Relationships linking emotional, motor, cognitive and GABAergic dysfunctions in dystrophin-deficient mdx mice

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Abstract

Alterations in the Duchenne muscular dystrophy (DMD) gene have been associated with enhanced stress reactivity in vertebrate species, suggesting a role for brain dystrophin in fear-related behavioral and cognitive processes. Because the loss of dystrophin (Dp427) reduces clustering of central γ-aminobutyric acid (GABA_A) receptors, it is suspected that local inhibitory tuning and modulation of neuronal excitability are perturbed in a distributed brain circuit that normally controls such critical behavioral functions. In this study, we undertook a large-scale behavioral study to evaluate fear-related behavioral disturbances in dystrophin-deficient mdx mice. We first characterized the behavioral determinants of the enhanced fearfulness displayed by mdx mice following mild acute stress and its association with increased anxiety and altered fear memories. We further demonstrated that this enhanced fearfulness induces long-lasting motor inhibition, suggesting that neurobehavioral dysfunctions significantly influence motor outcome measures in this model. We also found that mdx mice are more sensitive to the sedative and hypnotic effects of 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol hydrochlorid (THIP), a selective pharmacological activator of extrasynaptic GABA_A receptors involved in central tonic inhibition. Our results highlight that information on the emotional aspects of mdx mice are important to better understand the bases of intellectual and neuropsychiatric defects in DMD and to better define valuable functional readouts for preclinical studies. Our data also support the hypothesis that altered spatial localization of GABA_A receptors due to Dp427 loss is a pathological mechanism associated with brain dysfunction in DMD, suggesting that extrasynaptic GABA_A receptors might be candidate targets for future therapeutic developments.

Introduction

Recent studies have uncovered an unexpected role for dystrophin, the protein responsible for Duchenne/Becker muscular dystrophy (DMD/BMD), in fear and stress reactivity. This was first suggested by the enhanced fearfulness displayed by the dystrophin-deficient mdx mouse (1). Then, a high-throughput transcriptome analysis in Japanese quail lines revealed a significant association of the dmd gene with tonic immobility, an innate antipredator fear-related behavior, reminiscent of the phenotype of the mdx mouse (2). Gene expression profiles in tadpoles also suggested dmd as a candidate predation-threat responsive gene (3). Moreover, polymorphisms in the dmd gene and decreases in dystrophin expression have been associated with a novel stress syndrome in commercial porcine populations, sometimes resulting in death during regular handling and transport for slaughter or following anesthesia (4). Obviously, this may be an issue regarding the welfare, health and care of commercial animals, as well as for the handling of...
animal models used in research and preclinical testing of targeted therapies for muscular dystrophies (5–7). The phenotype appears to be independent from skeletal muscle impairment. Although a cardiac defect might be involved, a role for brain dystrophin in controlling emotional processes seems more likely, since fear responses can be significantly reduced in mdx mice by rescuing brain dystrophin expression with intracerebral administration of exon-skipping oligomers (1).

Characterizing the behavioral processes, influencing factors and consequences of the enhanced fearfulness resulting from defects in dystrophin is an important challenge, as this might constitute an overlooked endophenotype underlying part of the intellectual, behavioral and emotional disturbances associated with the DMD/BMD syndromes (8–11). Moreover, quantitative evaluation of fearfulness in animal models may provide a relevant readout in preclinical assessment of therapies targeting the central nervous system (12). Importantly, the expression of fearfulness is characterized by a strong motor inhibition with long-lasting freezing-like posture, which may represent a critical bias in the interpretation of functional outcome measures based on quantification of mobility or motor performance in animal models (http://www.treat-nmd.eu/resources/research-resources/dmd-sops/).

The central mechanisms underlying behavioral disturbances in mdx mice are still unclear. Dystrophin, or Dp427, is a 427 kDa protein normally expressed at the postsynaptic membrane in inhibitory synapses of principal neurons in several brain structures involved in cognition and emotional behavior, such as hippocampus, amygdala, cerebellum and sensory cortices. The loss of brain dystrophin in mdx mice induces aberrant molecular, structural and physiological changes in central inhibitory synapses (1,13–17), mainly characterized by alterations in the distribution, density and/or function of extrasynaptic GABA receptors involved in central tonic inhibition (20), we also investigated for the first time the effect that a selective activator of these receptors may have on emotional and motor performance in this model.

**Results**

**Robust and specific fear response following mild acute stress**

The duration of stress-induced tonic immobility, considered as a measure of unconditioned fearfulness (1,2,21), was analyzed in mdx and WT littermate male mice in several experimental conditions associated with variable degrees of stress (Fig. 1). In a first experiment, mice aged 9 months were gently handled by the tail and simply released in a novel cage without any prior restraint and their activity was video tracked for 5 min (Fig. 1A, Tail handling). The percent time spent immobile was <25% in both genotypes (F1,20 = 3.4, P > 0.05; NS), indicating that forced exploration of a small novel cage is not sufficient to induce fear responses in the mutants. On the next day, mice were submitted to a 15 s scruff-restraint (Fig. 1A, scruff restraint). This manual restraint, similar to that currently used by laboratory experimenters to immobilize mice for standard examination or intraperitoneal (i.p.) injection, did not result in any observable fear response in WT mice (time spent immobile still <25%). In contrast, scruff restraint induced a large increase in tonic immobility in mdx mice (>80%) characterized by a lasting freezing-like behavior (genotype effect: F1,20 = 275.4, P < 0.0001). Only short distance travelled and short episodes of mobility were observed in mdx mice over the 5-min testing period. When performed in younger (3-months old) mice, scruff restraint also resulted in high levels of freezing in mdx but not in WT mice (Fig. 1A; F1,26 = 312.3, P < 0.0001), indicating that duration of disease does not potentiate fear responses. In a second experiment, we showed that scruff restraint induced a rapid increase in adrenocorticotropic hormone (ACTH) circulating levels in both genotypes (blood samples taken <2 min after restraint) as compared with the baseline levels measured in home-cage controls (F1,1 = 21.9, P < 0.001; Fig. 1B). Although the basal level of ACTH was lower in the mutants (Home-cage condition; P < 0.05), the global effect of restraint on ACTH levels was comparable among genotypes (Genotype effect: F1,1 = 0.3, P > 0.5; genotype x condition: F1,18 = 1.6, P > 0.2) with similar levels of circulating ACTH in both genotypes after restraint. Scruff restraint is an acute stress regulator of ACTH production, and because intensity of stressful stimuli and magnitude of the adrenal response are considered to be correlated (22), the results suggest that mice from both genotypes underwent comparable stress.

