A deficit of brain dystrophin impairs specific amygdala GABAergic transmission and enhances defensive behaviour in mice

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Duchenne muscular dystrophy (DMD) is accompanied by cognitive deficits and psychiatric symptoms. In the brain, dystrophin, the protein responsible for DMD, is localized to a subset of GABAergic synapses, but its role in brain function has not fully been addressed. Here, we report that defensive behaviour, a response to danger or a threat, is enhanced in dystrophin-deficient mdx mice. Mdx mice consistently showed potent defensive freezing responses to a brief restraint that never induced such responses in wild-type mice. Unconditioned and conditioned defensive responses to electrical footshock were also enhanced in mdx mice. No outstanding abnormality was evident in the performances of mdx mice in the elevated plus maze test, suggesting that the anxiety state is not altered in mdx mice. We found that, in mdx mice, dystrophin is expressed in the amygdala, and that, in the basolateral nucleus (BLA), the numbers of GABA_A receptor α2 subunit clusters are reduced. In BLA pyramidal neurons, the frequency of norepinephrine-induced GABAergic inhibitory synaptic currents was reduced markedly in mdx mice. Morpholino oligonucleotide-induced expression of truncated dystrophin in the brains of mdx mice, but not in the muscle, ameliorated the abnormal freezing response to restraint. These results suggest that a deficit of brain dystrophin induces an alteration of amygdala local inhibitory neuronal circuits and enhancement of fear-motivated defensive behaviours in mice.

Keywords: GABAergic synapse; scaffolding proteins; dystrophin; defensive behaviour; amygdala

Abbreviations: BLA = basolateral nucleus of amygdala; DMD = Duchenne muscular dystrophy; IPSC = inhibitory postsynaptic current; IQ = intelligence quotient; NE = norepinephrine; PD = postnatal day; WT = wild-type
Introduction

Dystrophin is responsible for a severe muscle disease, Duchenne muscular dystrophy (DMD) (Hoffman et al., 1987). It is also expressed at relatively high levels in central neurons (Chelly et al., 1988; Lidov et al., 1990), but the role of dystrophin in brain function is not fully understood. Non-progressive intellectual and/or cognitive impairment is observed in about one-third of DMD patients (Emery, 1987; Bresolin et al., 1994; Wicksell et al., 2004). The verbal intelligence quotient (IQ) of DMD patients is significantly lower than that of normal controls, but their performance IQ is normal (Billard et al., 1992). Psychiatric disorders, such as autism (Komoto et al., 1999), and dysthymic and major depressive disorders (Fitzpatrick et al., 1986), are also observed in DMD patients, along with autistic symptoms, self-deprecation, marginalization, minor depression, signs of insecurity, hypochondria, high levels of anxiety and poor adaptation to the environment (Roccella et al., 1999). Abnormalities of GABAergic synapses in mdx mice have also been reported; the numbers of GABA_α receptor α1 and α2 subunit clusters are reduced in the hippocampi and cerebella, compared with control mice (Knuesel et al., 1999). This reduction is not accompanied by change in the number of gephyrin clusters (Knuesel et al., 1999). Dystrophin is dispensable for GABAergic synapse differentiation (Brunig et al., 2002; Levi et al., 2002). Because psychiatric symptoms are reported in DMD patients, as mentioned above, and because decreased GABA_α receptor clustering results in enhanced anxiety and a bias for threat cues (Crestani et al., 1999), information on the emotional aspects of mdx mice is likely to be significant.

In the present study, we performed three behavioural tests in adult mdx mice to examine the impact of dystrophin deficiency upon emotional behaviour, and found that defensive freezing behaviour is enhanced in these mice. We also found that, in mdx mice, dystrophin is expressed in the amygdala, and that, in the basolateral nucleus, specific GABAergic synaptic transmission is reduced. Furthermore, we found that the enhancement of defensive behaviour was ameliorated by expression of brain dystrophin via morpholino oligonucleotide-induced skipping of exon 23, suggesting that the enhanced defensive responses were attributed to the deficit of dystrophin in the brain.

