Upregulation of brain utrophin does not rescue behavioral alterations in dystrophin-deficient mice

Caroline Perronnet¹,², Carine Chagneau¹,², Pascale Le Blanc¹,², Nathalie Samson-Desvignes¹,², Dominique Mornet³, Serge Laroche¹,², Sabine De La Porte⁴ and Cyrille Vaillend¹,²,

¹Univ Paris-Sud, Centre de Neurosciences Paris-Sud, UMR8195, Orsay F-91405, France, ²CNRS, UMR8195, Orsay F-91405, France, ³INSERM, U1046, Université de Montpellier I, Université Montpellier II, Montpellier, France and ⁴CNRS, Institut de Neurobiologie Alfred Fessard, FRC2118, Laboratoire de Neurobiologie et Développement, UPR3294, Gif sur Yvette F-91198, France

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Dystrophin, the protein responsible for X-linked Duchenne muscular dystrophy (DMD), is normally expressed in both muscle and brain, which explains that its loss also leads to cognitive deficits. The utrophin protein, an autosomal homolog, is a natural candidate for dystrophin replacement in patients. Pharmacological upregulation of endogenous utrophin improves muscle physiology in dystrophin-deficient mdx mice, and represents a potential therapeutic tool that has the advantage of allowing delivery to various organs following peripheral injections. Whether this could alleviate cognitive deficits, however, has not been explored. Here, we first investigated basal expression of all utrophins and dystrophins in the brain of mdx mice and found no evidence for spontaneous compensation by utrophins. Then, we show that systemic chronic, spaced injections of arginine butyrate (AB) alleviate muscle alterations and upregulate utrophin expression in the adult brain of mdx mice. AB selectively upregulated brain utrophin Up395, while reducing expression of Up113 and Up71. This, however, was not associated with a significant improvement of behavioral functions typically affected in mdx mice, which include exploration, emotional reactivity, spatial and fear memories. We suggest that AB did not overcome behavioral and cognitive dysfunctions because the regional and cellular expression of utrophins did not coincide with dystrophin expression in untreated mice, nor did it in AB-treated mice. While treatments based on the modulation of utrophin may alleviate DMD phenotypes in certain organs and tissues that coexpress dystrophins and utrophins in the same cells, improvement of cognitive functions would likely require acting on specific dystrophin-dependent mechanisms.

INTRODUCTION

Dystrophins and utrophins are encoded by distinct genes but belong to a large superfamily of membrane-bound cytoskeletal proteins in which full-length products of ~400 kDa share over 80% structural homology and comprise four domains: N-terminal actin-binding domain, rod-shaped spectrin-like repeats, cysteine-rich and C-terminal domains. Several internal promoters encode distinct full-length proteins and at least three shorter proteins sharing the C-terminal, cysteine-rich and parts of the spectrin-like domains. Named according to their apparent molecular weight, the different dystrophins (Dp427, Dp260, Dp140, Dp116 and Dp71) and utrophins (Up395, Up140, Up113 and Up71) are expressed in a cell-specific manner in a variety of non-muscle tissues, including the brain. Dystrophins and utrophins directly interact with the transmembrane protein β-dystroglycan (β-DG), a central component of the dystrophin- and utrophin-associated scaffolding complexes that link extracellular matrix (ECM) proteins to the actin-based cytoskeleton and are involved in the clustering of receptors, ion channels and signaling proteins (1).
Genetic loss of the full-length dystrophin (Dp427) causes Duchenne muscular dystrophy (DMD), an X-linked recessive neuromuscular disorder characterized by muscle degeneration and cognitive impairments (2,3). The loss of Dp427 from brain regions involved in cognition, such as hippocampus, cerebellum and neocortex, is a common feature in all DMD patients and likely contributes to the cognitive deficits (2,4). The mdx mouse, which lacks Dp427, also displays cognitive and behavioral deficits (5–7), associated with alterations of synaptic plasticity (6,8–10), blood-brain barrier (BBB) dysfunction (11) and changes in neurons and synapse density and morphology (12–14). This multi-level phenotypic characterization of mdx mice provides neuropathological hallmarks to test whether new therapies developed to treat myopathy could alleviate brain alterations in DMD (15).

Strategies based on the regulation of endogenous utrophins were shown to alleviate myopathy in mdx mice. Thus, L-arginine, a precursor for synthesis of nitric oxide (NO), increases utrophin expression levels in muscle and favors its targeting to the sarcolemma, suggesting effective compensation or replacement of the missing dystrophin. This did not occur with D-arginine and could be prevented by oxadiazolo-quinoxalin-1-one (ODQ), an inhibitor of a soluble guanylate cyclase implicated in NO effects, suggesting an implication of NO (16). Moreover, various drugs implicated in NO-dependent mechanisms, including L-arginine, were shown to reduce myopathy in DMD mouse models (27).

In a recent study, L-arginine was combined with the HDACi, butyrate; the chronic administration of arginine butyrate (AB) at high levels phenotypic characterization of mdx mice provides neuro-pathological hallmarks to test whether new therapies developed to treat myopathy could alleviate brain alterations in DMD (15).

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In a recent study, L-arginine was combined with the HDACi, butyrate; the chronic administration of arginine butyrate (AB) at 250 mg/kg/day resulted in substantial utrophin upregulation associated with modest functional improvements in mdx mice, suggesting a need to develop distinct administration procedures to monitor the beneficial and toxic effects in vivo (28).

In the present study, we used high doses of AB (up to 800 mg/kg/day) but spaced series of peripheral (intraperitoneal) injections (three series of spaced injections for 6 weeks) to balance efficiency and minimal toxicity, and we tested the hypothesis that AB could induce utrophin expression in the brain and improve some of the behavioral deficits observed in mdx mice. Regulated expression of brain dystrophin and utrophin-gene products was assessed by semi-quantitative western blot and quantitative real-time polymerase chain reaction (RT-PCR) in both mdx and littermate control mice injected with AB or with a saline control solution. Moreover, an atlas of all brain utrophin-gene products was generated and compared between groups using immunofluorescence. Doses inducing high-level of full-length utrophin expression in mdx brain were selected to explore whether this could improve mice performance in a variety of behavioral tests in which mdx mice show impairments. The behavioral-test battery was designed to evaluate exploratory activity, emotional reactivity and long-term spatial and fear memories.