We then tested the possibility that tilting the animals during restraint could have been a main stressful stimulus, since in Japanese quails the restraint procedure used to induce tonic immobility only consists in tilting the animal on its back (23). However, we found that restraining mdx mice by grasping the scruff without tilting their body upside-down (“scruff-not tilted” condition) induced comparable levels of freezing as with the initial scruff-restraint procedure (>75%; Fig. 1C). When restraint consisted in placing mice individually in a narrow tube (3 cm in diameter) for 5 min, the freezing levels in mdx mice were lower than in the standard scruff-restraint condition (62%; P < 0.05; Fig. 1C, “tube” condition), suggesting that freezing amount varied depending on intensity of stressful events.

We then determined whether unconditioned fear responses could be modulated by repetition of the stressful stimuli. When scruff restraint was applied once a day during 6 days (Fig. 1D), the freezing response remained enhanced and stable in mdx mice (Genotype effect: F1,9 = 120.9, P < 0.0001; trial effect in mdx group: F5,25 = 0.6, P > 0.6; NS), indicating strong resistance to extinction of this behavior. In contrast, WT mice initially displayed a very low level of immobility (<10%), which progressively increased to ~35% (Trial effect: F5,20 = 5.8, P < 0.01), reflecting a typical decrease in their motivation for repetitive exploration. In another group of naïve mdx mice repetition of the stress procedure was applied in 18 trials within the same day (Fig. 1E). During the first block of six trials, the percent freezing increased from ~78% up to ~94%, reflecting a potentiation of the fear response. Then, the percent freezing decreased across next trials (F17,102 = 24.3, P < 0.0001), indicating that...
extinction may occur with extensive repetition of the stressful stimulus. This was associated with struggling attempts during restraint and slight renewal of vertical activity.

Enhanced fearfulness induces lasting motor inhibition

The lasting impact of fearfulness on mouse motor activity was addressed by comparing the circadian modulation of spontaneous locomotor activity following gentle handling (No stress condition) and scruff restraint (Stress condition). As shown in Figure 1F, the absence of prior stress resulted in comparable spontaneous horizontal activity in both genotypes over a 24 h cycle. A typical circadian rhythmicity was observed in both WT and mdx mice: Activity was increased during the first hour that followed handling and larger increases were further observed during the dark phase of the cycle. In marked contrast, the scruff-restraint stress, performed just before releasing the mice in actimeter cages, induced a strong and lasting motor inhibition in mdx mice. It is worth to note that horizontal activity was at a very low level in mdx mice during the first 5 h following stress, as compared to control mice. During the dark phase, locomotion in WT mice was globally higher in this stress condition as compared to the no stress condition. In mdx mice, the onset of darkness induced a renewal of mobility but their activity remained largely below WT levels throughout the dark phase. Comparable levels of activity between genotypes were only observed after >12 h. The temporal distribution of vertical activity followed a comparable, yet more variable, time course.

Figure 1. Fear-related tonic immobility. (A) Percent freezing in 5 min following gentle tail handling or brief scruff restraint (15 s) in 9-month-old (WT, n = 10; mdx, n = 12) and 3-month-old mice (n = 14 per genotype). (B) Plasma ACTH levels in home-cage control mice (n = 6 per genotype) and in mice submitted to scruff restraint (n = 5 per genotype). (C) Percent freezing when restraint was applied by grasping the scruff without tilting upside-down animal’s body (“scruff restraint—not tilted”; 5 WT and 7 mdx) or by placing individual mice in a transparent cylinder 3 cm in diameter (“tube”; 2 WT and 7 mdx). (D) Percent freezing following scruff-restraint in trials repeated once a day during 6 days (5 WT and 6 mdx). (E) Percent freezing in a group of 7 mdx mice when scruff restraint was applied in 18 trials distributed within the same day in 3 blocks of 6 trials (20-min ITI; 90-min interval between blocks). *P < 0.05; **P < 0.001. (F) Circadian modulation of spontaneous horizontal locomotor activity, evaluated from the number of consecutive beam breaks per 1 h bin during 24 h, following gentle tail-handling introduction of the mice in the actimeter (NO STRESS; n = 12 per genotype) or after scruff restraint (STRESS; n = 8 per genotype). Grey rectangles represent the 12 h periods of darkness; probabilities at the top show the genotype effects during the light and dark phases. Circadian modulation of vertical activity shown in Supplementary Material, Figure S1.
across successive trials (F1,26 = 14.2, P < 0.001). Locomotion and exploration of a novel environment (open-field test) were also drastically reduced in mdx mice (Fig. 2B and C): The distance travelled was constantly reduced by ~40% in mdx as compared to WT mice over the 30-min recording period (F1,26 = 15.7, P < 0.001; Fig. 2B) and the maximal speed shown by mdx mice during bouts of locomotor activity was also reduced (P < 0.01; Supplementary Material, Fig. S2A). Exploration of the center of the open field (OF), typically considered as an anxiety-related behavioral parameter, was lower in mdx compared to WT mice (Fig. 2C): Mdx mice displayed an increased latency to enter the center zone (F1,26 = 4.4, P < 0.05; Fig. 2C), a reduced number of entries into this zone (F1,26 = 9.2, P < 0.01; Fig. 2C) and the percent distance travelled in the center zone was also significantly reduced during the first 15 min of testing (F1,26 = 4.9, P < 0.05; Supplementary Material, Fig. S2B), suggesting a higher level of anxiety in the mutants.

Behavioral despair, typically characterized by episodes of immobility (freezing) in response to unescapable stressful situations, was assessed using two rodent models of depression, the tail-suspension test (TST) and the forced swimming test (FST). In both tests, mice initially engaged vigorous movements to escape from the stressful situation and then showed freezing episodes of increasing duration after a few minutes. A typical two-session procedure with a 24 h interval was used to induce a high and stable level of immobility. In both TST (Supplementary Material, Fig. S2C) and FST (Supplementary Material, Fig. S2D), the latency and duration of freezing were comparable between genotypes during each session, reflecting comparable motivation and active reactivity to stress in the two genotypes. The second testing session was characterized by reduced latencies and increased durations of freezing, reflecting learning of the unescapable nature of this aversive situation. In the FST, the ability to stay afloat was reduced in mdx compared to WT mice (F1,26 = 8, p < 0.01), while the time spent climbing (repetitive striking of the glass walls with forepaws) was longer in mdx mice (F1,26 = 6.7, p < 0.05; Fig. 2D). However, these two parameters did not show main variations within each genotype between the two sessions, suggesting that they reflected motor skills rather than behavioral despair or learning, as believed by others (25,26).

Fear memory was evaluated in a single-trial contextual fear conditioning paradigm. During familiarization to the testing box (habituation), motor activity was reduced in mdx mice (Supplementary Material, Fig. S1): Vertical activity was comparable in unstrressed WT and mdx mice, while it was largely smaller in mdx mice compared to WT mice in the stress condition. The data suggest long-lasting effects of fearfulness on mdx mice mobility but preserved circadian modulation of spontaneous locomotor activity.

