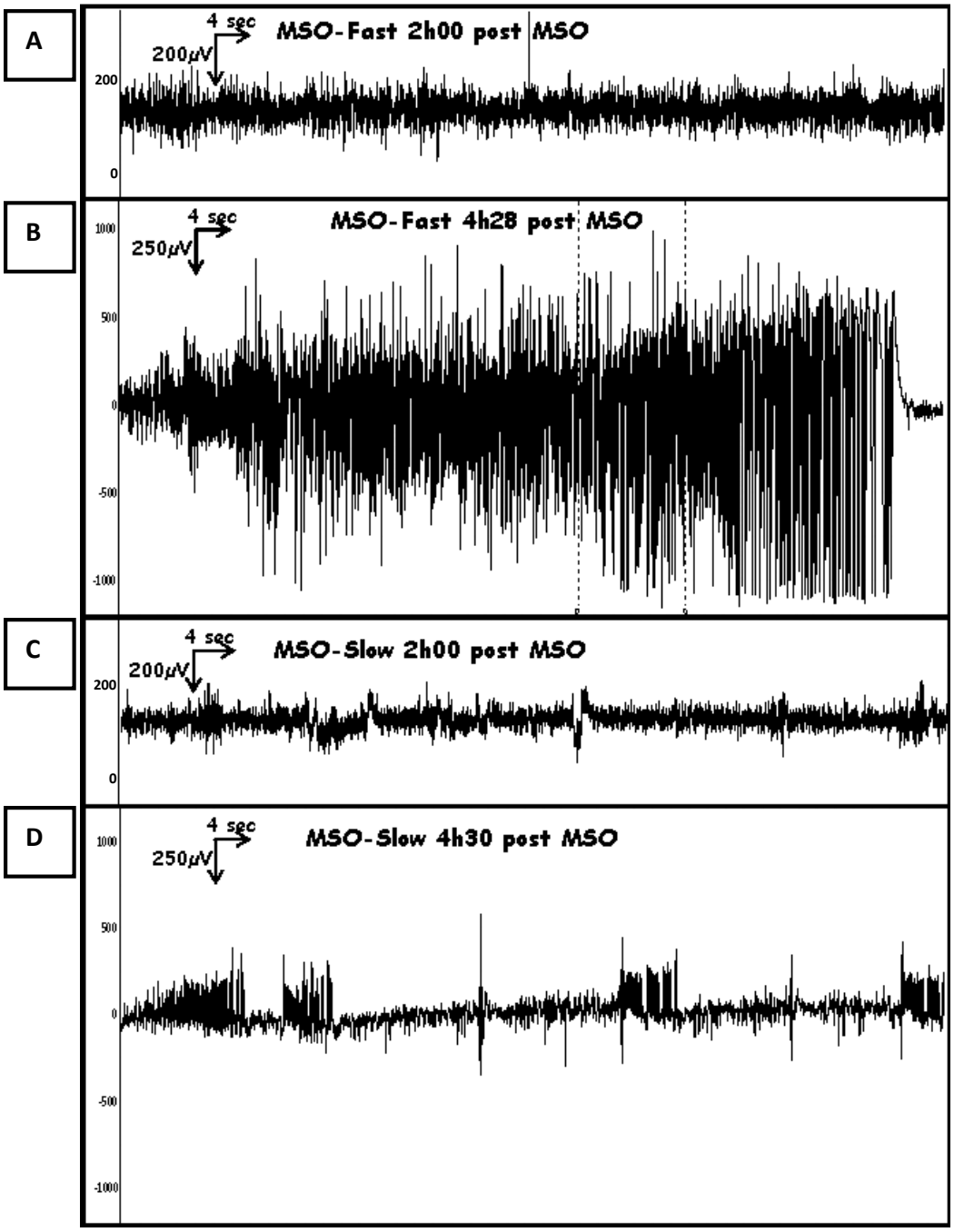

Figure 4. Typical electroencephalogram for MSO-Fast (A, B) and MSO-Slow mice (C, D) of both sexes at G6. EEG was continually recorded according to the method described either 2 hours after 75 mg/kg MSO (A, C) or 4½ hours after 350 mg/kg MSO (B, D). MSO-Fast and MSO-Slow mice were challenged with a dose of MSO derived from data presented Figure 3.

III.4.2. Responses of iMSO-Fast and iMSO-Slow to Other Convulsants and Anticonvulsants