RESULTS

Basal expression of utrophin and dystrophin proteins in the brain

The K7 antibody was used to detect all utrophins on the same immunoblot and the DRP2 antibody to specifically detect the full-length utrophin (Fig. 1A and B). We confirmed that Up113 is the main utrophin-gene product in the adult brain, followed by Up140 and full-length utrophin that also show quantifiable expression. Expression of Up113 and Up71 was decreased by 20% in mdx compared with wild-type (WT) mice (P < 0.05), while levels of the full-length utrophin were unchanged (Fig. 1C). Although two full-length forms of utrophin have been described, utrn-A and utrn-B (29), distinction of these isoforms could not be achieved with these antibodies. Quantitative RT-PCR analyses revealed no significant variations for utrn-A, utrn-B, Up140, Up113 and Up71 mRNAs (n = 6 per genotype; P > 0.05, NS, data not shown). This indicates that brain expression of full-length utrophins, including both utrn-A and utrn-B isoforms, is not changed in mdx mice.

The lack of variations of mRNAs encoding short utrophin-gene products suggests that the changes observed for Up71 and Up71 on immunobots resulted from yet unidentified posttranscriptional modifications (30,31).

Because the short dystrophin-gene products share critical C-terminal domains in common with the full-length dystrophin, they could also compensate for dystrophin (Dp427) loss in the brain of mdx mice (32,33). Dp260, Dp140 and Dp71 were all detected on immunobots in the two genotypes, while the nerve-specific Dp116 protein was not (Supplementary Material, Fig. S1A). As expected, Dp427 was only detected in brain samples from WT mice, while Dp71 showed the greatest level of expression in both genotypes. No basal increases in Dp260, Dp140 and Dp71 expression levels were found in mdx mice, suggesting a lack of compensatory mechanisms by C-terminal dystrophin-gene products...
AB upregulates brain full-length utrophins

As shown in Figure 2A, a 6-week period of intraperitoneal injections (three series of spaced injections) of AB (100, 200, 500 or 600 mg/kg/day) upregulated brain full-length utrophin in mdx mice in a dose-dependent manner (dose effect: F(4,15) = 10.36, P < 0.01). A large difference was shown in the group treated with a dose of 600 mg/kg/day when compared with NaCl-treated mdx mice (P < 0.05). This was further confirmed in a separate experiment in mdx mice injected with 500 mg/kg/day (250% of NaCl control, n = 6 per group, P < 0.02; data not shown). In contrast, expression of the short utrophins Up113 and Up71 was decreased by the treatment (P < 0.01) (Fig. 2B), and a negative correlation between Up395 and Up113 expression levels in the AB-treated group (r = −0.77, P < 0.02), but not in the NaCl-treated group (r = 0.36), suggested that Up113 downregulation accompanied Up395 upregulation (data not shown).

Treatment with AB also induced a slight increase in the expression of dystrophins Dp260 and Dp140 (15–20%), which was only significant for Dp140 (P < 0.03) (Supplementary Material, Fig. S1C).

Mechanisms of utrophin upregulation by AB

We first considered that AB could enhance utrophin transcription (34–36), but we found no significant upregulation of utrn-A and utrn-B mRNAs in brain samples from mdx mice treated with AB [utrn-A: ratio = 0.953 (0.431–1.922); utrn-B: ratio = 0.711 (0.201–1.745); P > 0.05; RT-PCR analyses]. Secondly, we tested the possibility that l-arginine could promote the production of NO and thereby inhibit activity of matrix metalloproteinases (MMP). MMP-9 activity normally induces cleavage of β-DG into a short 30 kDa form that loses capacity to bind the extracellular α-DG. This could reduce interactions with ECM proteins and possibly alter the stabilization of utrophin-associated complexes (37–40). Inhibition of MMP-9 activity could lead to the stabilization of utrophin complexes through a decrease in β-DG cleavage, as shown in muscles (23). Here, we characterized the full and cleaved forms of β-DG on the same immunoblots as two distinct bands of 45 and 30 kDa, respectively (Fig. 2C). Semi-quantitative comparisons did not reveal any difference between genotypes at a basal state (DG45/DG30 ratio; WT: 8.697 ± 0.722, n = 6; mdx: 8.397 ± 1.169, n = 5; NS). In mdx mice injected with AB (AB500: 500 mg/kg/day), DG expression levels were not changed compared with NaCl-injected mice (Fig. 2C), which does not support the hypothesis of a facilitated stabilization of utrophin complexes through this mechanism.

Functional outcomes of AB treatment in mdx mice

The functional impact of the 600 mg/kg/day dose of AB on general health and muscle histopathology was evaluated in both genotypes. AB had no impact on mouse body weight and did not induce any overt suffering or discomfort over a 15-week period of AB administration, suggesting good tolerance in mice. As expected (16,27,28), AB induced biochemical modifications indicative of increased membrane stabilization in the muscles of mdx mice, as shown by the changes in immunocytochemical markers observed in a group of mice sacrificed after completion of the behavioral test battery (AB600: 600 mg/kg/day, 8 injection series, 15 weeks of treatment) (Supplementary Material, Fig. S2A–D): There was a clear relocalization of Up395 along the muscle sarcolemma and a partial rescue of β-DG expression at the

Figure 2. Upregulation of full-length utrophin Up395 by AB. (A) Dose-dependent increase in Up395 expression levels in the brain of mdx mice treated with AB (100, 200, 500 and 600 mg/kg/day; black bars, n = 4 per group) compared with mdx mice injected with 0.9% NaCl (white bar, n = 4). Immunodetection was performed with the polyclonal K7 antibody. Expression was normalized to actin and changes in expression levels presented as percent of mdx-NaCl. *P < 0.05. (B) Decreased expression levels of Up113 and Up71 in brains from mdx mice treated with AB (600 mg/kg/day, black bar, n = 6) compared with mdx mice injected with 0.9% NaCl (white bar, n = 6). Immunodetection was performed with the polyclonal K7 antibody. Values are normalized to actin and presented as percent of mdx-NaCl. *P < 0.05. (C) Immunodetection of brain β-DG as 45 kDa full (DG45) and 30 kDa cleaved forms (DG30) using the polyclonal G5 antibody (left). Expression levels were normalized to GAPDH and the DG45/DG30 ratio in mdx mice treated with AB (500 mg/kg/day, AB500, black bar) is presented as percent of expression in mdx-NaCl mice (right, n = 6 per group, white bar).
fiber membrane. Moreover, this was associated with a significant improvement of muscle strength in treated mdx mice (Supplementary Material, Fig. S2E). In these mice, we also confirmed upregulation of brain full-length utrophins (140 ± 13% of NaCl; P = 0.02). In contrast, there was no significant variation of brain utrophin expression when AB was administered to WT mice (data not shown). The same dose of AB was used to evaluate putative effects of treatment on mdx mice behavioral deficits.