Materials and Methods

Animals

As the dystrophin gene is located on chromosome X in the mouse (Bulfield et al., 1984), male mdx mice and their WT littermates were obtained by mating a dystrophin heterozygote female (+/−) with a male WT mouse (C57BL/10J) and were kept at 2–5 mice/cage (genotype at random). The heterozygote female used was obtained by mating three pairs of male WT (C57BL/10J) mice and homozygote female mdx (C57BL/10Jmdx) mice, both of which were obtained from the Central Institute of Experimental Animals (Kanagawa, Japan). Littermate pairs were used in behavioural tests other than experiments using morpholino oligonucleotides. A tail sample was excised for genotyping after behavioural tests. Thus, experimenters were blind to genotype in behavioural tests. Genotyping was performed using previously described PCR methods (Amalfitano and Chamberlain, 1996). The experiments using morpholino oligonucleotides were performed using age-matched mice, the genotypes of which had been already identified and were known to the experimenters. Throughout experiments, including examination of developmental changes in the sensitivity to restraint, each mouse was used for one test only, and repetitive uses in other tests were avoided. Animal care and ethics approval for the animal experiments are described in Supplementary data.
**Behavioural test-1 ‘restraint’**

Mice were restrained by the experimenter by placing the neck between the thumb and index finger, and putting the tail between the third and little fingers. After 10 s, the mouse was released to a cage (24 × 17 cm, surrounded by a 12-cm high wall) containing wood chips that had been placed inside the observation box (illuminated with 80 lx). A camera on the ceiling of the box transferred video to a personal computer. Locomotion and freezing were calculated from the image files obtained during the 5 min after restraint using Image OF (O’Hara & Co. Ltd., Tokyo, Japan), modified software based on the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Complete immobilization of the mouse, except for respiration, was regarded as a freezing response (Blanchard and Blanchard, 1972).

**Behavioural test-2 ‘electrical footshock’**

Electrical footshock was applied to mice in the same apparatus that we previously used for contextual fear conditioning (Zushida et al., 2007). After a 3-min habituation to this apparatus (pre-footshock freezing test), an electrical footshock was delivered from the grid (0.8 mA; 2-s duration; scrambled, twice with a 1-min interval), followed by a 3-min post-footshock freezing test. Twenty-four hours later, the mice were introduced to the same apparatus without shock to test contextual learning for 3 min.

**Behavioural test-3 ‘elevated plus maze’**

The elevated plus maze test was performed as described previously (Yamada et al., 2002), and the detail is described in Supplementary data.

**Immunostaining**

Halothane-anaesthetized male mice (70- to 100-days old) were perfused transcardially with 0.1 M phosphate-buffered saline (pH 7.4, PBS) for dystrophin staining. The brains were immediately removed and frozen. Coronal brain sections (20 μm thick) were prepared using a microtome (Leica, Wetzlar, Germany). Sections were post-fixed with 4% paraformaldehyde (PFA, 20 min), washed with PBS (5 min) and treated with 0.1% Triton X-100 in PBS (10 min). After incubation with 1% H2O2 in PBS (60 min), sections were incubated with PBS solution containing 1% blocking reagent (TSA kit, Molecular Probes, Carlsbad, CA, USA) for 60 min and with the same solution containing mouse to mouse blocking reagent (ScyTek Laboratories, Logan, UT, USA) for an additional 60 min. Sections were then incubated with monoclonal anti-dystrophin antibody (MAB1692; 1:20 dilution, Chemicon, Temecula, CA, USA) in 1% blocking reagent, washed with PBS and incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG, Molecular Probes). Sections were stained using a tyramide signal amplification kit (Molecular Probes) and examined using a confocal laser-scanning microscope (FV-1000, Olympus, Tokyo, Japan).

Immunostaining for GABA<sub>A</sub> receptor α2 subunit and gephyrin was performed using the method previously reported by Fritschy et al. (1998) with minor modifications, and the detail is described in Supplementary data.

**Electrophysiology**

Slice patch clamp recordings were performed as reported previously (Zushida et al., 2007; Amano et al., 2008), and the detail is described in Supplementary data.

**Rescue experiments**

The methods for microinjection of morpholino oligonucleotides, analysis of the expression of truncated dystrophin by means of Western blotting, and confirmation of exon-skipping by RT–PCR are described in Supplementary data.

**Statistical analysis**

Statistical differences between two data groups were assessed using the two-tailed Student’s t-test.