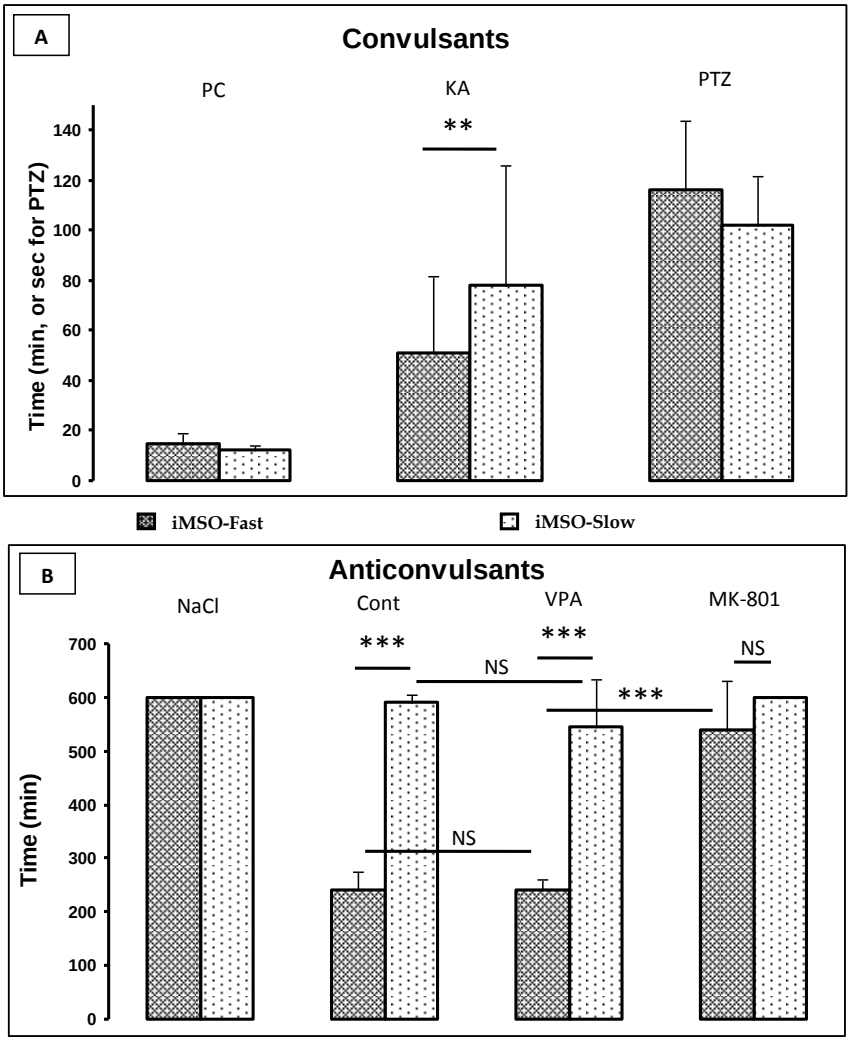

Figure 5. Latency towards various convulsants (A) and effect of two anticonvulsants (B) in MSO-Fast and MSO-Slow mice (n=20, 10 ♂, 10 ♀). MSO was i.p. administered to iMSO-Fast and iMSO-Slow at doses of 75 and 350 mg/kg, respectively. A: Mice were given convulsants pilocarpine (PC, 300mg/kg), kainic acid (KA, 25mg/kg), pentylenetetrazole (PTZ , 75mg/kg). The latency was determined and mice not convulsing were given a score of 60 minutes for pilocarpine, 120 minutes for kainic acid, and 180 seconds for PTZ. B: Thirty minutes before MSO mice were given saline (cont), or MK-801 (1mg/kg) or valproic acid (VPA, 250mg/kg). The latency was determined and mice not convulsing were given a score of 600 minutes for MSO (NaCl, given instead of anti-convulsant). Values are expressed as mean ± SEM, and significance between latencies in MSO-Fast and MSO-Slow inbred mice was determined using Student *t*-test as $p < 0.05$: *; $p < 0.001$: ***.

Figure 5 shows for iMSO-Fast and iMSO-Slow mice their responses towards other convulsants as well as their responses to anti-convulsants after a convulsive dose of MSO. The only convulsant, other than MSO, which demonstrated a significant difference between

the two inbred MSO-strains was kainic acid. Latencies toward seizures induced by kainic acid were indeed longer in iMSO-Slow mice than in iMSO-Fast. Moreover, only MK-801 was able to significantly antagonize the MSO-dependent seizures in iMSO-Fast mice. From these data, we suggested that MSO might act through the glutamatergic pathway rather than the GABAergic one. These data were consistent with the putative mechanism of action for MSO as previously reported (Meister, 1978, Eid et al.., 2008, Perez et al.., 2012).

III.4.3. Brain Glutamine Synthetase Activity

Since the data indicated a preferential involvement of the glutamatergic pathway during the selection process of MSO-Fast and MSO-Slow mice, the activity of the main enzyme responsible for transforming glutamate to glutamine, characteristics of glutamine synthetase (GS), were evaluated in cerebral cortices of both MSO-Fast and MSO-Slow mice (Figure 6). V*max* (Figure 6A) was therefore determined by increasing the glutamate concentrations, and in this condition the V*max* for GS from iMSO-Fast was significantly higher compared to that of iMSO-Slow. When Km values for glutamate and K*i* for MSO were addressed also (Figure 6B), no differences were observed between iMSO-Fast and iMSO-Slow mice.

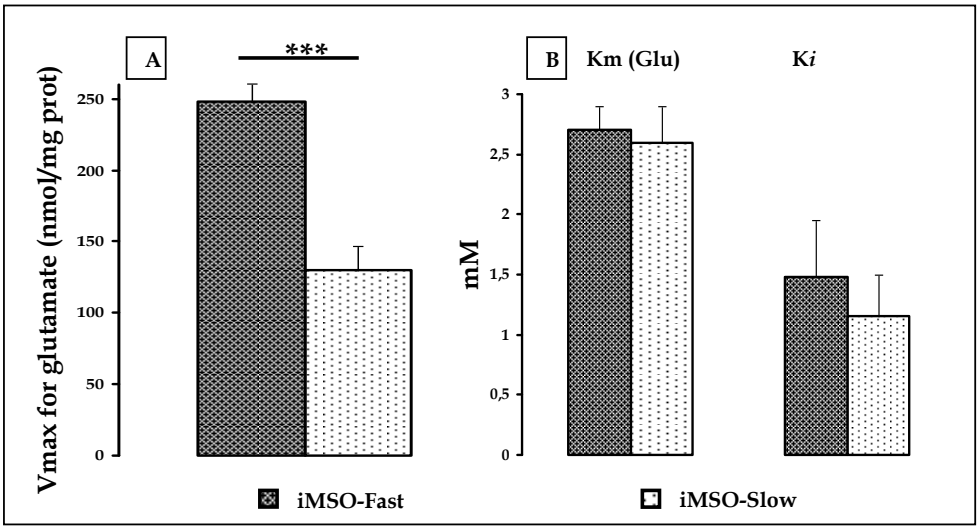

Figure 6. Glutamine synthetase (GS) activity of cerebral cortices of iMSO-Fast and iMSO-Slow mice. GS activity was determined in cortices of both inbred mice as previously described (Blin et al.., 2002). A: V*max* values were determined with increasing concentrations of glutamate ranging from 0.2 to 50 mM. B: Determination of Km value for glutamate using varying glutamate concentration from 0.2 to 50 mM, and K*i* value for MSO using increasing concentration of MSO from 1.5 µM to 25 mM with saturating glutamate concentration of 50 mM. Values are expressed as mean ± SEM and significance between iMSO-Fast (n=10) and iMSO-Slow (n=10) inbred mice of both sexes, was assumed using Student *t*-test as $p<0.001$: ***.

These data suggested either an intrinsically higher GS activity, or a higher level of enzyme expression in the iMSO-Slow lines compared to iMSO-Fast ones. Similarly, since no significant difference for GS-inhibition by MSO was observed between the two strains of mice, it could be concluded that the sensitivity of GS toward MSO might not be different among the strains and was therefore not responsible for the differing responses towards MSO-induced seizure activity. Moreover, since the activity of GS is significantly different between

the two strains of mice, the glutamate/glutamine cycle may be involved in the process of MSO-dependent seizures within.

III.4.4. MSO Induced Variation in Neurotransmitter Contents in Brain Cortices

The above results suggested that the observed differences in GS-activity may lead to different concentrations of glutamine derived neurotransmitters such as Glu and GABA as well as others; we therefore analyzed their cerebral contents in both iMSO-Fast and iMSO-Slow mice either after MSO administration or under control conditions (Figures 7 and 8).