Relationships among motor, emotional and cognitive performance

The young mice (3 months old) tested for unconditioned tonic immobility in Figure 1A were further submitted to a behavioral test battery aimed at evaluating putative dependence among behavioral variables measuring motor, emotional and memory performance. The inverted grid test (Fig. 2A) confirmed that mdx mice fail to maintain a grip onto a grid placed upside down, which is believed to result from their inability to support their own body by grasping the grid due to muscle weakness (24). Their fall latencies were consistently shorter than in WT mice across successive trials (F1,26 = 14.7, P < 0.001). The data suggest long-lasting effects of fearfulness on mdx mice mobility but preserved circadian modulation of spontaneous locomotor activity.

Figure 2. Motor, emotional and memory performance in a longitudinal behavioral study. (A) Muscle strength evaluated by fall latencies in mice placed upside-down on an inverted grid across three successive trials. (B) Ambulation in the OF for 30 min, expressed as distance travelled by 5-min bins. (C) Two representative track plots on the left show exploration paths cumulated over the 30-min recording period in the OF, with an overt reduction of exploration of the center zone in mdx mice. The following plots show the latency of the first entry (s) and the total number of entries in the center zone. (D) Time spent staying afloat (s) and time spent climbing (s) during the two sessions of the FST. (E) Single-trial contextual fear conditioning. The bar plot shows the percent freezing recorded during the habituation (Hab) and acquisition sessions (BS and AS: before and after shock delivery, respectively). The line plot shows the percent freezing expressed in 30-s bins during the first 3 min of the retention session performed 24 h later. All tests, n = 14 per genotype. **P < 0.01; ***P < 0.001; *P < 0.05. Supporting data in Supplementary Material, Figure S2.
compared to WT, as evidenced by analysis of the distance travelled (P < 0.001), maximal speed (P < 0.0001) and vertical activity (P < 0.001). Slight hypoactivity was also present in mdx mice before shock delivery during the acquisition session. Delivery of a single electric shock during the acquisition session was associated with increased freezing in both genotypes (Fig. 2E, left panel) and there was no more genotype effect in all activity parameters, suggesting comparable acquisition in the two groups of mice. On the following day, mice placed in the same context expressed fear responses (freezing) reflecting retention of contextual fear memory (Fig. 2E, right panel). However, the quantity of freezing expressed during the first 30 s was significantly lower in mdx than in WT mice (P < 0.05), suggesting a delayed recall of contextual fear memory. Moreover, when freezing behavior was normalized to basal immobility recorded during habituation (21), then the genotype difference in fear memory performance was significant throughout the 5-min testing period (F1,26 = 6.3, P < 0.02; not shown). Impaired performance could not be attributed to overt changes in the behavioral expression of the fear response, as excessive running, jumping, or motor stereotypes were not observed during this retention session.

To evaluate putative relationships among the diverse behavioral disturbances displayed by mdx mice, we selected seven main variables that enabled identification of significant genotype effects in this longitudinal study and performed a multivariate factor analysis to highlight interrelated variables in each genotype (Table 1). The principal component method enabled extraction of three factors with eigenvalues >1, which together explained >75% of the total variance in each genotype (mdx: 87%; WT: 77.2%). Each factor was represented by a linear combination of a relatively independent subset of highly correlated variables (27). Interpretation of each factor was based on the identification of the variables that highly correlated with this factor but not with the other extracted factors. In mdx mice, the first factor, which explained the largest amount of group's variance (38%), was characterized by a high and inverse correlation between variables representing either exploration or fearfulness. This likely reflects a strong relationship between the enhanced fearfulness and reduced exploratory activity during open-field testing in mdx mice. Factor 2 was characterized by a coherent subset of variables related to motor function, highlighting that large amounts of climbing behavior correlated with short time spent floating in the FST and short fall latencies in the inverted grid test. This confirms that the genotype effects revealed in the FST reflect a motor impairment rather than altered behavioral despair. Interestingly, variables associated with motor functions only correlated with factor 2 whilst fear memory performance correlated with factor 3. Overall, this suggests that reduced exploration and locomotion in the OF were mainly influenced by fear reactivity, while the other motor and cognitive deficits in this mutant were relatively independent from emotional disturbances. Distinct variable interdependencies were found in WT mice. First, there was no relationship between exploratory behavior and fear reactivity, as the related variables were highly correlated to independent factors (Factor 1 and 3, respectively). Second, fear memory performance was partially correlated to factor 2 and 3, suggesting that both motor performance and stress reactivity influenced the level of freezing upon memory recall in the fear conditioning task in WT mice. This further suggests that the functional relationships linking motor, emotional and cognitive functions in mdx mice were disorganized.

**Anxiety-related behavior and aversive cue-outcome associative learning**

We further evaluated the presence of anxiety-related responses in independent groups of WT and mdx mice in standard anxiety tests. In the light-dark box test, mice had the choice to explore a brightly lit anxiogenic compartment or to stay in a more secure dark compartment. As shown in Figure 3A, the time spent in the lit compartment was shorter in mdx mice compared to WT mice (F1,30 = 10.4, P < 0.01), suggesting a higher level of anxiety in the mutants. In contrast, no main genotype difference was detected in the elevated plus-maze test, in which anxiety results from the threat induced by void in elevated open arms (Fig. 3B). The number of entries and time spent in open arms were comparable between genotypes (P > 0.4; NS). Moreover, both genotype performed a comparable number of head dips at exit of closed arms (Protected head dips; F1,15 = 0.5, P > 0.4; NS) and during exploration of open arms (Unprotected head dips; F1,15 = 0.7, P > 0.4; NS), indicating that risk assessment behavior was unaltered in mdx mice.

Aversive cue-outcome associative learning was studied in auditory-cued fear conditioning and conditioned taste aversion. Mice were gently handled every day for a week before being submitted to tasks, in order to avoid any stress before testing. As shown in Fig. 3C, acquisition of fear conditioning was significantly delayed in mdx mice (F1,29 = 5.6, P < 0.03), whereas freezing amount during inter-trial intervals (after footshock delivery) was comparable in the two genotypes (F1,29 = 1.4, P > 0.2; not shown), suggesting a specific deficit in learning the predictive value of the conditioned stimulus (CS) (tone). Recall of fear memory 24 h post-acquisition was also strongly delayed in mdx mice, as shown by the small amount of freezing induced by the delivery of the CS alone during the retention session (F1,29 = 9.1, P < 0.01). In contrast, no genotype difference was detected in conditioned taste aversion. For this test, the mice were maintained for 3 days under a water-restriction regimen before conditioning started; mice in both genotypes displayed comparable body-weight loss (mdx: 10.8 ± 0.4%; WT: 10.6 ± 0.3%; F1,21 = 0.04, P > 0.8) and total water intake (mdx: 1.7 ± 0.08 ml; WT: 1.6 ± 0.06 ml; F1,21 = 0.9, P > 0.3). During the acquisition session, comparable sucrose intake was quantified in the two genotypes (mdx: 1.3 ± 0.07 ml; WT: 1.2 ± 0.1 ml; F1,21 = 1, P > 0.3). During the retention session (Fig. 3D), i.e. 24 h after sucrose consumption had been paired with LiCl-induced nausea, both mdx and WT mice consumed less of the sucrose solution as compared to unconditioned controls injected with NaCl (Genotype effect: F1,19 = 0.3, P > 0.5; treatment: F1,19 = 29.8, P < 0.0001, genotype × treatment interaction: F1,19 = 1.5, P > 0.2). This other form of long-term associative memory therefore appeared to be unimpaired in mdx mice.