In the open-field test, the distance run was lower in mdx than in WT mice throughout a 20 min period of observation (mdx, 71 ± 6.7 m; WT: 94 ± 5.4 m, P < 0.02; Fig. 3A) and the time spent immobile was conversely larger (mdx, 231 ± 27 s; WT: 138 ± 17 s, P < 0.01, data not shown), thus confirming reduced locomotion and exploration in the mutants (6). As shown in the Figure 3A, AB600 had no effect on locomotor activity in any genotypes and therefore did not alleviate the deficit displayed by mdx mice. The percent distance run in the centre zone of the open field was also reduced in mdx (21 ± 1.8%) compared with WT mice (24.8 ± 1.7%; P < 0.01) (Fig. 3B), suggesting that enhanced anxiety-like responses could partly account for the reduced activity. Here again, AB600 did not reduce the deficit. However, in treated mdx mice, the percent distance run in the centre zone was positively correlated with Up395 expression levels (r = 0.68, P < 0.05, Supplementary Material, Fig. S3B) and negatively correlated to Up113 levels (r = −0.71, P < 0.03, Supplementary Material, Fig. S3D), suggesting opposite influence of Up395 and Up113 expression on this behavioral parameter. In contrast, there were no such correlations in mdx mice injected with NaCl (Up395, r = 0.005; Up113, r = −0.13; Supplementary Material, Fig. S3A and C). As Up395 upregulation by AB was associated with a slight downregulation of Up113, the treatment and Up395 overexpression apparently tended to reduce anxiety-related behavior in mdx mice. Utrophin expression was not correlated to any other behavioral parameter.

Unconditioned fear responses were assessed by videotracking mice in an empty novel cage for 5 min after short manual restraint (7). As expected, the time spent freezing was larger in mdx (50.8 ± 5.7%) than in WT mice that only showed few periods of immobility (9.5 ± 1.2%, P < 0.0001, Fig. 3C). Although the percent freezing expressed by mdx mice in this experiment was smaller than in another study (7), a control experiment (Supplementary Material, Fig. S4) showed that the percent freezing is smaller in mdx mice injected for 6 weeks with a NaCl solution compared with non-injected mice naïve to experimental conditions. This suggests that
intensive handling in our study partially reduced unconditioned fear responses. In any cases, the unconditioned response was large enough in mdx mice to quantify putative effects of chronic AB administration. As shown in Figure 3C, however, the percent time spent freezing was unaltered in mdx mice treated with AB600, compared with NaCl-injected mice. Conditioned fear was assessed in a contextual fear conditioning paradigm in which mice receive one single footshock (0.4 mA) during acquisition and are exposed 24 h later to the same experimental context. During habituation, mice of both genotypes exhibited few episodes of immobility, although the percent time spent immobile was larger in the mdx (WT: 10.7 ± 2.4%, mdx: 29.2 ± 5.2%, P < 0.005). During acquisition, freezing increased after foot-shock delivery in a comparable manner in both genotypes. During retention, however, freezing was larger in WT than mdx mice (P < 0.005) (Fig. 3D), suggesting impaired fear memory in mdx mice. Treatment with AB did not modify the amount of freezing in any of the groups and did not improve fear memory in mdx mice (Fig. 3D).

Performance during massed training in a water maze was comparable in all groups of mice. The swim speed was lower in mdx than in WT mice (WT: 0.173 ± 0.002 m s⁻¹, mdx: 0.145 ± 0.002 m s⁻¹, P < 0.0001), but the distance swum by mice to reach the platform significantly decreased as training progressed (F(4,236) = 37.2; P < 0.0001), with no main group differences (genotype effect: F(1,59) = 0.152; NS, genotype × session interaction: F(4,236) = ; NS, Fig. 3E), as reported previously (6). When memory retention was assessed 24 h later, the percent distance spent in the target quadrant was significantly above the chance level in WT mice (AB: 32 ± 2.5%, P < 0.026; NaCl: 31.3 ± 3%, P < 0.036) but not in mdx mice, whether they received a treatment with AB or not (AB: 29 ± 2.5%, P > 0.1; NaCl: 27 ± 1.4%, P > 0.1, Fig. 3F).

Treating the mice with a higher dose of AB (800 mg/kg/day) resulted in a larger upregulation of Up395 (160 ± 15% , P < 0.03) compared with AB600 (140 ± 13%, P < 0.02), but this also failed to compensate for behavioral deficits in mdx mice (Supplementary Material, Fig. S5).

Localization of utrophin and dystrophin proteins in the brain

The regional and cellular expression of brain utrophins was analyzed in the main brain structures and nuclei defined in the mouse brain atlas (41). Expression of utrophins was mainly localized in blood vessels among all brain structures, as shown in the sample images from the CA1 hippocampal region in Figure 4I. Utrophin immunoreactivity (IR) was only detected along walls of blood vessels, not in neurons or synaptic structures, as in untreated mice of both genotypes. Cell nuclei were counterstained with DAPI (blue). Scale bars (A–H): 200 μm; (I): 500 μm.

Figure 4. Immunolocalization of brain utrophins in mice. (A–H) Utrophins IR revealed by the K7 pan-utrophin antibody was characterized by intensely stained blood vessels in all the brain structures included in this analysis. Strong staining was found in epithelial cells of the choroid plexus and along the walls of the lateral ventricle (A) and in the glia limitans (B). Utrophin IR was also localized along the cell surface in subpopulations of neurons in cortex (C, arrow), thalamus (D) and in some brainstem nuclei such as in the spinal trigeminal nucleus (E, arrow shows stained soma and proximal dendrite of a neuron) and the reticular nucleus (F). There was no utrophin IR in pyramidal neurons of CA1 hippocampus (G) and in Purkinje cells of cerebellum (H). (I) Utrophin staining in CA1 hippocampus in an mdx mouse treated with AB (500 mg/kg/day), using the DRP2 antibody that only binds the full-length utrophins. IR (red) was only detected along walls of blood vessels, not in neurons or synaptic structures, as in untreated mice of both genotypes. Cell nuclei were counterstained with DAPI (blue). Scale bars (A–H): 200 μm; (I): 500 μm.
some brainstem nuclei (Fig. 4E and F). In utrophin-positive neurons, immunostaining outlined the membrane of the soma and proximal dendrites (Fig. 4C–F). In contrast, no utrophin stain was found in hippocampal pyramidal cells (Fig. 4G and I) and in Purkinje cells (Fig. 4H), as reported previously (42). Punctuate immunostaining of synaptic structures was never observed (Fig. 4I). Urophin pattern of expression was comparable in WT and mdx mice. Moreover, analyses with the K7 and DRP2 antibodies revealed comparable immunostaining patterns, suggesting that full-length utrophin expression overlays that of the short utrophins.