**Results**

**Altered unconditioned and conditioned defensive behaviours in mdx mice**

We first applied restraint to examine the emotional responses of mdx mice. Restraint is widely used as an emotionally aversive stimulus (Miller and McEwen, 2006). After restraint, mice were released to a cage containing wood chips. These tests were performed with the experimenters blind to genotype (see Materials and methods section). Figure 1 shows the locomotion (A) and freezing (B) behaviours of mice over a 5-min period after very brief (10 s) restraint. We found that wild-type (WT) and mdx mice (120- to 130-days old) behaved quietly different in response to restraint. All mdx mice displayed very little locomotor activity (Fig. 1A) and a strong freezing response (Fig. 1B, see also Supplementary Video 1). The frozen posture intermittently persisted longer than 30 min in mdx mice, after which time the mice commenced normal movement. In almost all cases, freezing was conducted at the edge or corner of the cage; it appeared that mdx mice selected a specific location at which to show freezing responses (see Supplementary Video 1). By contrast, all WT mice began moving immediately after restraint (Fig. 1A and B). The mdx mice did not freeze when simply transferred without restraint to the measuring field, and the locomotion time course was essentially identical to that in WT mice (Fig. 1C), suggesting that restraint induces freezing in mdx mice. In these simple transfer experiments, total locomotion during a 5-min test session was not significantly different between WT and mdx mice (Fig. 1D).

Next, we tested the sensitivity of mdx mice to electrical footshock. The freezing response immediately after footshock is utilized as an index of fear-motivated unconditioned defensive response (Pentkowski et al., 2006). WT and mdx mice (100- to 130-days old) did not show freezing responses when they were introduced into a footshock apparatus (Fig. 1E-Pre), but immediately after footshock, they showed freezing responses (Fig. 1E-Post). The amount of time mdx mice spent frozen during a 3-min session was significantly longer than the amount of time WT mice spent in this state (P = 0.027, n = 10 and 9 for WT and mdx mice, respectively,
two-tailed Student’s t-test). These conditioned mice were returned to their home cage and, after 24 h, re-exposed to the same apparatus for 3 min to examine their conditioned freezing responses (Fig. 1E-24 h). The freezing rate in this test was also significantly higher among mdx mice than among WT mice ($P = 0.004$). These results suggest that fear-motivated unconditioned and conditioned defensive responses were enhanced in mdx mice.

Performance in the elevated plus maze is normal

In order to examine anxiety-motivated behaviour in adult mdx mice, we used an elevated plus-maze test. The total amount of time the mice spent in the arms, regardless of whether they were open or closed (Fig. 2A), and the total number of entries into arms regardless of their being open or closed (Fig. 2B), were not significantly different between WT and mdx mice ($P = 0.303$ and 0.087 for time and entries, respectively), suggesting that mdx mice have normal exploratory activity in this test. The percentage of time the mouse spent in the open arm and the percentage of arm visits made to the open arm were not significantly different between WT and mdx mice (Fig. 2C, $P = 0.339$ and 0.561 for time and entries, respectively). We also counted the number of times that the mouse adopted a stretched attend posture in the central platform as a risk assessment behaviour (Yamada et al., 2002), because risk assessment behaviour is reported to be a defensive behaviour (Blanchard et al., 2003). The frequency of stretched attend postures was not significantly different between WT and mdx mice (Fig. 2D, $P = 0.642$). These results suggest that WT and mdx mice behave identically in the elevated plus maze.

Expression of dystrophin and a reduction in the number of GABA<sub>A</sub> receptor clusters in the amygdala