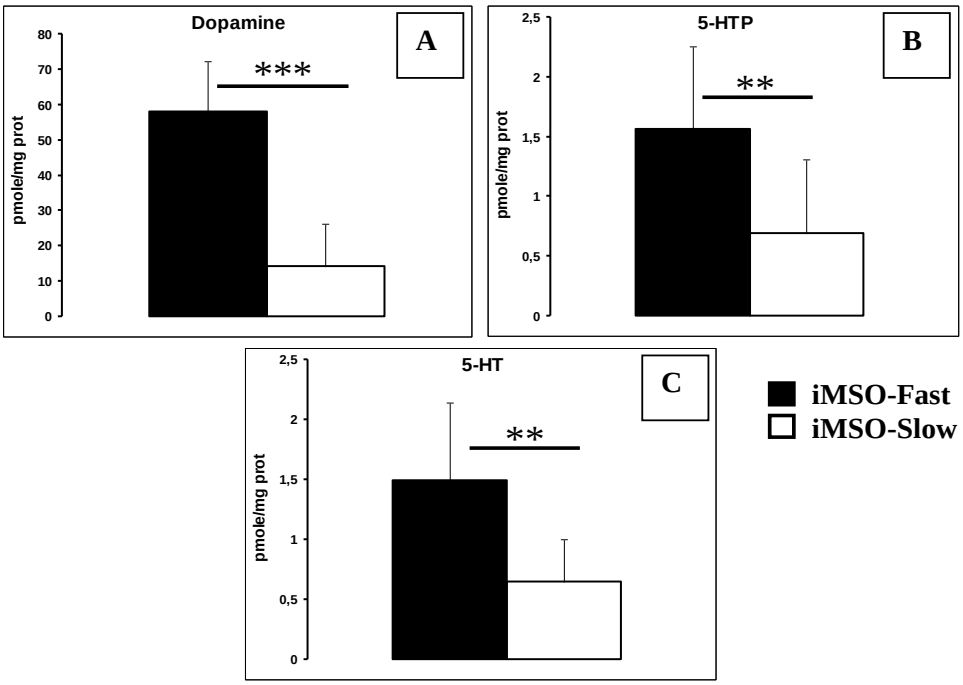

Figure 7. Variation of brain cortex basal content of DA (A), 5-HTP (B) and 5-HT (C) in inbred iMSO-Fast and iMSO-Slow mice. Mice were sacrificed by decapitation and neurotransmitters were determined using a reverse-phase HPLC coupled to an amperometric detection as previously described in the section of materials and methods (Cloix et al.., 2010a). Values are expressed as mean ± SEM, and significance was assumed *t* test $p<0.01$: **; $p<0.001$: ***, for n=8, (4 ♂ and 4 ♀).

Basal levels of cerebral neurotransmitters (Figure 7) demonstrated significantly higher levels in iMSO-Fast than in iMSO-Slow for DA (Figure 7A), 5-HTP (precursor of 5-HT; Figure 7B) and 5-HT (Figure 7C) only. The cerebral levels of other neurotransmitters were not significantly changed. Therefore, in basal conditions, cerebral contents of DA, 5-HTP, and 5-HT itself were significantly increased in iMSO-Fast mice. The high 5-HT content might be relevant in iMSO-Fast mice since 5-HT had been reported as an anti-convulsant (Blizard and Balkoski, 1982) and described as modified in epileptic models (King and LaMotte, 1989, Pranzatelli and Nadi, 1995). We hypothesize therefore that 5-HT was increased in MSO-Fast mice in order to cope with a higher risk of hyperexcitation even under normal conditions.

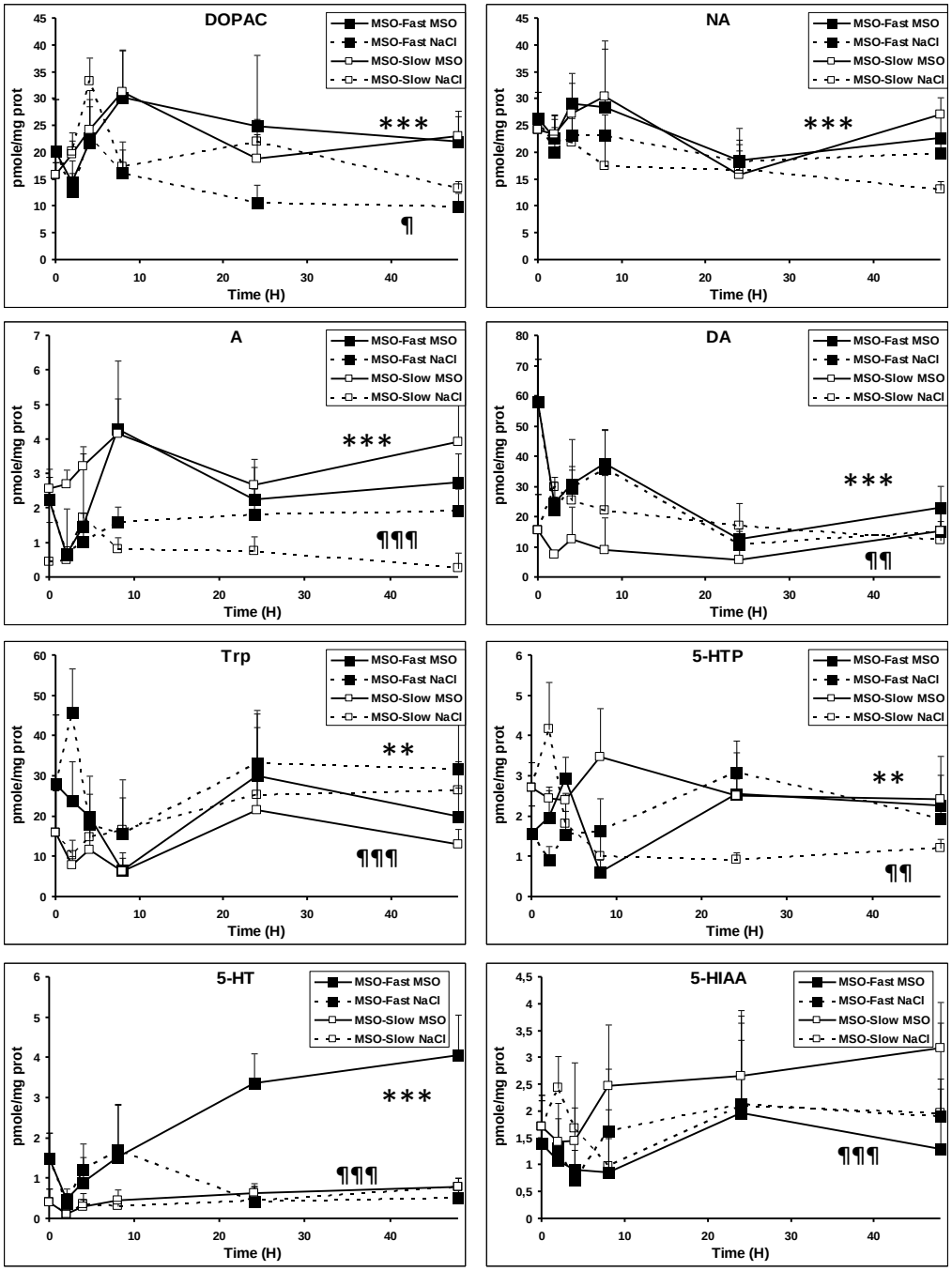

Figure 8. Variation of brain cortex content of monoamines in inbred iMSO-Fast and iMSO-Slow mice after administration of either 75 mg/kg MSO (n=10) or NaCl (n=10). Mice were sacrificed by decapitation at various indicated time and neurotransmitters were determined using an HPLC coupled to an amperometric detection as previously described in the section of materials and methods (Cloix et al.., 2010a). Values are expressed as mean ± SEM, and significance was assumed using repeated ANOVA test: $p<0.01$: **; $p<0.001$: *** when considering effect of treatment, i.e. MSO *vs* NaCl, in both inbred lines; and $p<0.05$: ¶; $p<0.01$: ¶¶; $p<0.001$: ¶¶¶ when considering differences between iMSO-Fast and iMSO-Slow, in both treatments.