AB does not induce relocalization of utrophins in dystrophin-deficient brain structures

Table 1 summarizes expression of utrophins in neuronal structures. The immunoreactive patterns were remarkably similar in all groups of mice, with no apparent changes in the pattern of expression in neural and non-neuronal structures. The utrophin upregulation observed in western blot experiments could not be attributed to a specific structure. The staining of utrophin in treated mdx mice thus likely occurred in the same regions and cell types where utrophins are normally expressed in WT and untreated mdx mice. In particular, no punctuate staining was observed in the dendritic field of pyramidal cells in hippocampus (Fig. 4I) or cortical pyramidal cells, nor in Purkinje-cell dendritic areas in the cerebellum, suggesting that AB did not induce expression of utrophins in dystrophin-deficient synaptic structures.

DISCUSSION

Pharmacological compounds designed to target various organs and tissues with limited injection sites, as opposed to more invasive gene-replacement and repair strategies, offer the prospect to alleviate both brain and muscle alterations in syndromic neuromuscular diseases. While both arginine (19,22,23,26) and HDACi (27) reduce myopathy, a 6-month chronic administration of AB compound in mice recently showed modest beneficial effects (28). As suggested by clinical studies in distinct genetic diseases, spaced injections may allow administration of higher doses of treatment without major toxicity (43). Based on results on the effects of chronic but intermittent administration of AB on mdx muscle pathophysiology, showing in particular significant reduction in diaphragm pathology and improved respiratory functions (Hua Yu et al., manuscript in preparation), we evaluated the potency of spaced injections of AB to induce brain utrophin expression and to alleviate some of the behavioral deficits reported in mdx mice. As dystrophin structural homologs might be regulated at the basal level in dystrophin-deficient tissues (29,44,45), we first characterized expression of dystrophins and utrophins in the brain and then evaluated whether and how AB could modulate their expression and impact on behavioral functions.

Basal expression of brain utrophins and dystrophins in mdx mice

The dystrophin- and utrophin-gene products (Dps and Ups) bear specific sequences but also share common structural domains, and a putative interplay between the two families of proteins might contribute to spontaneous compensatory mechanisms in mdx mice. Here, we report the first quantitative study assessing expression of all brain utrophin-gene products simultaneously on the same immunoblots. We found no variations in expression of the full-length utrophins in brains of mdx mice, whereas a significant decrease in Up113 and Up71 expression was observed. This appears at variance with certain results reported previously. For example, in a recent study, upregulation of both full-length utrophins (A and B) was reported in selective brain structures in mdx mice (45). Although the comparisons were limited as the two full-length utrophins were analyzed separately, the results suggested a structure-specific upregulation of utrophins, which could not be easily detected in whole-brain extracts in our study. In contrast, another study by Culligan et al. (44) suggested that all utrophins, including the short forms, are not regulated in the brain of mdx mice.

Here, levels of utrophins mRNA were comparable to control levels in our RT-PCR analysis, suggesting that changes in Up71 and Up113 protein expression reflected protein posttranslational modification and/or regulation. For example, increased MMP activity, as reported in the brain of mdx mice (46,47), could lead to disruption of utrophin-associated complexes due to β-DG cleavage, thus leading to the
degradation of utrophin proteins. However, we found no significant increase in the level of β-DG cleavage in the brain of mdx mice. This suggests that distinct MMP targets, such as laminin, could alternatively affect the stabilization of dystrophin- and utrophin-associated complexes in this tissue (46,48). Also, a lack of correlation between utrophin protein and mRNA levels has been previously linked to a putative involvement of utrophin mRNAs in priming DNA replication through interactions with origins-of-replication proteins (49).

The short dystrophin-gene products also share critical C-terminal domains with the full-length dystrophin, suggesting that they could compensate for the Dp427 loss in mdx mice (32,33). Thus, the expression of Dp71 appears to be decreased specifically in brain microvessels of mdx mice (11), whereas one study also reported impaired oligomerization of this protein (44). Here, we found a decrease in Dp140 in the brain of mdx mice. Dp140 is mainly expressed in fetal brain but also to a lesser extent in the adult brain, where it has been detected in the walls of blood vessels (50). The subcellular localization of Dp140 is unknown and this is the first indication that the loss of Dp427 could alter expression and/or stabilization of Dp140 in the adult brain.

Immunolocalization analyses confirmed that the full-length dystrophin and utrophin have distinct cellular and subcellular localizations in the adult brain (42). Dystrophin is normally localized in the postsynaptic density of inhibitory synapses in pyramidal cells of hippocampus, cortex and amygdala, and in cerebellar Purkinje cells, and this is typically reflected by punctuate synaptic labeling around neuronal soma and in dendritic fields (7,51–54). In contrast, utrophins are not expressed in hippocampal, cerebellar and amygdala neurons, where immunostaining appears to be restricted to microvessels. Here, we show an apparent overlap among the different utrophin products at the glialvascular interface. However, it is possible that some utrophin-gene products were expressed in perivascular astrocyte endfeet while others were in the facing endothelial cells. In any cases, although we detected utrophin labeling along the somatic and dendritic membrane in some neurons of cerebral cortex or brainstem nuclei, we did not see any punctuate staining, suggesting lack of expression in synaptic structures. Hence, utrophins do not replace the missing dystrophin in synaptic structures of mdx mice, as suggested by others (42,44,55), which supports the hypothesis of distinct regulatory mechanisms of expression for these two related families of proteins.

**AB modifies expression of utrophins and dystrophins**

As expected from previous studies (16,27,28), AB treatment resulted in an upregulation of the full-length utrophin protein in muscles of mdx mice, also characterized by relocalization to the sarcolemmal membrane along with β-DG. Here, we show that AB also induces utrophin upregulation in brain tissues in a dose-dependent manner, and we selected for subsequent functional studies the dose of 600 mg/kg/day that resulted in high utrophin overexpression in both muscle and brain tissues of mdx mice.