**Behavioral effects of the extrasynaptic GABAA-receptor agonist, THIP**

4,5,6-Tetrahydroisoxazolo[5,4-c]pyridin-3-ol hydrochlorid (THIP), also called Gaboxadrol, is a selective activator of extrasynaptic 6-subunit-containing GABAA receptors, which can induce a range of dose-dependent anxiogenic, sedative/ataxic and hypnotic effects following i.p. injection (28–30). We first investigated the effects of two doses of THIP (3 and 10 mg/kg) on exploratory activity in the OF, 30 min post-injection. Saline control as well as THIP-injected mdx mice were less active than WT mice receiving comparable treatments in this test, as shown by decreased distance travelled and decreased ambulation speed.
in all mdx subgroups (Fig. 4A and B, respectively; \( P < 0.001 \)). There was a trend in THIP-treated mdx mice, but not in WT mice, to display dose-dependent decreases for these motor parameters. However, this apparent difference between genotypes was not significant (drug \( \times \) genotype interaction: NS). Both doses of THIP only induced a slight reduction in vertical activity in WT mice (Fig. 4C). In contrast, 10 mg/kg THIP induced a drastic decrease in vertical activity in mdx mice, but not in WT mice.

Table 1. Relationships among motor, emotional and memory performance. In each genotype (\( n = 14 \) per genotype), seven variables that revealed significant genotype effects in the behavioral test battery were used: the percent time spent in tonic immobility following scruff restraint (Fearfulness), the total distance travelled and the number of entries in the center zone in the open-field test (OF distance; OF center entries), the fall latency in the inverted grid test (Grid fall latency), the time spent staying afloat and climbing in the FST (Staying afloat; Climbing) and the percent freezing during the first 30-s bin during the retention session of contextual fear conditioning (Fear memory). The seven variables were reduced to three extracted factors in each genotype. For each factor, the eigenvalue (EV) and percent of total variance explained by the factor are shown at the top of the columns. Interpretation of each factor is indicated below the variance values, based on the correlation of the seven variables on this factor. Strong correlation of a variable (\( > 0.75 \)) with only one of the three factors is highlighted in grey. Correlations comprised between 0.5 and 0.75 are underlined. Note the strong inverse correlation between fearfulness and OF exploration parameters in mdx, but not in WT mice (Factor 1), while other motor parameters (grid fall latency, staying afloat and climbing) and memory performance independently correlated with factors 2 and/or 3 in both genotypes.
Figure 4. Behavioral responsiveness to the extrasynaptic GABA_A receptor agonist, THIP. (A–F) Effects of THIP administration on WT and mdx mouse behavior during OF exploration for 10 min (n = 9 per treatment group in each genotype). Several behavioral parameters were analyzed in THIP-injected (3 mg/kg; 10 mg/kg) and saline-injected mice of both genotype: (A) distance travelled, (B) travelling speed, (C) vertical activity, (D) time spent circling along walls, (E) number of entries in the center area, (F) percent distance travelled in the center area. The main genotype difference ("P < 0.01 in C) was the drastic reduction of vertical activity observed in mdx mice, but not in WT mice, following administration of 10 mg/kg THIP. (G) Number of entries in the lit compartment during light-dark choice testing. (H) and (I) Hypnotic effect of 30 mg/kg THIP administration (WT, n = 8; mdx, n = 10) characterized by loss of the righting reflex (LORR) and expressed as LORR latency (H) and LORR duration (I). Genotype differences: "P < 0.01; ""P < 0.001.
mdx as compared to WT mice (Fig. 4H), while the LORR duration was drastically increased in mdx mice (>4-fold increase; Fig. 4I), thus reflecting a strongly delayed righting reflex recovery in the mutants. In all, mdx mice showed a higher responsiveness to THIP at doses which usually induce sedative (10 mg/kg) and hypnotic (30 mg/kg) effects in mice.

Discussion
In the present study, we have further characterized the determinants of the enhanced fearfulness displayed by dystrophin-deficient mdx mice in response to mild acute stress and we deciphered its potential impact on motor, emotional and cognitive performance. Moreover, we provide the first evidence that mdx mice have an enhanced sensitivity to the behavioral effects induced by a selective agonist of extrasynaptic GABA_A receptors, suggesting that a defect in central tonic inhibition might contribute to the behavioral disturbances associated with DMD.

Determinants of enhanced fearfulness
The potent freezing response in mdx mice, expressed as lasting tonic immobility, is reminiscent of the natural defensive death-feigning posture that animals normally enter when confronted to a predator or other extreme threat, which is believed to involve a brain circuit that includes the amygdala (1,31). The precise brain mechanisms responsible for this phenotype remain to be fully elucidated, but the present study constitutes an important step forward to better understand the bases of this abnormal behavior. Here, we show that the enhanced fearfulness displayed by dystrophin-deficient mdx mice is robust and resistant to extinction, as it can be stably induced and repeatedly expressed over days. This phenotype might therefore constitute a main readout for longitudinal studies assessing the dynamics of therapeutic agents targeting brain dysfunctions in this model of DMD. Here, we have used singly-housed mice that we have released in novel environments following scruff restraint stress. However, it is likely that group-housed mice released in a familiar environment may express reduced fear responses, as suggested by one study showing that the presence of familiar olfactory cues in the cage reduces the amount of freezing in stressed mdx mice (32).

It seems unlikely that a peripheral dysfunction contributes to the expression of fearfulness in mdx mice. Indeed, the magnitude of the fear response does not increase as a function of disease duration from 3 to 9 months of age, and a substantial extinction of the fear response can occur if presentation of the stressful event is extensively repeated within a single day, which does not seem compatible with a cardiac defect. Accordingly, severe cardiomyopathy has only been reported in mdx mice older than 18 months (33,34) or in the specific mdx^cn^ model of cardiomyopathy (35). Performing restraint stress without tilting animal's body did not reduce the amplitude of the fear response, which rules out contribution of a vestibular defect. A greater pain sensitivity is also unlikely, as freezing was also observed after a short stay in a tube restrainer and changes in nociception were not detected in mdx mice in a footshock threshold test (36). Mdx mice did not show any characteristic features of spontaneous catalepsy during tonic immobility, such as rigidity of the extremities or maintenance of a position of the body forced by the experimenter (37), and we verified that sudden noises or touching the animal could interrupt freezing and induce motion.