The significant effects on neurotransmitter changes after challenging both inbred MSO-strains with MSO compared to saline are shown in Figure 8. Time variation of DOPAC (3,4-dihydroxyphenylacetic acid), NA (norepinephrine), A (epinephrine), DA (dopamine), Trp (trytophan), 5-HTP (5-hydroxytryptophan) and 5-HT (5-hydroxytrypamine or serotonin) were significantly increased by MSO treatment when compared to control mice in both inbred strains. In terms of difference in the variation of monoamines between iMSO-Fast and iMSO-Slow mice, a significant difference between the two strains was observed for DOPAC, A, DA, Trp, 5-HTP, 5HT and 5-HIAA (5-hydroxyindoleacetic acid). The MSO-induced changes of these monoamines were higher in iMSO-Fast mice than in iMSO-Slow ones, except for Trp and DA which underwent similar alterations in both strains.

All these results indicated that the serotinergic pathway was more activated in iMSO-Fast mice when compared to iMSO-Slow, whereby MSO induced an increase in both strains. Taken together it suggested that the basal synthesis and utilization of 5-HT is enhanced in iMSO-Fast mice compared to iMSO-Slow. The latter may imply a role for a 5-HT related mechanisms which became activated in order to cope with MSO-dependent seizures.

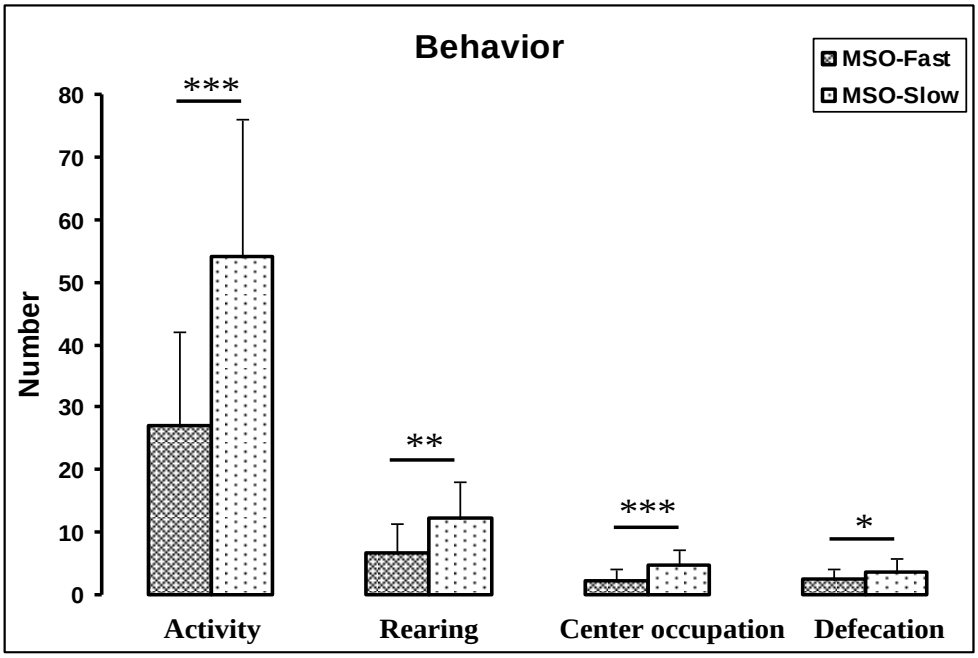

Figure 9. Behavior of iMSO-Fast and iMSO-Slow mice. Mice were put in the central square of the open-field and observed for 5 minutes. Activity corresponded to the numbers of crossed lines by the shoulder of mice. Center occupation corresponded to the numbers of return to the central square. Defecation corresponded to the number of feces pellet. Values are expressed as mean ± SEM and significance between iMSO-Fast (n=40, 20 ♂, 20 ♀) and iMSO-Slow (n=40, 20 ♂, 20 ♀) inbred mice, was assumed using Student *t*-test as $p<0.05$: *, $p<0.01$: **, $p<0.001$: ***.

III.4.5. Behavioral Studies

The spatial memory and the anxiety of iMSO-Fast and iMSO-Slow mice were compared. No significant differences between the selected strains could be observed in terms of spatial

learning capacities (data not shown), and the data on anxiety analyzed by the open-field test are presented in Figure 9.

In general, iMSO-Slow mice were more active than iMSO-Fast, which had been validated by the significantly lower values, observed for iMSO-Fast than for MSO-Slow, in terms of number of squares visited and rearing, the time spent in the central case before mice entered the neighbor case, and the number of defecation determined in the open-field (Figure 9). These values were considered as an index of anxiety for mice (Belzung and Griebel, 2001, Crusio, 2001, Einat et al.., 2003). It seems quite obvious that iMSO-Fast mice were more anxious than the MSO-Slow. This might not be directly related to acute MSO-dependent effects and could be a consequence of the selection process. In the latter case, MSO challenging in the course of producing these two strains (MSO-Fast and MSO-Slow) the strain which suffered more may have already inherited a phenotypic more pronounced anxiety behavior which has to be addressed in future studies especially to obtain information on whether epigenetic factors are related to this phenomenon.

Our data were in agreement with reports underlining the involvement of the glutamatergic system in learning capacity and in anxiety. Therefore, we can assume that the data indicating higher GS activity in iMSO-Slow than in iMSO-Fast could possibly contribute to a higher level of anxiety in MSO-Fast as compared to MSO-Slow (Mohler, 2006, Hashimoto et al.., 2007, Venault and Chapouthier, 2007b, Kanner, 2009, Duvoisin et al.., 2010a, Duvoisin et al.., 2010b). Furthermore, for MSO-induced seizures in rats, focal changes in neurotransmitters binding have been described for hippocampal formation as well as for the amygdala, the latter of which is a key structure within the cerebral anxiety circuitry (Cremer et al.., 2009, Cremer et al.., 2010, Pape et al.., 2010, Pape and Pare, 2010) which may indicate that even during the selection process of the strains alterations within the amygdala may have led to differences observed in these two inbred strains.