The mechanisms underlying utrophin upregulation by AB are unclear. While both arginine and HDACi such as butyrate may potentially facilitate gene transcription in adult tissues (34–36), we observed no quantitative changes in utrophins mRNA expression. This was similarly observed in muscle tissues in a previous study (28). However, we have assessed mRNA expression several days or weeks after the start of AB administration and with a 24 h delay after the last injection, while there is evidence showing that activation of the transcription machinery after intraperitoneal injections of HDACi is rapid (56). This may explain that mRNA modulation could not be detected in our experimental conditions.

Alternatively, the observed changes in utrophin expression levels might reflect improved stabilization of the protein at the membrane. The capacity of L-arginine to induce NO may have multiple consequences in the brain (57). NO can inhibit brain MMPs, which may reduce β-DG cleavage and thereby stabilize utrophin-associated complexes (23). Our data suggest that modification of β-DG cleavage by AB is unlikely to explain utrophin stabilization, but we cannot exclude the contribution of MMP acting on other major ECM protein targets (46). Besides, NO may regulate the phosphorylation of certain proteins. Increased expression of utrophin by arginine in muscles of mdx mice has been linked to activation of a NO/cGMP pathway that stimulates PKG and inhibits PKC (16). It has been then hypothesized that NO induction could improve interaction of utrophin with actin, thereby stabilizing the utrophin-associated complex (30).

Upregulation of full-length utrophin in AB-treated mdx mice was also accompanied by a significant decrease in Up113 and Up71 expression, with no changes in mRNA expression. This suggests that upregulation of full-length utrophin could be counterbalanced by a reduced expression of the short utrophin-gene products in the brain. Our immunofluorescence study revealed that the full-length and short forms of utrophin are expressed in the same brain structures and neuronal-cell types and could therefore be in competition for interaction with the utrophin-associated complex and/or actin. Such hypothesis has been suggested by others showing that exogenous Dp71 can compete with full-length dystrophin and probably utrophin for the binding to the dystrophin-associated proteins in muscles (33,58). Here, upregulation of full-length utrophin could thus tip the balance toward the stabilization of full-length utrophin-containing complexes. In contrast, in WT mice injected with AB, the expression of utrophins was not modified, probably because brain dystrophin (Dp427) is still expressed.

In mdx mice, putative compensations of dystrophin loss by other C-terminal products of the DMD gene were also investigated. We found that the only dystrophin-gene product upregulated by AB treatment in mdx mice was Dp140. Interestingly, Dp140 is the major dystrophin protein expressed in the fetal brain, suggesting that AB, which bear HDAC inhibition properties, induced expression of this protein that is normally expressed at very low levels in the adult brain. In the human condition, the loss of Dp140 contributes to the genesis of mental retardation (2). We showed that Dp140 expression is decreased by ~15% in mdx mice, and one may hypothesize that this could participate to the mild cognitive deficits displayed by this DMD mouse model. However, treatment with AB increased expression of Dp140 by ~20%, thus normalizing Dp140 expression levels in mdx mice. Because AB treatment did not alleviate the behavioral and cognitive
deficits in mdx mice, it seems unlikely that this slight down-regulation of Dp140 contributes to the behavioral alterations in this mutant. Alternatively, utrophin overexpression may interfere with a putative effect of Dp140 upregulation, as the two proteins are expressed near to the same cell functional domains along the walls of blood-brain vessels.

**Why AB does not alleviate behavioral deficits in mdx mice**

Our study confirms the presence of behavioral and cognitive deficits in mdx mice, which include decreased locomotor activity and exploration, enhanced emotional responses and impaired spatial and contextual fear memory. Two main hypotheses are currently considered to explain the cognitive deficits in mdx mice. First, dystrophin loss in mdx mice alters the clustering of GABA<sub>A</sub> receptor (51), and this may lead to altered GABAergic inhibition and synaptic plasticity (8,10,13,59,60). Secondly, the loss of dystrophin is associated with altered BBB function in mdx mice (11,61). This may secondarily result from hypoxic episodes contributing to BBB opening and changes in expression and activity of MMPs (47,62). Defective hippocampal neurogenesis has also been reported in mdx mice, which could also contribute to the long-term memory deficits (63).

Because dystrophin and utrophin share structural and functional homologies, we tested the hypothesis that induction and/or overexpression of brain utrophin-gene products could functionally compensate for the loss of brain Dp427, as could be anticipated from the beneficial effects of several utrophin inducers, including AB on dystrophin-deficient muscles in mdx mice. Additionally, HDAC inhibitors can compensate for certain types of memory deficits (64) and may have some antidepressant effects (56,65), suggesting that AB could also induce some utrophin-independent improvements of behavioral and cognitive functions in mice. Moreover, it is likely that NO activation by AB has some beneficial effects on adult neuronal differentiation and/or physiology that may also compensate memory deficits (63). However, while increasing doses of AB correlated with increases in brain full-length utrophin expression in the present study, the treatment did not result in any improvement of behavioral performance in mdx mice. This suggests that either utrophin-dependent or -independent mechanisms resulting from AB treatment have no significant impact on cognitive and functional behaviors.

While one may hypothesize that AB reactivated or maintained a developmental state in which utrophin expression predominates, which could be viewed as a protective effect, this was apparently not sufficient to restore the brain functions that are under the specific governance of dystrophin. The significant utrophin upregulation quantified from whole-brain samples suggests a global regulation of utrophin expression in all brain structures. However, this might be insufficient to restore brain alterations if a larger expression of the protein is needed in specific Dp427-deficient structures involved in cognitive functions, such as in the hippocampus. Because utrophin upregulation was not associated with changes in β-DG, we also suggest that utrophin stabilization after treatment with AB may involve interactions with other ECM proteins, which could result in inappropriate utrophin addressing, a condition without benefit for brain function. Alternatively, overexpressing utrophin in the brain of adult mice may not be relevant if the protein normally has a role during developmental and/or early postnatal stages. While utrophin and dystrophin were found colocalized in fetal-cultured neurons (66), their cellular expression in the adult brain does not seem to overlap anymore. One may therefore hypothesize that AB could show a greater impact when administrated in younger mice, when developmental plasticity is still occurring in the immature brain. The situation is different in muscle tissues of mdx mice, as they may undergo chronic regeneration cycles involving utrophin expression. A better understanding of the dynamic regulation of the dystrophin and utrophin proteins during brain development and maturation is needed to help defining an appropriate time window for AB administration and to ascertain the relevance of this strategy for alleviating the cognitive status in the human condition.