We further addressed the possibility that neophobia or depressive-like behaviors could contribute to enhanced fearfulness in mdx mice (38). However, unstressed mdx mice showed normal exploratory activity in a novel environment (actimeter) and the behavioral responses induced by unescapable stressful situations (attempts to escape, despair) were comparable in adult mdx and WT littermate mice in both TST and FST, two standard tests used in depression studies in mice models (39,40). Therefore, enhanced fearfulness could not be attributed to a main alteration of the processes involved in active reactivity to stress or of a motivational component of emotionality. We also quantified the levels of the circulating adrenocorticotropic stress hormone (ACTH) in home cage and after scruff restraint. Home-cage levels of ACTH measured close to the time of circadian nadir were lower in mdx compared to WT mice, suggesting a slight alteration of the passive (glucocorticoid degradation) or active processes (feedback inhibition) that terminate hypothalamo-pituitary-adrenocortical responses following stressor suppression (41). However, a large rise in ACTH level was induced by scruff restraint and comparable net levels were detected in both genotypes, suggesting that they underwent comparable stress responses, in line with the comparable levels of corticosteroids detected by others following the same protocol (32).

The enhanced fearfulness in mdx mice likely reflects a mal-adaptive coping strategy, as maturational and environmental factors normally favor progressive decrease of such unconditional responses. Often considered as an innate behavior, unconditioned fear is also viewed as a cognitive process since it involves evaluation of emotional valence, representations, attention and arousal, and outcome predictions that enable anticipatory, coordinated and dynamic response to fearful stimuli (42). Our results support the hypothesis that dystrophin loss alters the amygdala-dependent processing of threat cues (1) or, more largely, the functional integrity of the brain fear circuit that normally regulates fear responses and fear-related motor behavior (42-44).

Impact on motor functions
Importantly, we found that enhanced fearfulness may have a strong and long-lasting impact on locomotion and exploratory activity in mdx mice. Indeed, we demonstrate that the strong stress-induced motor inhibition in mdx mice may last several hours after termination of the restraint when mice are immediately placed in a novel environment (actimeter). Even though circadian rhythmicity was preserved and a renewal of mobility was induced following onset of the dark phase in the actimeter, both horizontal (ambulation) and vertical (rearing, leaning) activities were strongly reduced in mdx mice compared to WT mice for about 12 h. In contrast, locomotion and vertical activity were comparable in the two genotypes in the absence of prior stress. Such a long-lasting effect of restraint stress has also been observed in Japanese quails in which a significant association of the dmd gene with tonic immobility was determined (2). In our longitudinal study, in which mice were submitted to a behavioral test battery starting with a scruff restraint episode, we also observed a drastic reduction in mobility during exploration of an OF, even though this test was performed five days after restraint. Reduced ambulation of mdx mice during open-field testing has been previously reported (45), but this is at variance with the normal locomotion observed in naive mdx mice released in a novel cage or OF without prior stress, such as
shown here in the actimeter or in previous open-field studies undertaken by other research groups (1,38). Our results suggest that any period of restraint may have a long-lasting effect on exploratory behavior and ambulation in mdx mice, yet differences in housing, handling and experimental conditions could explain variability among laboratories (32).

Our longitudinal study revealed a strong inverse correlation between stress-induced freezing and global locomotor activity during open-field testing (Table 1), thus confirming the relationship between exploratory behavior and prior stress experience in our conditions. In contrast, other motor parameters reflecting poor muscle strength and/or motor incoordination, such as climbing ability and capacity to stay afloat in the forced swim test, or the fall latency in the inverted grid test, did not correlate with enhanced fearfulness. Hence, acute stress in mdx mice has a lasting inhibitory effect on exploration and locomotion in novel environments (cage, actimeter, OF), but not on other outcomes of motor function tests related to muscle strength and motor coordination. Poor mobility in these mice, sometimes attributed to extreme fatigue due to muscle wasting (24), should therefore be interpreted with caution as it may in part result from enhanced stress reactivity. Scruff restraint is a routinely used method in animal facilities to verify mouse identification and for intra-peritoneal injections. We demonstrate here that this apparently mild stress associated with routine mouse handling may have profound and confounding effects on locomotion parameters that are often used to assess treatment effects in preclinical studies using the mdx mouse model.

Impaired fear learning and memory

In Pavlovian fear conditioning, good memory performance is characterized by rapid occurrence and long duration of freezing responses that reflect associations between aversive stimuli and predictive cues. A chronic enhancement of emotional arousal may result in a bias for threat cues and facilitation of fear learning and memory, as in mouse models with disrupted clustering of GABA A receptors (46). However, despite the reduced GABA A-receptor clustering and high fear reactivity reported in the mdx mouse, this model rather displays performance deficits in several amygdala-dependent fear-learning paradigms (21,36). Freezing responses upon recall of fear memories are reduced in mdx mice compared to WT mice, whether animal's motion and freezing responses are quantified by video-tracking methods or using a weight transducer system. Analyses of different behavioral parameters did not reveal any clue that fear could be expressed through different behavioral responses in mdx mice. A pronounced impairment was found during auditory-cued fear conditioning, in which association of fear with a predictive auditory stimulus requires an intact amygdala (47). In contrast, long-term memory of a conditioned taste aversion was unimpaired, suggesting that only a subset of amygdala-dependent aversive-outcome associative processes are affected by dystrophin loss (48,49). The recall of contextual fear memory was also impaired in mdx mice. This performance, recorded in a longitudinal study, did not correlate with the tonic immobility recorded in the scruff-restraint test, suggesting that these two phenotypes relied on separate mechanisms. This is in agreement with current neurobehavioral models highlighting that distinct nuclei and subnuclei within the amygdala, as well as connections to different subcortical structures and specific cellular and signaling mechanisms, differentially contribute to unconditioned fear and to different forms of fear memory (42,50).

Because memory deficits are detected in mdx mice even in tasks for which good performance is normally reflected by heightened immobility, they likely result from the formation of inappropriate fear associations and/or alterations of consolidation processes, as suggested earlier (36). Hippocampal function is altered in mdx mice (51,52) and it is worth to note that the hippocampus is also engaged with amygdala during contextual fear conditioning (53,54), as well as in the modulation of fear-related motor behavior (43). A recent study using cell-specific conditional knockouts of the GABA A receptor α2 subunit in distinct hippocampal subfields unveiled that a specific microcircuit in the CA1 subfield takes part in the expression of fear responses (55). It is thus likely that dystrophin loss, which has been associated with impaired clustering of α subunit-containing GABA A receptors in various cortical and subcortical structures, may elicit variable alterations in distinct amygdalar nuclei as well as in connected structures involved in the circuits of fear, anxiety and emotional learning, giving rise to the complex phenotypes characterized herein.