III.4.6. MSO Induced Variation in Glycogen Contents in Cerebral Cortices

Basal glycogen content and variation of glycogen as a function of time are shown in Figure 10. The basal content of glycogen in cerebral cortices (Figure 10A) was significantly elevated in iMSO-Fast mice compared to iMSO-Slow. Consequently, the variation of brain glycogen content after MSO administration is higher in iMSO-Fast mice than in iMSO-Slow (Figure 10B). In parallel, glycogen content of iMSO-Fast and iMSO-Slow mice did not change after saline administration. These data demonstrated that in iMSO-Fast mice the glycogen level as well as the changes in the level of glycogen in response to MSO was higher compared to iMSO-Slow mice. Therefore, in iMSO-Fast higher amounts of cortical glycogen could theoretically be used to support neurons during their more pronounced seizure activity. iMSO-Fast mice could use the elevated glycogen-produced G-6-P to provide neurons with directly utilizable metabolites to support their energy demand. Conversely, iMSO-Slow either could not provide neurons with energy or the use of glycogen-derived G-6-P was more rapidly utilized to postpone seizures. However, it should be recognized that in iMSO-Fast mice the basal cerebral amount of glycogen was increased when compared to their ancestral generation MSO-Fast G6 (Figs. 1 and 10). Therefore our data do not support the previously reported view that increasing amounts of glycogen may be directly involved in a prolongation of the latency period. This is because latency toward MSO-induced seizures was significantly shorter in iMSO-Fast despite their high basal glycogen content and their more pronounced glycogen enhancement iMSO-Fast mice provide clear evidence that higher glycogen content

on its own is not directly involved in the suppression of mechanisms involved in seizure generation.

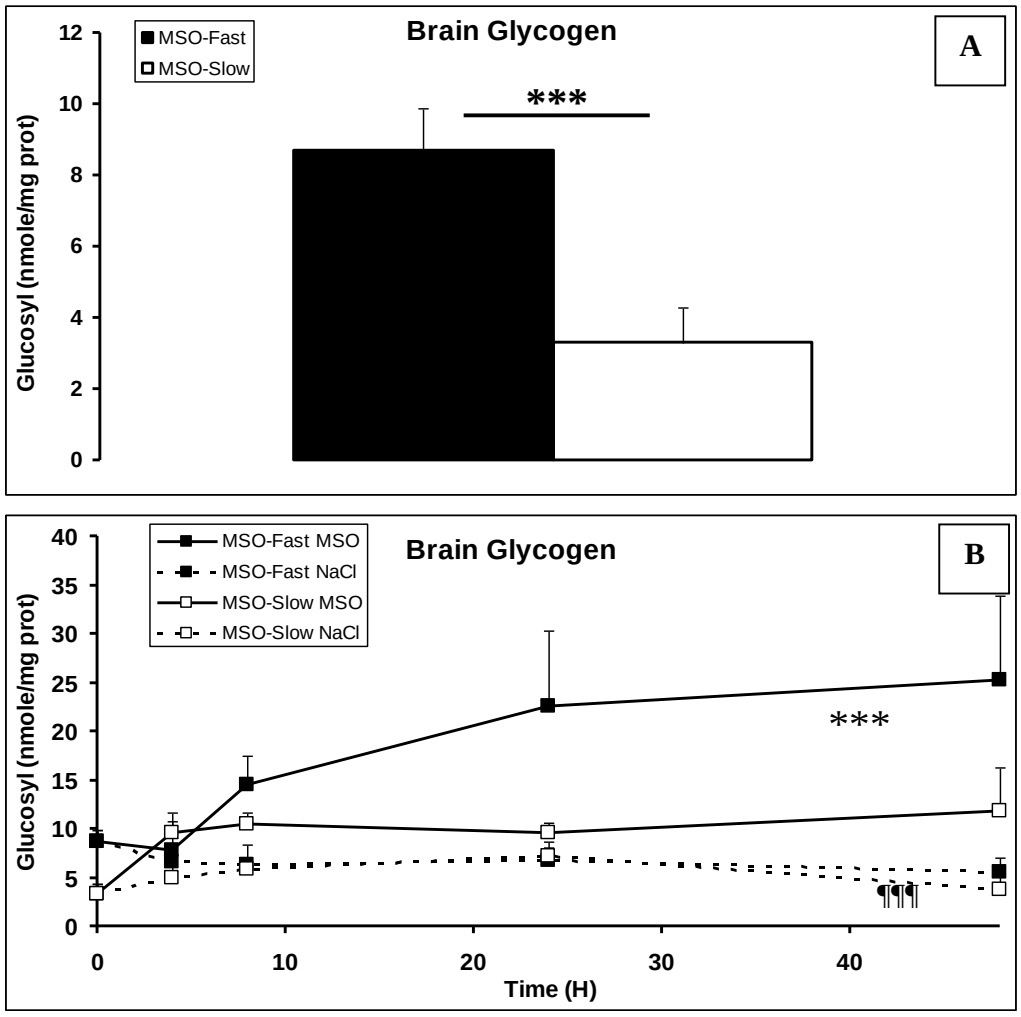

Figure 10. Brain glycogen content determined in basal conditions (A) and after administration of either 75 mg/kg MSO of saline (B) to iMSO-Fast and iMSO-Slow mice. Values are expressed as mean ± SEM (n=10), and significance was assumed using either Student *t* test (A) or repeated ANOVA test (B), when considering effect of treatment, i.e. MSO *vs* NaCl, in both inbred lines: $p<0.001$: ***; and when considering effect of ID, i.e. MSO-Fast *vs* MSO-Slow, in both treatments: $p<0.001$: ¶¶¶, respectively.

III.5. MSO Induced Variation upon Astrocyte Cell Surface in Cerebral Cortices

Astrocytes are able to accumulate glycogen in the brain which could be transformed down to G-6-P and various other glucose metabolites. Xenobiotics such as MSO sometimes modify signaling thereby inducing major changes in cells. As glycosylation is an important way to cell signaling we tried to evaluated the glycosylation of astrocyte membrane proteins

in order to look for a possible involvement in altered astrocyte metabolism. For this cell membrane glycans of primary astrocyte cultures were analyzed by glycoprofiling (Figure 11). Data were standardized using the PHA-E signal as reference, and the effect of 10 mM MSO was analyzed 7 hours after its addition to the cultures. The obtained ratios for the interaction between astrocytes treated with 10mM MSO or saline is represented Figure 11. MSO induced higher interactions (ratio >100%) for astrocytes derived from iMSO-Slow mice with WFA, MPA, AIA and MAA lectins whereas a higher ratios of interaction (ratio>100%) from astrocytes of iMSO-Fast mice were observed with WFA, AIA and GSL-II. These data indicated that the above listed lectins recognized glycans which could be involved in MSO-induced changes in the processing of astrocytic cell membrane glycoconjugates. Therefore, we hypothesize that MSO affects extracellular matrix components and related intercellular communication the latter of which could also be contributing to the induction of seizures.