One main conclusion from our study is that upregulation of brain utrophin by AB has no major functional impact in the mature brain, probably because it is not associated with a relocalization of utrophin to brain structures and cell types that lack Dp427 in mdx mice. In all groups of mice, whether they were injected with NaCl or AB, utrophins were mostly localized in microvessels throughout the brain, but not in the main neuronal and synaptic structures that normally express Dp427. This suggests that dystrophin and utrophin are involved in distinct brain mechanisms and/or cell types during brain development and in the adulthood. Again, this is a major difference with the processes involved in muscle physiopathology, as in skeletal muscles utrophin expression precedes that of dystrophin in immature fibers, while both proteins are still expressed in the same populations of mature muscle fibers. In the present study, butyrate theoretically acted as a potent HDACi enabling activation of a large variety of genes. However, upregulation of utrophins mRNA could not be demonstrated, suggesting that the effects of AB rather involved posttranscriptional mechanisms. Future studies in the search for transcriptional activators controlling utrophin expression may aid defining alternative strategies to activate and/or redirect utrophin expression specifically in neurons. Several regulators of the utrophin-gene promoters have been identified, some of which enabling synapse-specific expression of utrophin (15,67,68). This is the case for the nerve-derived trophic factors agrin and heregulin that can activate Ets-related GABP transcription factors which in turn transactivate the utrophin gene via the N-box/EBS motif of the utrn-A promoter (69–71).

The recent development of artificial zinc finger-based transcription factors, acting as strong transcriptional activators of the utrophin A promoter (72,73), and of high-throughput screening assays aimed at identifying new compounds capable of activating transcription from the utrophin promoter (74,75), could potentially yield new means of achieving utrophin upregulation in dystrophin-deficient neurons at the transcriptional level.

With AB in the condition of this study, compensation of dystrophin loss by utrophin overexpression may only be a potent strategy in tissues where these two proteins are normally coexpressed in the same cells (1,76). Although this is mostly not the case in the adult brain, coexpression of both dystrophin-gene and utrophin-gene products could be found...
in other parts of the mature nervous system, such as in the Müller glial cells of the retina where utrophin is coexpressed with Dp71 (77) and in the Schwann cell of the peripheral nerves where utrophin is coexpressed with Dp116 (37). In Müller glial cells, utrophin upregulation and redistribution has been observed in Dp71-null mice, suggesting spontaneous compensation (77,78). In such biological systems and mouse models, the capacity of AB treatment to upregulate utrophins and to compensate for the absence of dystrophin products should be examined. In the adult brain, utrophin and Dp71 are not expressed in the same cell types, the former being expressed in endothelial cells and the latter in glial end feet. However, the glial-vascular interface is a site of expression of several forms of dystrophins and utrophins, which are likely involved in complex, and still poorly understood, functional interplays. Hence, AB-induced changes in the vascular expression of utrophins might have some beneficial effects on the glial-vascular alterations in adult brain tissues, a hypothesis that could be investigated in mice lacking dystrophin products normally expressed at the glial-vascular interface, such as Dp71 or Dp140.

Conclusions

The potential of exogenous micro-utrophin delivery on muscle status has been demonstrated in mdx mice (79), giving rise to the current hypothesis that utrophin may functionally compensate for dystrophin loss. The use of pharmacological treatments combining a molecule that upregulates transcription in selected structures and another molecule that enables to upregulate and/or stabilize endogenous utrophin remains an important challenge in the search for a therapeutic strategy for DMD, with the major advantage of allowing widespread treatment delivery to various organs and tissues from single, peripheral, injection sites. Despite high structural homologies in dystrophins and utrophins, putative functional redundancies in brain tissues remain to be demonstrated. Here, we show that AB significantly upregulates expression of the brain full-length utrophin. But at variance with the capacity of upregulated utrophin to take dystrophin’s place at the sarcolemmal membrane in muscles, AB does not induce novel expression of utrophin in adult brain structures that lack dystrophin. This obviously reduces the possibility to replace dystrophin and alleviate brain alterations, which may rather require acting on distinct and specific dystrophin-dependent mechanisms (54,80). The brain expresses a large variety of proteins from both the dystrophin and utrophin genes, which constitutes a far more complex environment compared with muscle. Our data highlight that several of these proteins may be spontaneously or experimentally modulated, suggesting a putative functional interplay between these two families of proteins, which could involve both independent and synergic regulatory mechanisms of expression. Therefore, there is a need to better understand the specific transcription mechanisms that in brain control the tissue and cell-specific expression of the multiple proteins that belong to the dystrophin–utrophin superfamily, in order to define specific targets for pharmacological approaches in this tissue. Moreover, we suggest that AB treatment could favor functional compensation in other tissues or organs that normally coexpress distinct forms of dystrophins and utrophins, as in some retinal, peripheral-nerve and endothelial cells, which might open new routes to further explore the functional effects of utrophin inducers in dystrophinopathies.

MATERIALS AND METHODS

AB preparation

l-Arginine (Sigma, France) was dissolved in milliQ water and n-Butyric acid (Riedel-de Haën, Germany) was added to obtain a 12.5% stock solution (0.76 m arginine/1 m butyrate; pH = 5.5). This formulation was adapted from FDA recommendations and corresponds to that currently used for clinical trials in patients with beta-haemoglobinopathies (S.P. Perrine, personal communication), another genetic condition for which induced expression of a homolog of the missing protein alleviates pathology (43,81). Stock solutions were diluted before use in 0.9% NaCl to obtain injectable solution.

Animals and genotype

Experiments were conducted in male mice of the C57BL/10ScSn-Dmd/mdx (mdx) mutant line and littermate controls (WT) bred in our laboratory (6). Heterozygous females were mated with WT males to obtain progenies in which male siblings were either of the mutant or WT genotype. Male siblings were kept in groups (two to eight mice per cage) under a 12 h light–dark cycle (light on: 7.00 a.m.) with food and water ad libitum. All experiments were undertaken under constant room temperature (22–23°C) and homogeneous illumination by an experimenter blind to the genotype and in accordance with the recommendations of the European Communities Council Directive (86/609/EEC) for animal care and experimentation.