Anxiety-related behaviors

Higher reactivity to acute stress may be associated with alterations in other aspects of emotionality such as anxiety (56,57). Here, mdx mice showed a reduction of exploration of the central zone of an OF, an indication of enhanced anxiety (21,38), and a higher level of anxiety in a light-dark choice anxiety test. As reported by others (1), however, mice of both genotypes displayed comparable performance and risk assessment strategies in the elevated plus maze test. The cause of these discrepancies observed among distinct anxiety tests remains unclear but cannot be attributed to differences in the motor demand, as enhanced anxiety-related responses were observed in both low-(light-dark choice) and high-motor demand tests (OF exploration), but not in the elevated plus maze which also involves a high-motor demand. The nature or strength of the anxiogenic stimulus could be a possible factor modulating emotional reactivity in mdx mice, as well as the different novelty-seeking motivation and attention states associated with distinct experimental situations. Anxiety is a complex phenomenon that cannot be described in a single test and several studies demonstrated that the effects of anxiolytic compounds may greatly vary depending on the targeted neurotransmission system, mouse genetic background and experimental conditions (58). Although an alteration of amygdala GABAergic circuits remains a major hypothesis to explain emotional disturbances in mdx mice, additional alterations of central serotonin (38,59,60) and cholinergic functions (61) might also induce specific modulations of anxiety-related behaviors in distinct testing conditions (62,63).

What role for extrasynaptic GABA A receptors?

In mdx mice, the number of α subunit-containing postsynaptic GABA A-receptor clusters is reduced by ~50% in central inhibitory synapses of hippocampus, amygdala and cerebellum. Consequently, a reduction in GABAergic inhibition has been reported in these structures (1,17,20,64) and associated with altered synaptic plasticity (52,65–67). In the basolateral amygdala of mdx mice, norepinephrine (NE)-induced GABAergic transmission is reduced (1). As NE is normally released in
response to stressful stimuli such as restraint and footshocks, this alteration in mdx mice might perturb the control of neuronal excitability in this structure and inappropriately modulate the brain aversion system and neuronal circuits involved in unconditioned and conditioned fear.

The reduced clustering of synaptic GABA\(_A\) receptors in mdx mice is not associated with significant changes in the total amount of GABA\(_A\)-receptor protein, suggesting that dystrophin is dispensable for GABA\(_A\)-receptor anchoring but rather participates to the dynamic stabilization of large synaptic receptor clusters (15,64,68,69). The loss of dystrophin would thus result in a partial loss of synaptic clusters due to the lateral diffusion of unstable receptors to extrasynaptic sites, with putative changes in the combination of specific receptor subunits leading to complex alterations of GABA\(_A\) receptor pharmacology.

mdx mice were more sensitive to higher doses of THIP that mdx mice, suggesting that altered tonic GABAergic inhibition underlie part of the phenotype of DMD patients, such as intellectual disability and epilepsy, and to the phenotypes of mdx mice, such as anxiety (72), stress adaptation (73–75) and fear conditioning (76).

Because of its higher selectivity for the \(\delta\) subunit, THIP constitutes a unique tool to determine whether altered expression of extrasynaptic GABA\(_A\) receptors plays a role in DMD pathophysiology. One major finding in the present study is the demonstration that mdx mice are more sensitive to the behavioral effects of THIP, which strongly supports this hypothesis. As many other pharmacological modulators of GABAergic function, THIP may induce distinct functional alterations in a dose-dependent manner, including hypnotic, sleep-promoting, antinociceptive, anticonvulsant, sedative and anxiolytic effects (28–30). We therefore compared the effects of three doses of THIP (3, 10 and 30 mg/kg) known to have main effects on anxiety, sedation and hypnosis, respectively. While the lowest dose of THIP induced comparable effects in both genotypes, we found that mdx mice were more sensitive to higher doses of THIP that usually mediate sedative/atletic and hypnotic effects in mice. The main effect of 10 mg/kg THIP in mdx mice was a drastic reduction of vertical activity, a parameter that is typically altered in stressed mdx mice, suggesting that altered tonic inhibition might participate to motor inhibition processes in this model. This suppression of vertical activity in mdx mice may reflect a change in arousal, motivational and/or emotional states, but putative alterations of central mechanisms controlling nociception, balance and/or motor/muscle functions cannot be ruled out (77). The hypnotic effect of a higher dose of THIP (30 mg/kg), characterized by shorter latency and longer duration of the loss of righting reflex (LORR), was drastically enhanced in mdx mice. Interestingly, this was comparable to the phenotype reported in mice overexpressing \(\gamma\) subunit-containing extrasynaptic receptors (30) but opposite to that of mice lacking the extrasynaptic \(\delta\) subunit (78). As subsets of both synaptic and extrasynaptic receptors are affected in mdx mice, there is a need for future studies to specify the molecular and functional consequences of defective GABAergic inhibition in this model. It is likely that the molecular alterations in mdx mice are subject to local and temporal modifications due to the homeostatic competition between tonic and phasic inhibition to maintain total inhibition (79) and because receptor composition and diffusional trafficking between synaptic and extrasynaptic sites also varies during network activity and plasticity to finely tune inhibitory sensitivity (80–82). Our present results suggest that the altered tonic conductance mediated by \(\delta\)GABA\(_A\) extrasynaptic receptors in mdx mice may regulate important behavioral and physiological functions. Therefore, this subtype of GABA\(_A\) receptors might be a relevant therapeutic target for DMD and animal models of this disease.

Conclusions

Although the increased prevalence of anxiety and mood disorders in DMD/BMD patients may partly result from their clinical condition and quality of life, mouse studies as well as recent neuropsychological studies have given greater consideration to the contribution of a central component in the genesis of emotional and neuropsychiatric disturbances in these syndromes (11,83–85). Our present data are in line with recent clinical studies suggesting that emotional disturbances are common in DMD patients with mutations that specifically impede expression of Dp427 dystrophin (11). Information on the emotional aspects of mdx mice is therefore important to further understand DMD neuropathology and may also provide valuable functional readouts in preclinical studies (12). However, the nature and severity of the cognitive impairments in DMD may vary depending on individual’s mutation profile, as distal mutations may alter expression of shorter dystrophin-gene products having distinct functions in brain (11,51). Our present results suggest that loss of full-length dystrophin alters the functioning of the neuronal circuit of fear, which includes the amygdala and connected subcortical and cortical structures, leading to enhanced unconditioned fears, anxiety and deficits in fear conditioning and memories. Recognizing, avoiding and adapting to aversive situations are central aspects of mammalian cognition. Dysfunctions in these processes may result in severe changes in emotional reactivity, maladaptive social behaviors and decreased intellectual functioning (42,86,87). Moreover, we show that enhanced fearfulness in mdx mice induces long-lasting motor inhibition. This highlights that brain and behavioral dysfunctions may also significantly influence motor outcome measures in this model, which might lead to an overestimation of motor deficits and/or be confused with responses of lack of responses to treatments in preclinical studies. Our data also support the current hypothesis that altered spatial localization of GABA\(_A\) receptors and altered tonic GABAergic inhibition underlie part of the central disturbances due to Dp427 loss, suggesting that extrasynaptic GABA\(_A\) receptors should be further considered as candidate targets for future therapeutic developments.