Previous studies have suggested that the organization of an unconditioned freezing response is performed in a brain aversion
system composed of the medial hypothalamus, the dorsal periaqueductal grey (dPAG) and superior and inferior colliculi (Brandao et al., 1999). The hippocampus and amygdala modulate this brain aversion system to alter the extent of the unconditioned freezing response (Pentkowski et al., 2006; Ruiz Martinez et al., 2006). In addition, projections from the CA1 field and subiculum of the ventral hippocampus to the basolateral nucleus of amygdala (BLA) are involved in contextual fear (Maren and Fanselow, 1995). As mentioned above, dystrophin expression is not detectable in the hypothalamus and most of the brainstem, including the PAG and the superior and inferior colliculi. This information prompted us to assume that the hippocampus and/or amygdala may be affected in mdx mice, and that this leads to the emergence of abnormal defensive behaviours. In particular, considering the importance of efferent projections of the amygdala to the PAG in the freezing response induced by fear conditioning (LeDoux et al., 1988), the participation of the amygdala was suspected. The expression of dystrophin in the CA1 field of the hippocampus has already been reported, as mentioned above, but there has been no description of dystrophin expression in the amygdala. In order to elucidate whether dystrophin is expressed in the amygdala, we performed immunohistochemical staining of the amygdala using an anti-dystrophin antibody. There was fairly abundant expression of dystrophin (green) in the BLA and the lateral nucleus of the amygdala (LA) in adult WT mice (Fig. 3A). Dystrophin staining in the BLA was punctate along the somatic membrane and processes (Fig. 3B). This pattern is similar to that reported previously for the cerebral cortex (Lidov et al., 1990). The BLA and LA of adult mdx mice were devoid of immunocytochemical signals (Fig. 3C), suggesting that the signals in WT mice originated from full-length 427-kDa dystrophin, because mdx mice lack only full-length dystrophin, but express its shorter isoforms, Dp140 and Dp71 (Im et al., 1996). The distribution of dystrophin-specific immunocytochemical signals other than in the amygdala was in good agreement with that described in previous reports (Lidov, 1996). In particular, dystrophin expression in the hippocampus was hallmark, being positive in the CA1–CA3 regions and negative in the dentate gyrus (Fig. 3D).

As mentioned in the Introduction section, a previous study showed that the number of GABA<sub>A</sub> receptor α2 subunit clusters is reduced in the hippocampi and cerebella without being accompanied by change in the number of gephyrin clusters (Knuesel et al., 1999). We investigated whether a reduction in the number of GABA<sub>A</sub> receptor clusters was evident in the BLA of mdx mice. For this purpose, coronal brain slices from adult mice were stained with antibodies specific for the GABA<sub>A</sub> receptor α2 subunit (green in Fig. 3E and F). Staining with an antibody specific for gephyrin was also performed (red in Fig. 3G and H) for comparison. The GABA<sub>A</sub> receptor α2 subunit is abundantly expressed in the amygdala (Persohn et al., 1992). The α1 subunit is expressed in the amygdala (Persohn et al., 1992). The α1 subunit is expressed in the amygdala (Persohn et al., 1992).
is also expressed in the amygdala (Persohn et al., 1992), but the cells expressing this subunit are reported to be parvalbumin-containing non-pyramidal neurons (MacDonald and Mascagni, 2004), not pyramidal neurons. Because we found a reduction in GABAergic synaptic transmission in BLA pyramidal neurons (Fig. 4), an analysis of α2 subunit clusters was performed in the present study. As shown in Fig. 3E and F, the immunopositive dots were apparently fewer in the BLA of mdx mice compared with WT mice. In contrast, no outstanding differences were evident in the staining of gephyrin (Fig. 3G and H). In the case of the GABA<sub>A</sub>...
receptor α2 subunit, the number of immunopositive dots counted in a 6400 μm² area in the centre of the BLA was significantly (P = 0.018) fewer in mdx mice than in WT mice (Fig. 3I, data from five mice of each genotype). In the case of gephyrin, there was no significant difference between mdx and WT mice in the number of immunopositive dots similarly counted (Fig. 3I, data from three mice in each mouse model). These results suggest that the number of clusters of the α2 subunit were reduced in the BLA of mdx mice without being accompanied by a decrease in the number of clusters of gephyrin.