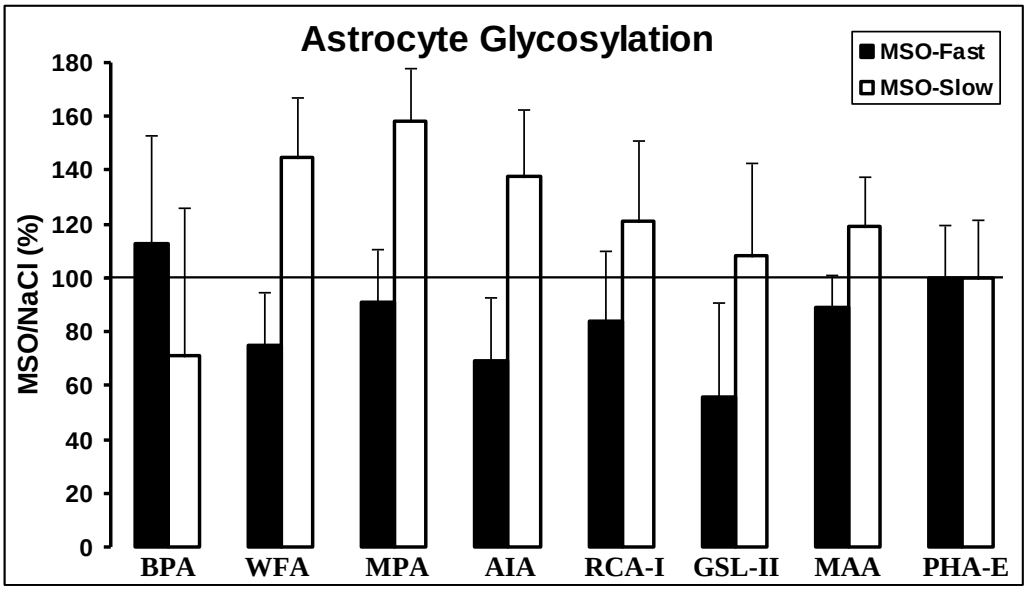

Figure 11. Glycosylation profile of cultured astrocyte cell membrane either after NaCl or 10 mM MSO incubation to both iMSO-Slow and iMSO-Fast mice. The more relevant lectins are shown. Data are expressed as ratio between MSO and NaCl effects standardized to PHA-E variation. Values are expressed as mean ± SEM in 5 different astrocyte cultures.

IV. Discussion

IV.1. Glycogen Changes in Cerebral Cortices

IV.1.1. Glycogen Changes in Cerebral Cortices during the Selection Process

The present data strongly suggest, in terms of seizures and brain glycogen accumulation, that our two lines of mice selected for their latency to MSO-dependent seizures (MSO-Fast and MSO-Slow) are the most extreme mouse strains actually available. The two other strains, CBA/J and C57BL/6J, are in between MSO-Fast and MSO-Slow inbred mice. In terms of latency toward MSO-dependent seizures, indeed the order of responses starting from the

higher responding strain is as follows: MSO-Fast > CBA/J > C57BL/6J > MSO-Slow. Regarding glycogen variation in brain cortices induced by MSO, the order is as follows: MSO-Fast ≈ CBA/J < C57BL/6J < MSO-Slow. Therefore our set of data obtained for the ancestral generations of MSO-Fast and MSO-Slow mice strongly suggested a relationship between the two MSO-induced phenomena.

IV.1.1. Glycogen Changes in Cerebral Cortices After the Inbreeding Process

In terms of involvement of cerebral glycogen in MSO-induced seizures in MSO-Fast and MSO-Slow, i.e. after the inbreeding process of the two strains of mice, we can assume that an increase in brain glycogen content may constitute a way to deal with MSO-dependent seizures, i.e. higher brain glycogen content, higher MSO-dependent seizure latency. The hypothesis, that the ability to increase brain glycogen content may constitute a way to postpone the MSO-dependent seizures, has already been proposed according to our data obtained with CBA/J and C57BL/6J (Bernard-Hélary et al.., 2000, Bernard-Hélary et al.., 2002) and with the ancestral MSO-Fast (G6) and MSO-Slow (G6) mice during their selection process (Cloix and Hevor, 2009, Cloix et al.., 2010b). However, our new strains of iMSO-Fast and iMSO-Slow allowed us to address the role of cerebral glycogen of cortices even further and indeed our data (Figure 10) show higher glycogen levels for iMSO-Fast mice than for iMSO-Slow. Because since iMSO-Fast is still the strain with the faster response toward MSO-induced seizure activity we were able to show for the first time that glycogen by itself does not postpone seizures and may rather be used to adapt the brain in a way so that it can provide the energy needed to support seizure prone neurons in a more optimal way. With the present data, it seems that the previous hypothesis may now be abandoned. Therefore, the inbreeding process resulting in iMSO-Fast mice has led to an adaptation towards a higher energy demand in the newly generated iMSO-Fast strain when compared to iMSO-Slow, whereas the mechanisms contributing to the faster appearance of MSO-induced seizures in the iMSO-Fast strain remained almost unaltered, despite the fact that both strains increased their glycogen content in response to the convulsive dose of MSO. The transposition of this hypothesis to other models of epilepsy is not yet validated, neither is its extension to human disease. Nevertheless, quantification of brain glycogen is now considered as a putative diagnostic and therapeutic tool (Cloix et al.., 2008, Cloix and Hevor, 2009). Indeed, a great deal of effort is being made to determine brain glycogen content using non invasive methods, both in animals and humans (Gruetter, 2002, Gruetter et al.., 2003, Oz et al.., 2003, Oz et al.., 2007, van Heeswijk et al.., 2010). Moreover, as noted earlier, brain glycogen content increases in human epilepsy (Dalsgaard et al.., 2007). Tagliabracci and co-workers implicated abnormal glycogen accumulation as the cause of Lafora disease, a disorder characterized by progressive myoclonic epilepsy (Delgado-Escueta, 2007, Tagliabracci et al.., 2008). These reports are in agreement with the efforts made to determine brain glycogen content in humans, and with the proposed hypothesis of involvement of brain glycogen content in either the control of epilepsy or as an adaptation to higher energy demand within the epileptic brain as may be concluded according to results obtained for iMSO-Fast mice current study.

Taking our new data into account they could be quite consistent with the reported elevation in human brain glycogen in epileptic patients (Dalsgaard et al.., 2007) because also the long lasting time of ongoing seizures may lead to an additional, plastic increase in brain glycogen content in perifocal cerebral tissue in order to better support neurons which have to cope with an increased risk of hyperexcitation.