Materials and Methods

Animals and experimental groups

Mice of the C57BL/10ScSn-Dmd\(^{mdx}\)/J (mdx) and C57BL/10ScSn strains were originally purchased from Charles River Laboratories.
Human Molecular Genetics, 2017, Vol. 26, No. 6 | 1051

(France) and Harlan (UK), respectively, and then bred in our laboratory. Dystrophin-deficient mdx and wild-type littermate male mice (WT) were obtained by mating heterozygous mdx females with C57BL/10ScSn males. Siblings were kept in group (two to five per cage) under a 12-h light-dark cycle (light on: 7:00 a.m.) with food and water ad libitum. Only the cages containing mice of both genotypes were selected for experiments. Behavioral testing was performed blind to the genotype by a male experimenter. Mouse genotype was verified by PCR and confirmed by post-mortem histological analysis of quadriceps muscle (21). Animal care and experimental procedures complied with the European Communities Council Directive (CEE 86/609/EEC), EU Directive 2010/63/EU, French National Committee (87/848) and ethic committee (Paris Centre et Sud, N°59).

Mice were changed to individual caging 1 week and given daily handling during at least 3 days before testing. A first group of mice aged 3 months (n = 14 per genotype) was submitted to a series of behavioral tests with intervals of 3–5 days between tests, in the following order: restraint-induced unconditioned fear, OF activity, forced swim test, tail-suspension test, single-trial contextual fear conditioning and inverted screen test. Independent groups of naïve mice aged 4–6 months were submitted to the other tests. One group of 9-month old mice was tested for restraint-induced stress to assess aging effects on this behavioral response. Pharmacological experiments (effects of THIP on behavior) were performed in 3-months old mice.

**Behavioral tests**

**Restraint-induced unconditioned fear.** The mouse was restrained by grasping the scruff and back skin between thumb and index fingers, while securing the tail between the third and little fingers and tilting the animal upside-down in order that the ventral part of its body faced the experimenter. After 15 s, the mouse was released to a novel cage (24 × 19 cm, with 12-cm high walls) containing clean sawdust and was then video-recorded for 5 min under dim illumination (80 lx) using the Any-maze software (Stoeling, USA). Unconditioned fear responses induced by this short acute stress were characterized by periods of tonic immobility (freezing) and quantified during a 5-min recording period. Complete immobilization of the mouse, except for respiration, was regarded as a freezing response (88). The percent time spent freezing was calculated for group comparisons. In separate groups of mice, fear responses were analyzed following restraint applied by grasping the scruff without tilting upside-down animal’s body (“scruff—not tilted” condition), or by placing individual mice horizontally in a transparent polypropylene cylinder (3 cm diameter, 10 cm long) provided with ample air holes for ventilation (tube condition). Extinction of the freezing response was analyzed by repeating scruff restraint either once a day during 6 days (5-min recording each day) or during 18 trials distributed within the same day and grouped in three blocks of six trials (20 min intertrial interval (ITI); 90 min interval between blocks; 3 min recording per trial).

**OF activity.** The test box was a square OF (50 × 50 × 50 cm) with black walls and a floor covered with sawdust. Experiments were undertaken under constant room temperature (22–23°C) and homogeneous dim illumination (50 lx). Each mouse was released near the wall and video-recorded for 30 min using the Any-maze software. Recorded xy positions were used to generate tracking plot of the exploration paths and to calculate the distance travelled, speed and time spent in distinct zones of the box, i.e. in the whole apparatus, in a virtual corridor (width: 10 cm) along the walls and in the remaining central area, referred to as the center area. Latency of the first entry, number of entries, percent distance travelled in center area and circling sequences along walls were calculated as relative measures of anxiety.

**Elevated plus-maze anxiety test.** The maze had two facing arms enclosed with high walls (20 × 8 × 25 cm), two open arms (20 × 8 cm) and a central area (8 × 8 cm) forming a plus sign 65 cm above the floor. Illumination was 150 Lx in open and 30 Lx in enclosed arms. Mice were individually placed at the center of the maze with the head facing an open arm. The number of entries and time spent in open or enclosed arms were recorded for 5 min. Head-dipping over sides of open arms were counted and classified as protected head dips, when the rest of the mouse’s body remained in a closed arm, and as unprotected head dips when the whole mouse’s body was located in the open arm.

**Light-dark choice.** The apparatus had 20-cm-high Plexiglas walls and consisted of a brightly lit white compartment (40 × 15 cm; illumination: 600 Lx) connected by a trap door (6 × 6 cm) to a dark compartment (15 × 15 cm; illumination < 10 Lx). Each mouse was placed in the dark compartment for 10 s, the trap door was then opened and mice allowed to freely explore the whole apparatus for 5 min. Step through latency, number of entries and total time spent in the lit compartment were scored.

**Forced swim test.** Each mouse was lowered in an inescapable glass cylinder (Diameter: 11 cm; height: 23 cm) filled with 18 cm water at 25°C (39). Room temperature was 25°C. Behavior was recorded on video for 5 min each day in two sessions separated by a 24 h delay. Video were analyzed offline using event-recorder keys in Any-maze to quantify the latency and duration of three main parameters (25,26): climbing, staying afloat and immobility (freezing). Climbing was considered when mice had a vertical position of the spine with the forepaws striking the glass walls while hind paws showed repetitive movement in water. Staying afloat corresponded to movements simply performed to keep the head above water. Immobility was defined by a complete immobilization of the body for at least 1 s. The time not spent performing any one of these activities represented either unspecified uncoordinated movements or swimming activity involving horizontal spine position with legs treading water and producing a clear displacement of body.

**TST.** Each mouse was suspended by adhesive tape placed 2 cm from the tip of the tail, 35 cm above a bench top during a 6 min period (40,89). Behavior was recorded on video during two sessions separated by a 24 h delay. The latency to the first bout of immobility (freezing latency) and the duration of freezing were quantified offline using event-recorder keys in Any-maze. Complete immobility for > 2 s was regarded as freezing. Inverted screen test. Mice were placed individually on a cage wire screen above 35 cm above a table. After slowly inverting the screen upside-down to 180° the ability to maintain a grip was monitored (grip latency) and a maximum score of 120 s given if the animal did not fall. Testing was repeated three times with 10-min ITIs.