**Electrophysiological abnormalities in BLA pyramidal neurons**

In order to examine the effects of dystrophin deficiency on neural activity in the BLA, whole-cell patch clamp recording was performed on pyramidal neurons in acute brain slices. Pyramidal neurons were identified by spike-frequency adaptation in response to depolarizing pulses (Sah et al., 2003; Fig. 4A). We elicited action potential-induced GABAergic inhibitory postsynaptic currents (IPSCs) in pyramidal neurons (voltage-clamped at −75 mV) by application of norepinephrine (NE) in the presence of CNQX and MK-801 to block AMPA and NMDA receptors. It has been reported that NE consistently facilitates GABAergic IPSCs in rat BLA pyramidal neurons, mainly via activation of adrenergic receptors on the somatodendritic regions of GABAergic interneurons (Braga et al., 2004). In our experiments also, application of NE reversibly facilitated the generation of transient inward discharges in all WT BLA neurons tested (Fig. 4B, n = 17 from 10 WT mice, mean resting membrane potential = −65.7 ± 0.8 mV, also see Fig. 4E and Table 1). Both the baseline and NE-induced IPSCs were completely blocked by 100 μM picrotoxin, suggesting that these discharges were mediated by GABAA receptors (data not shown). In the presence of 1 μM tetrodotoxin (TTX), the baseline IPSCs were abolished in all neurons tested (five WT and five mdx neurons), and subsequent application of NE did not induce the generation of IPSCs (Fig. 4C). This suggests that the majority of IPSCs were action potential dependent. In contrast to WT neurons, some neurons from mdx mice were insensitive to NE (Fig. 4D). Scatter plots of baseline frequency versus the frequency in the presence of NE indicated that there are two types of neurons in mdx mice (Fig. 4E). NE-insensitive neurons comprised ~61% (11 out of 18 neurons, data from 11 mdx mice, mean resting membrane potential = −65.2 ± 0.7 mV, n = 18) of the neurons tested and NE-sensitive neurons comprised 39% of the population (7 out of 18 neurons). Table 1 summarizes the mean (±SEM) frequencies, amplitudes and decay time constants of IPSCs recorded from 17 WT, 11 NE-insensitive mdx and 7 NE-sensitive BLA pyramidal neurons. The increase in the frequency of IPSCs induced by NE was significant in WT neurons (1.5 ± 0.5 versus 8.0 ± 1.3) and NE-sensitive mdx neurons (5.2 ± 1.0 versus 17.2 ± 2.2), but not in NE-insensitive mdx neurons (1.8 ± 0.6 versus 1.7 ± 0.6). NE had no significant effect on the amplitudes or decay time constants of the IPSCs in WT neurons or either type of mdx neuron (Table 1). The absence of an effect upon amplitude was also suggested from the cumulative amplitude histogram (Fig. 4F). In the NE-sensitive mdx neurons, the baseline (without NE) frequency of IPSCs was about three times higher than that in WT neurons (6.2 ± 1.0 versus 1.5 ± 0.5) and the baseline decay time constant was accelerated compared with WT neurons (6.2 ± 0.6 versus 10.6 ± 0.8). This suggests that NE-sensitive mdx neurons are also influenced by the lack of dystrophin, although they are sensitive to NE as well as WT neurons. In term of sensitivity to NE, these results suggest that the induction of IPSCs by NE is reduced in the BLA of mdx mice.

There are two possible explanations for the reduction of NE-sensitive pyramidal neurons in mdx mice. One is a decrease in the number of normal functional GABAergic synapses between NE-responsive interneurons and pyramidal neurons, and another is the impairment of the mechanism by which NE depolarizes interneurons beyond the threshold of action potentials. To clarify this, we recorded from non-pyramidal BLA neurons in brain slices. There are several types of non-pyramidal neuron in the BLA, with respect to their pattern of action potential generation in response to a current pulse (Rainnie et al., 2006). We surveyed several cells in WT mice and found that NE induced depolarization with action potentials in so-called regular spiking neurons (Fig. 4G and I). This result is consistent with a report that the major neurons in rat frontal cortex that respond to NE with action potential firing are regular-spiking non-pyramidal neurons (Kawaguchi and Shindou, 1998). Accordingly, we selected these neurons by applying current pulses, and their sensitivity to NE was compared between WT and mdx mice. The regular spiking neurons showed a high input resistance (Rainnie et al., 2006) in both WT (337.9 ± 58.4 MΩ, n = 10) and mdx (332.2 ± 46.1 MΩ, n = 10) mice. In five out of 10 regular-spiking WT neurons (resting membrane potential = −62.2 ± 1.0 mV, n = 10), the NE-induced depolarization was accompanied by action potential firing (Fig. 4H). Similarly, NE induced depolarization with action potentials in five out of 10 mdx interneurons tested (Fig. 4I), resting membrane potential = −59.3 ± 0.9 mV, n = 10). These results suggest that the proportion of regular-spiking non-pyramidal BLA neurons that respond to NE with action potential firing is identical in WT and mdx mice.

Overall, these electrophysiological results suggest that the number of normal functional GABAergic synapses between NE-responsive interneurons and pyramidal neurons is decreased in the BLA of mdx mice.