However, since in both strains iMSO-Fast and iMSO-Slow cerebral glycogen increased which was more pronounced in iMSO-Fast it may be possible that the iMSO-Slow strain does not only increase cerebral glycogen but at the same uses it more efficiently during the preconvulsive period which may contribute to a delay in seizure appearance. Therefore, future experiments have to be focused on developing techniques which will overcome currently existing methodological constraints to address this question in a reliable manner.

IV.2. Glycosylation Profiles

This high level of brain glycogen content in MSO-Fast inbred mice could contribute to the observed differences between MSO-Fast and MSO-Slow inbred mice in terms of glycosylation profile. Cell surface glycosylation can indeed be modulated or modified after injection of a pharmacological compound and lectins are a tool to observe these modifications (Braza-Boils et al.., 2006). In cultured astrocytes from iMSO-Slow inbred mice, MSO induces an elevation of the proportion of glycans while a diminution is observed for astrocytes derived from iMSO-Fast. First of all these in vitro data provided clear evidence that the astrocytes of both strains responded differently to MSO under otherwise similar conditions. The phenotypic difference in the glycosylation response between the two inbred lines indicated that the resistance to MSO of iMSO-Slow and the sensitivity of iMSO-Fast involves two different mechanisms in terms of glycosylation. These data would strongly support an involvement of the extracellular matrix which relates to the glia-neuron interface and its discussed role in epilepsy including also G protein-coupled receptors, whose expression is mostly regulated by N-glycans (Lanctot et al.., 2006) and/or changes related to extracellular matrix controlled perisynaptic neurotransmitter diffusion affecting perisynaptic receptors (Vargova and Sykova, 2008, Zamecnik et al.., 2012).

IV.3. Role of Neurotransmitters in Cerebral Cortices

The implication of neurotransmitters in the control of glycogen level has been largely documented and demonstrated; particularly, for 5-HT and brain glycogen content (Leonard, 1975, Magistretti, 1988, Darvesh and Gudelsky, 2003, Verleysdonk et al.., 2005). Moreover, hypofunctional serotonergic neurotransmission was described in Lafora epilepsy, and 5-hydroxytryptophan administration was found to be beneficial for patients (Pranzatelli et al.., 1995). Reduced levels of serotonergic receptors have been reported in temporal epileptic foci (Liew et al.., 2009), and 5-HT could play an important role in modulating seizure activity because it appears to inhibit the expression of overt seizure behaviors (Trindade-Filho et al.., 2008). Furthermore, the involvement of 5-HT in the control of MSO-dependent seizures has been suggested (Blizard and Balkoski, 1982); and the present data indicate that 5-HT might be involved in the response toward MSO-induced seizures in all tested strains. The basal level of 5-HT is indeed lower in iMSO-Slow mice compared to iMSO-Fast ones. This might be particularly true considering the potential of 5-HT to antagonize seizures, and the high utilization of 5-HT by synapses to respond to the changes brought about by seizures. In view of the literature discussed above it was expected that iMSO-Fast mice having such high 5-HT levels would be better equipped to suppress seizures compared to iMSO-Slow because the

latter seem to be 5-HT deficient. However, like for the MSO-induced glycogen response (see above) our results show highest 5-HT in the strain with the shortest latency. Therefore, it could be assumed that the mice most prone to excitation may have adapted plastic cerebral changes towards mechanisms to increase 5-HT faster in order to cope more efficiently with the sequelae brought about by hyperexcitation. Whereas the adaptational pressure of the seizure resistant strain iMSO-Slow remained lower and accordingly 5-HT responses toward a convulsive dose of MSO remained low. The latter assumption may be also supported also by the observation that in iMSO-Fast mice not only the MSO-induced increase was affected but also basal 5-HT level was significantly higher compared to iMSO-Slow (see Fig. 7). Otherwise one would have to assume that iMSO-Slow mice use and catabolise their synthesized 5-HTP and 5-HT much faster than iMSO-Fast in order to delay seizures during their prolonged latency period. Our results need further experimental evaluations in order to find out whether the MSO challenge during the selection process may indeed have altered 5-HT level and 5-HT - utilization in brains of selected mice.

IV.4. Possible Involvement of Glutamatergic Pathway in MSO-Induced Seizures

The better sensitivity of the MSO-Fast line to the analogue of glutamate, KA, as compared to the GABA antagonist, PTZ, supposes an involvement of glutamatergic pathways in the action of MSO. Therefore our data indicate that MSO-induced seizure activity acts at least partly through a similar mechanism as KA and those mice which are better adapted to cope with MSO-induced epileptic activity such as iMSO-Slow are using the same mechanism to reduce the effects caused by KA. Therefore one part of the explanation why MSO-Fast mice are more prone to seizures than MSO-Slow relates to their difference in sensitivity towards glutamate. The analyses of KA-dependent latency showed that MSO-Slow mice are more resistant to KA-induced seizures than MSO-Fast ones, which is in agreement with a lower involvement of glutamatergic pathways in MSO-Slow lines. Such an idea is reinforced by the anticonvulsant effect of the MK-801, which blocked selectively NMDA glutamatergic receptors, while less anticonvulsant action was observed with VPA. The present data strongly suggest less involvement of GABAergic pathways as compared to glutamatergic ones in the mechanisms of MSO-dependent seizures. This observation is corroborated by the higher level of GS activity in MSO-Slow compared to the MSO-Fast line, with no significant variation in terms of affinity for glutamate and constant inhibition by MSO. These data indicate a higher conversion of glutamate into glutamine leading to a putative, yet to be demonstrated, lower level of glutamate in MSO-Slow line. The fate of brain glutamate under the influence of MSO, which was utilized to select the mice, is unclear. Nevertheless, the powerful inhibition of GS by MSO was thought to trigger the seizures, as glutamate was supposed to increase as a consequence of GS inhibition. However until today this view is heavily debated, since a decrease in brain glutamate level was observed after the administration of convulsive doses of MSO (Engelsen and Fonnum, 1985, Fonnum and Paulsen, 1990) whereas no variation had been described by Somers and Beckstead (1990). Still an unilateral deficiency of hippocampal GS, induced by MSO, causes seizures in rats (Eid et al.., 2008). In addition, in rats with PTZ-induced repetitive epileptic seizures the regions showing a strong glial heat shock response correspond with reduced GS-activity and GS-nitration, which together are