**Circadian modulation of locomotor activity.** The apparatus was a computerized soundproof multi-box infrared-sensitive motion detection system (Imetronix, Pessac, France). It was composed of eight individual cages (30 × 15 × 18 cm, with sawdust on the floor), each containing a food dispenser and a water bottle and two parallel horizontal lines of infrared captors mounted along the longer side walls. Cages were illuminated 12 h per day starting from 7 a.m. Each mouse was introduced into an activity cage at 3:30 p.m. and left undisturbed for 24 h. Horizontal and vertical activities were measured by beam breaks in 1 h bins.
Conditioned taste aversion. Procedure was as previously described (90). Three days prior to testing, mice were placed on a water-restriction regime with access to water for 30 min/day, from two identical bottles placed in their home cages. Mice were handled and weighted each day to assess general effects of restriction regime. The bottles were weighed to evaluate fluid consumption. On the conditioning day, mice had free access to a 15%-sucrose solution for 30 min, in two identical bottles. One hour later, mice were injected (i.p.) with either 0.9% saline, or lithium chloride (LiCl: 0.3 M, 2% body weight). Twenty-four hours later, mice were given a two-bottle choice test between the water and sucrose for 30 min. The relative position of the two bottles was counterbalanced between mice. Conditioned taste aversion was expressed as the percent sucrose solution consumed over total fluid intake.

Contextual fear conditioning. Contextual fear was studied in a conditioning box consisting of a grid floor (30 x 30 cm) enabling delivery of electric footshocks as unconditioned stimuli (US), and clear Plexiglas walls (45 cm in height) with no ceiling in order to allow full observation and videorecording. Experiments were performed under moderate illumination (80 Lx) and the box was washed with absolute ethanol before each test. A 5-min habituation session was followed on the next day by a 5-min acquisition session during which a footshock (0.4 mA, 2 s) was delivered at 2.5 min as the US. Retention of conditioned fear was measured 24 h later, by placing the mouse in the same context for 5 min. Freezing was analyzed from videorecording plots (Any-maze, Stoelting), whereas the number of leanings (standing upright on the hind legs with one or two forepaws against the wall) was recorded using event-recorder keys and referred to as vertical activity. To evaluate fear memory while taking into account basal activity during habituation, the freezing behavior during retention was also normalized to the duration of immobility during habituation (23).

Auditory-cued fear conditioning. The conditioning procedure was carried out using the StartFear system (Panlab S.L., Barcelona). The conditioning chamber (25 x 25 x 25 cm) had three black methacrylate walls, a transparent front door, a grid floor connected to a shock scrambler and a speaker mounted on the ceiling used to deliver audible tones as CS. The conditioning chamber rested on a high sensitivity weight transducer system to generate an analogical signal reflecting animal’s movement. The chamber was confined in a ventilated soundproof enclosure (67 x 53 x 55 cm) on an anti-vibration table with a surrounding 60-dB white noise. Interchangeable floors and walls (i.e. plain floor and white walls) were used to analyze retention of cued fear in a novel context. On the first day (acquisition), a 2-min baseline period was recorded before delivery of five CS-US pairs (Tone: 80 dB, 10 kHz, 30 s; footshocks: each at 0.4 mA for 2 s) with variable and pseudo-randomly distributed intervals between pairs of stimuli (60, 120 and 180 s). On the next day (retention), the session started by placing the mouse in a different context for 2 min (baseline) before delivery of four CS (80 dB, 10 kHz, 30 s) separated by intervals of variable durations (60, 90 and 120 s). Animal’s movements were sampled at 50Hz for quantitative analysis (FREEZING software, Panlab S.L., Barcelona). Freezing was analyzed during delivery of the CS (periods of 30 s) to specifically reflect associative learning performance (36).

Determination of ACTH response
Mice were either taken from their home cage directly, for baseline measures, or 1 min after a 15s scruff restraint. Mice were then anesthetized with isoflurane (Forene, Abbott-France) and blood plasma samples collected rapidly (< 1 min) by intracardiac puncture. All samples were collected on the same day between 9 and 10.30 a.m. Blood taking from distinct genotypes and groups (basal, restraint) was counterbalanced. ACTH concentration was determined in duplicate from ethylenediaminetetraacetic acid plasma samples using an automated enzyme chemiluminescent immunoassay (Immulite 2000, IDEXX-Allfort laboratory, France).

Behavioral effects of the extrasynaptic GABA<sub>A</sub>-receptor agonist, THIP
The behavioral effects THIP, also called Gaboxadol, were analyzed following i.p. injections with distinct doses (3, 10 and 30 mg/kg; Sigma-Aldrich, France) or with 0.9% NaCl (saline control groups). To reduce stress reactivity in this experiment, the i.p. injections were performed without scruff restraint, by lifting up the rear body while the mouse was holding a grid with its forepaws. Low and moderate doses of THIP (3 and 10 mg/kg) were used to evaluate effects on motor and emotional parameters, by testing mice 30 min postinjection in the OF for 10 min or in the light-dark choice for 5 min. All tests were performed between 9 and 12 a.m. A higher dose (30 mg/kg) was administered to a third group to evaluate THIP-induced hypnosis, expressed by the LORR latency and duration. The LORR was determined every 4 min by turning the mouse in a supine position in 3 consecutive trials (5 s duration each) on a V-shaped polycarbonate trough. LORR was quantified when the mouse could not right itself in any of the three consecutive trials, while righting reflex recovery was considered when shown in at least one of the three trials.

Statistical Analyses
Data are presented as means ± SEM. Behavioral parameters were analyzed with the Statview 5.0 software (SPSS, USA). One or two-way analysis of variance were used, with genotype as the between-group factor and temporal variables (time, trial, session) as the within-group factor for repeated-measure comparisons. Multivariate analysis of interrelated variables recorded in the behavioral test battery was undertaken as described (91) using a factor analysis based on the principal component method. Extraction performed in each genotype separately was followed by an orthogonal rotation (Varimax) and the weight of each variable (i.e. their correlation coefficient) on extracted factors was calculated.

Supplementary Material
Supplementary Material is available at HMG online.

Acknowledgements
This work was supported by grants from the Association Francaise contre les Myopathies (AFM, France; MED2 2008) and Agence Nationale de la Recherche (ANR, France; ANR-14-CE13-0037-01 DYSther) to C.V. and by a PhD fellowship from Ministère de l’Enseignement Supérieur et de la Recherche (France) to R.C. The authors wish to thank P. Leblanc-Veyrac for intracardiac punctures, S. Granon for providing access to actimetric platform in Orsay for mouse breeding, care and genotyping.

Conflict of Interest statement. None declared.
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