Genotypes were determined by PCR using genomic DNA from tail tips. Three primers (Invitrogen, France) were used: one reverse primer for the WT (CACTCAGATTGGAAGCGATTTTG–R1) and one for the mdx mice (CACTCAGATTGGAAGCGCATTTTA–R2), and a forward primer in common with the two genotypes (CTCATCAATATGCCTGTTAGTGT). Two PCRs were performed with the R1 and R2 primers in each mouse. Genotype was confirmed by post-mortem histological analyses of quadriceps muscle (5).

General procedure

At the age of 12–15 weeks, mice were treated by intraperitoneal injections (10 µl/g of body weight) of either saline solution (0.9% NaCl) or AB. We used an intermittent treatment protocol with series of injections repeated every 2 weeks: each series consisted of an injection performed each day during 4 days. In all experiments, mice were sacrificed by cervical dislocation 24 h after the end of the last series of injections and their brains were dissected out; one brain hemisphere of each mouse was used for western blot analyses and the other for immunohistochemistry or quantitative RT-PCR analyses.

Dose–response analysis. The mice received three series of injections; the WT mice were injected with NaCl, while the
mdx mice were divided into five subgroups of four animals, each subgroup receiving a specific dose of AB (100, 200, 500 and 600 mg/kg/day) or NaCl. The brains were processed for western blots and immunofluorescence analyses.

Effects of AB on behavior. Doses of AB (600, 800 mg/kg/day) were selected for their capacity to induce high levels of brain utrophin expression. The first set of mice received doses of 600 mg/kg/day (AB600) (WT: NaCl, n = 12; AB600, n = 10; mdx: NaCl, n = 11; AB600, n = 9) and was submitted to successive behavioral tasks between series of injections as follows: unconditioned fear and open field were done after three series of injections (6th week), spatial learning in a water maze after five series (10th week) and contextual fear conditioning after 7 series (14th week). Mice were then sacrificed for protein and mRNA analyses on week 15 after a last (8th) series of injections. A second set of mice (n = 6 per group) was only tested in a spatial learning task in a water maze after four series of injections (8th week) and brains were processed after the fifth series of injections. A third set of mice (n = 6 per group) was used to evaluate a higher dose of AB (800 mg/kg/day) in a subset of behavioral tests.

Protein analyses

Antibodies. Urophins were detected with the K7 polyclonal antibody (gift from D. Mornet, Montpellier) directed against the common C-terminal end of all utrophin-gene products and with the DRP2 monoclonal antibody against a specific N-terminal sequence of the full-length utrophin (NCL-DRP2, Novocastra, UK). Dystrophins were detected using the H4 polyclonal antibody (gift from D. Mornet, Montpellier) against the common C-terminal end of all dystrophins. For immunoblots, we also used the polyclonal G5 antibody (gift from D. Mornet, Montpellier) to detect β-DG, the monoclonal anti-actin (AC-40, Sigma) and anti-GAPDH antibodies (Sigma). K7, H4 and G5 polyclonal antibodies were produced as described (82). For immunofluorescence in muscle tissues, we used the monoclonal C19 antibody to detect full-length utrophin (C19, Santa Cruz Biotechnology, USA) and the monoclonal anti-β−DG (NCL-β-DG, Novocastra, UK). Secondary antibodies in immunofluorescence studies were monoclonal anti-rabbit and anti-mouse antibodies conjugated to Cy3, and polyclonal anti-goat antibodies conjugated to Cy2 (Jackson Immunoresearch, USA). For chemiluminescence analyses of immunoblots, we used secondary sheep anti-mouse (Jackson Immunoresearch) and goat anti-rabbit antibodies (Sigma) linked to horseradish peroxidase.

Immunoblot analyses. Proteins were extracted from whole-brain samples in lysis buffer (pH 6.8; 10 mM Tris, 1 mM EDTA, 10% SDS) supplemented with 2.5% protease inhibitor cocktail (Sigma) using a homogenizer (Precellys24, Bertin Technologies, France). Total protein concentration was determined according to DC Protein Assay protocol (Bio-Rad, France) and proteins were separated by SDS–PAGE on NuPAGE 4–12% Bis–Tris gels (Invitrogen) and electrotransferred onto immobilon-P polyvinyl membranes (Sigma). After blockade with 4% non-fat milk powder and 0.4% Tween 20 in Tris-buffered saline, the membrane was incubated 2 h at RT or overnight at 4°C with different primary antibodies: K7 (1/300), H4 (1/5000), G5 (1/600), anti-actin (1/500) and GAPDH (1/40000). Membranes were then incubated with secondary antibodies for 1 h at RT. Immunoreactive products were revealed by a chemiluminescence reaction (ECL, GE Healthcare, France). Actin (43 kDa) and GAPDH (37 kDa) were used as loading controls. Image acquisition was realized using the Bio-Vision 1000 imaging system (Vilber Lourmat, France) and quantification of immunoreactive band intensity with the TotalLab TL120 software (Nonlinear Inc., Durham, NC, USA). Results were normalized to controls.

Immunofluorescence. Brain were rapidly excised, frozen with powdered dry ice and stored at −80°C. Then, 10 μm thick coronal cryostat sections were cut, mounted onto SuperFrost Plus slides (Roth, France) and stored at −80°C. After brief thawing, sections were fixed in cold solution of acetone/methanol 1:1 for 10 min at −20°C, incubated 1 h at RT with a blocking solution (10% normal goat serum, 0.3% Triton X-100 and 1% bovine serum albumin in phosphate buffer saline) 2 h at RT with the K7 (1/200) or DRP2 (1/60) primary antibodies and 45 min at RT with fluorescent secondary antibody (1/500). The slides were cover-slipped with anti-fade Dapi-fluoromount-G medium (Clinisciences, France) and observed using an Olympus BX60 fluorescence-imaging microscope equipped with a CoolSNAP camera (Roper Scientific, USA). Images were analyzed using the Mercator software (Explora Nova, France). Sections incubated with antisera preabsorbed with corresponding antigen or with fluorescent secondary antibodies alone were devoid of specific staining.

For muscle tissue analyses, cryostat sections (7 μm thick) were fixed in cold methanol (+4°C for 10 min), incubated 5 h at RT with C19 (1/100) and NCL-β-DG (1/100) primary antibodies, and then for 1 h with the corresponding fluorescent secondary antibodies (polyclonal anti-goat Cy2, 1/1000 and monoclonal anti-mouse Cy3, 1/8000). Tissue sections were observed using a Leica DM RXA2 fluorescent-imaging microscope (Leica Microsystems, Germany) equipped with a CoolSNAP camera (Roper Scientific) and Openlab software (Improvision, UK).