clear indicators of a nitrosative stress response (Bidmon et al.., 2008) and a recent study shows MSO-induced glutamate accumulation in hippocampal astrocytes (Perez et al.., 2012). As GS is confined to astrocytes, Phelps (Phelps, 1975) suggested that glutamate might be massively drawn on to build the glycogen molecule, since glycogen accumulates to tremendous levels in the brain under the effect of MSO (Folbergrova et al.., 1969, Hevor, 1994, Cloix and Hevor, 2009, Cloix et al.., 2010a). For these reasons, the actual role of the glutamatergic system in the epileptogeny generated by MSO remains unclear and needs further investigations. In addition and in contrast to PTZ-induced seizure (Cremer et al.., 2009) no significant changes in ligand binding towards glutamate receptors of $GABA_A$ receptors had been observed in MSO challenged rats which showed only slight significant alterations for adenosine receptors within hippocampus and amygdala (Cremer et al.., 2010). Taking into account the data discussed above such as minor alterations in cerebral glutamate content and a lack of adaptive receptor alteration, one possible explanation could be that GS the enzyme responsible for glutamate catabolism is solely confined to astrocytes. As already suggested for nitrotyrosine-inhibted GS (Bidmon et al.., 2008) GS inhibition caused by MSO could lead to a pathological microcompartimentalization of glutamate in time without altering the total glutamate concentration within the affected tissue, a view which is supported by the MSO-induced unilateral glutamate accumulation reported by Perez et al.. (2012). The resulting dysbalance in glutamate distribution could cause epileptic activity either by an episodic excessive glutamate overflow from astrocytes or by other changes which may be related to astrocytic glutamate accumulation such as glycogen accumulation and altered protein glycosylation as discussed above.

IV.5. Behavioral Changes

The relationship between brain glutamatergic pathways and epilepsy has already been reported for human epilepsy as well as for animal models of epilepsy (Armijo et al.., 2005, Andrade and Minassian, 2007, Sierra-Paredes and Sierra-Marcuno, 2007, Vincent and Mulle, 2009). Mood and psychiatric disorders in patients have been described as consequences of *status epilepticus* (Kanner, 2008, 2009, Mula and Monaco, 2009). In addition, the population of epileptic patients is more prone to commit suicide (Kanner, 2009); but whether mood and/or psychiatric disorders are related to seizure genesis, or the inverse, remains an unsolved question (Kanner, 2008, 2009). In our experiments the behavior of the two inbred lines of mice was different and indicate that iMSO-Fast mice were more anxious than MSO-Slow. Many reports underline the involvement of the glutamatergic system in learning capacity and in anxiety (Mohler, 2006, Hashimoto et al.., 2007, Venault and Chapouthier, 2007b, Kanner, 2009, Duvoisin et al.., 2010a, Duvoisin et al.., 2010b). In addition, receptors of AMPA and kainate types have been reported to be implicated in memory processes (Perkinton et al.., 1999, Zilles et al.., 2000), while NMDA and metabotropic glutamatergic receptors (mGluR) have been described as involved in anxiety (Nowak et al.., 1995, Platenik et al.., 2000, Duvoisin et al.., 2010a, Duvoisin et al.., 2010b). Moreover, one example is the direct binding of glutamate to cell membranes: glutamate binding to hippocampal membrane fractions of fully kindled rats was significantly higher when compared to controls, the fully kindled rats having poorer learning performance, whereas binding of not fully kindled ones did not differ from that of controls (Rossler et al.., 2000). In our experiments, the difference in GS activity

may account for the difference in anxiety behavior between the two lines due to the pivotal role of the latter enzyme in the glutamate/glutamine pathway. Indeed, even though GS is confined to astrocytes, the latter provide glutamine to neurons, which transform it into glutamate and GABA. Astrocytes withdraw glutamate released by glutamatergic neurons in the synaptic cleft; thus the magnitude of GS activity may influence the glutamate/glutamine cycle in the brain. As this enzyme activity is statistically different between the two inbred strains of mice, it is highly probable that this impacts the behavior of each line differently. In future experiments, blocking glutamate receptors may help to clarify the relationship between the glutamatergic system, behavior, and seizure genesis. Moreover, we reported a higher level of serotonin in the cortex of MSO-Fast compared to MSO-Slow mice (Cloix et al.., 2010a) which could also contribute to the anxiety developed by MSO-Fast mice. This might fit with what is known in depressed patients that have elevated expression of neuronal tryptophan hydroxylase 2 mRNA and protein in midbrain serotonergic neurons, as well as increases in brain serotonin turnover (Lowry et al.., 2008) indicating that both our observed alterations in the amount of 5-HTP and 5-HT might contribute to the observed behavioral differences.

The involvement of the glutamatergic system in the mechanism of MSO epileptogenic activity had been strongly suggested years ago (Griffith and Meister, 1978); but more recent data point to the prominent role of mGluR in various psychiatric disorders in human and in models of mood disorders (Wieronska and Pilc, 2009, Duvoisin et al.., 2010a, Duvoisin et al.., 2010b, Gravius et al.., 2010, Krystal et al.., 2010). Although the role of glutamate was disclaimed in the epileptogenic action of MSO, because of the lack of increase in glutamate content (Engelsen and Fonnum, 1985, Fonnum and Paulsen, 1990, Somers and Beckstead, 1990), recent data (Berlicki, 2008, Eid et al.., 2008, Zou et al.., 2010), together with the present results, this strongly suggest the necessity of a revalidation of the glutamate pathways in the mechanism of action of MSO.

The design of the investigation used during the selection process of iMSO-Fast and iMSO-Slow mice has been described in another model of epilepsy based on the GABAergic system. β-Carboline, an inverse agonist of GABAergic receptors, was used to select two inbred lines of mice having different sensitivity toward seizure genesis (Chapouthier et al.., 1998). Data obtained with this mouse model suggested that the two strains differed in their motor activity, anxiety, aggression, and learning capacity (Guillot et al.., 1999, Rossler et al.., 2000, Suaudeau et al.., 2000, Venault et al.., 2006, Venault and Chapouthier, 2007a). Previous data, in addition to the present work, underlines a parallelism between an impairment of behavior and seizure genesis. The basis of the actual relationship between these two physiological effects remains to be established.

Conclusion

The present investigation compares some characteristics of two commercially available inbred strains of mice, CBA/J and C57BL/6J, with two selected strains of mice, iMSO-Fast and iMSO-Slow, according to their latency toward MSO-dependent seizures. The main result describes a relationship between seizures latency and the accumulation of brain glycogen, the iMSO-Fast and iMSO-Slow mice being the more extreme. This allowed us for the first time to address the role of glycogen accumulation related to epileptic activity in a different way

since in our inbred animal model higher glycogen accumulation is associated with a faster and more severe seizure response which contradicts the current discussion about the functional relation between glycogen and epileptic activity. The implication of brain 5-HT in the responses to MSO, both in terms of seizures and glycogen, seems to be relevant. In addition, iMSO-Fast mice seem more anxious than the MSO-Slow ones. We believe that the use of inbred mice, and particularly the mice newly selected for their sensitivity toward MSO, may be very helpful to better understand the mechanisms underlying the responses to MSO, and could also be useful to extrapolate data obtained with animal models to human disease.

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