RT-PCR assays

RNA extraction was conducted using a standard protocol with Trizol reagent (Invitrogen). The quality and concentration of RNA were determined by spectrophotometry (OD at 260 nm). After treatment by Turbo DNase (Ambion), the cDNA was synthesized using the Thermoscript RT-PCR system (Invitrogen) with random hexamers. PCR was then performed using the StepOne Real-Time PCR System (Applied Biosystems, France). The forward and reverse primer sequences (Supplementary Material, Table S1) were as described previously (37,45,83–85) or designed using the primer3 software (86). Each primer pair was tested and melt curves were constructed and analyzed to ensure that only a single amplicon was generated. PCR was performed in plates in a final volume of 20 μl, containing 2× Fast SYBR Green Master Mix, 0.5 μM of each primer and cDNA corresponding to 50 ng of mRNA. Standard PCR conditions were used for the Applied Biosystems assays: 50°C for 2 min,
95°C for 10 min and 40 cycles at 95°C for 15 s alternating with 60°C for 1 min. All samples were assayed in duplicate for each target or reference gene and the averaged values were used as Cycle Threshold. Changes in the relative expression of genes of interest were calculated; data were normalized to endogenous controls (UBC, HPRT1 and PGM1). The Relative Expression Software Tool [REST\(^\text{\textregistered}\), (87)] was used for statistical analysis of the data from RT-PCR reactions, which incorporates the variability of data from both the housekeeping and target genes to calculate statistical significance.

**Behavioral testing**

*Muscle strength in the inverted grid test.* Mice were placed individually on a cage wire grid \(\sim 35\) cm above a table. After slowly inverting the grid upside-down to 180°, the ability of the mice to maintain a grip into the grid was monitored (grip latency); with a maximum score of 120 s being given if the animal did not fall.

*Open-field activity.* The test box consisted of a square open field (50 \(\times\) 50 \(\times\) 50 cm) with black walls and a white floor covered with sawdust. Mice were placed near the wall and allowed to explore freely for 20 min. A video-recording camera was placed above the arena and mice videotracked allowed to explore freely for 20 min. A video-recording covered with sawdust. Mice were placed near the wall and was released to a cage containing sawdust (25 cm between the fourth and little fingers. After 15 s, the mouse between the thumb and index fingers, while securing the tail the experimenter grasping the scruff and back skin of the mouse. Restraint was achieved by the

*Unconditioned fear response.* Restrained was achieved by the experimenter grasping the scruff and back skin of the mouse between the thumb and index fingers, while securing the tail between the fourth and little fingers. After 15 s, the mouse was released to a cage containing sawdust (25 \(\times\) 17 cm, with 14 cm high walls; illumination: 80 lux) and it was videotracked for 5 min (ANY-maze software). Freezing was defined as complete immobilization of the body except for respiration (88). The percent time spent immobile was calculated and immobility was regarded as a freezing response if at least 90% of the mouse body remained static for a period of 1 s or more, which typically enables reliable quantification of mouse freezing episodes in our hands.

*Contextual fear conditioning.* The test box consisted of a transparent Plexiglas box (30 \(\times\) 30 cm, with 45 cm high walls) with a metallic grid floor connected to a shock generator monitored by a computer. The test box was cleansed between animals with ethanol to reduce olfactory cues. Mice were placed in the apparatus for a 5 min habituation session. On the next day, they were submitted to a 5 min acquisition session during which a footshock (0.4 mA, 2 s) was delivered at 2.5 min as an unconditioned stimulus. Retention of contextual conditioned fear was measured 24 h later by placing the mouse in the same context for 5 min. During all sessions, animal’s activity was recorded and analyzed using the ANY-maze software. The percent time spent freezing was calculated as above. To evaluate fear memory on the retention session and to minimize putative bias due to the slight basal hypoactivity displayed by the mutants during habituation, the freezing behavior during retention was normalized to that recorded during habituation.

*Spatial learning in a water maze.* Apparatus and procedure were as previously described (6). The maze was a circular water tank (1.5 m diameter) filled with water (22°C) to 15 cm below the edge of wall and made opaque by addition of a white paint (Acosul, OP301 Opacifier, Brenntag, France). It was placed in a well-lit room containing several extramaze cues and was surmounted by a video camera connected to a computer located in an adjacent room. The maze was divided into four virtual quadrants defined by the four cardinal points (N, E, S and W). A non-visible circular escape platform (10 cm diameter) was placed 0.5 cm below the water surface, laid in the center of the maze during pretraining or in the center of a quadrant (35 cm from the wall) during training. The platform position was assigned for each mouse in one of the four virtual quadrants of the maze, such that the four positions were equally used in both groups of mice.

After daily handling for 1 week, mice were successively submitted to a pretraining phase, a training phase and a probe test. Mice were introduced in the maze from different starting points (NE, NW, SE and SW), with position and sequence counterbalanced among individuals. On the first day, mice were submitted to two identical pretraining sessions (morning/afternoon) of four trials, each session starting with the mouse standing on the platform for 60 s in the center of the maze. Next trials started by introducing the mouse in the maze facing the wall at one of the four designated start positions. The mouse was gently guided by hand to the platform and allowed to remain on it for 60 s. A 2 h training period started on the next day and consisted of a massed-trial procedure (6,89): Mice were submitted to five blocks of five trials with an interval of 15–20 min between blocks, during which the mice were returned to home cages. Mice failing to find the platform after 60 s were guided to it and a maximum escape latency of 60 s was recorded. Mice remained 120 s on platform before the start of the next trial. Probe tests were performed 24 h after the last training trial and consisted of a single trial during which the platform was removed. Mice were allowed to swim freely for 60 s. Mouse swim paths were recorded using ANY maze and used to calculate the averaged swim speed, time and distance swum in the four quadrants. Performance in probe tests was evaluated by comparing to chance (25%) the time spent in the quadrant that previously contained the platform.

**Statistical analyses**

All data are presented as means ± SEM. Behavioral parameters were analyzed with the Statview 5.0 software (SPSS, USA). Factorial two-way analysis of variance with genotype and treatment as between-group factors and a temporal variable as the within-group factor for repeated-measure comparisons were used. Significant main effects were further analyzed by *post hoc* comparisons using the Fisher’s test. Two-group mean comparisons were done using the unpaired
Student’s t-test. Ratios were compared with the theoretical chance level using univariate t-tests. Significant linear correlations were determined using the r to z Fischer’s test. Statistical significance was set at a P-value < 0.05.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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