### Table 1 Frequencies, amplitudes and decay time constants of IPSCs recorded from BLA pyramidal neurons

<table>
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<tr>
<th></th>
<th>n</th>
<th>Frequency (Hz)</th>
<th>Amplitude (PA)</th>
<th>Decay (ms)</th>
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<tr>
<td><strong>WT</strong></td>
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<tr>
<td>−NE</td>
<td>17</td>
<td>1.5 ± 0.5</td>
<td>19.8 ± 1.8</td>
<td>10.6 ± 0.8</td>
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<tr>
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<td>21.9 ± 2.2</td>
<td>10.9 ± 0.6</td>
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<tr>
<td><strong>Mdx-NE-insensitive</strong></td>
<td>11</td>
<td>1.8 ± 0.6</td>
<td>20.9 ± 2.8</td>
<td>8.1 ± 1.6</td>
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<tr>
<td>−NE</td>
<td></td>
<td>1.7 ± 0.6</td>
<td>19.7 ± 2.5</td>
<td>10.9 ± 3.0</td>
</tr>
<tr>
<td>+NE</td>
<td></td>
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<td>19.5 ± 1.4</td>
<td>6.2 ± 0.6</td>
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<tr>
<td><strong>Mdx-NE-sensitive</strong></td>
<td>7</td>
<td>17.2 ± 2.2</td>
<td>22.0 ± 2.9</td>
<td>7.1 ± 0.9</td>
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*P = 0.008, **P < 0.001, versus corresponding values in WT mice.

*P < 0.001, **P = 0.001, versus corresponding values in −NE.
Expression of truncated dystrophin by morpholino oligonucleotide and amelioration of the behavioural phenotype

Our behavioural experiments suggested that defensive behaviour elicited by restraint or footshock is enhanced in mdx mice. However, because mdx mice lack dystrophin not only in the brain, but also in muscle, there is no direct evidence that these behavioural abnormalities could be attributed to the lack of dystrophin in the brain. In order to elucidate this issue, we took advantage of a morpholino oligonucleotide to induce recovery of dystrophin expression in its truncated form. This morpholino nucleotide induces skipping of exon 23, which includes an aberrant stop codon in the mdx allele (Alter et al., 2006), producing a 71 amino acid deletion in the mid-rod domain of dystrophin. The rod domain is thought to be a long spacer linking an actin-binding domain near the N-terminus and a dystroglycan-binding domain near the C-terminus of dystrophin, and dystrophin with a deletion within the rod domain is thought to retain the physiological function of full-length dystrophin. Indeed, muscular injection of this morpholino oligonucleotide efficiently ameliorates the muscular pathology of mdx mice (Alter et al., 2006). In our study, the morpholino oligonucleotide was administered intracerebroventricularly using an osmotic infusion pump that persistently released the drug solution for 1 week. First, we determined the timing of morpholino administration by examining the developmental time course of behavioural abnormalities in mdx mice. The restraint-induced freezing response progressed with postnatal development in mdx mice (Fig. 5). The freezing response was weak and not significant on postnatal day (PD) 20 (P = 0.100 versus WT mice), but was evident on PD36, and gradually increased thereafter. Dystrophin is expressed in the rat cerebral cortex beginning on PD10, and its expression gradually increases thereafter (Kim et al., 1992). Therefore, postnatal development of the freezing response coincides with the time course of brain dystrophin expression. The infusion cannula was implanted in mdx mice on PD30, the age of onset for the behavioural abnormality.

Intracerebroventricular administration of antisense (indicated as ‘A’) morpholino oligonucleotide from PD30 induced the appearance of an immunoreactive signal (detected by chemiluminescence) corresponding to dystrophin in Western blots of the postsynaptic density fraction prepared from brain tissue (including the cerebral cortex and amygdala) (Fig. 6A). The immunoreactive signal was evident on PD65 (5-weeks postoperatively) and PD80 (7-weeks postoperatively), but only trace immunoreactivity was detected on PD55 (3-weeks postoperatively) and PD110 (11-weeks postoperatively). On PD65 and PD80, the intensity of the band corresponding to dystrophin was estimated to be 27.6 ± 10.9% (n = 3 mice) of the level in WT mice (rightmost lane in Fig. 6A). This estimation, samples from WT and mdx mice were analysed on the same membrane, and the condition that did not induce saturation of the chemiluminescence signal in WT mice was chosen by stepwisely changing the exposure time for signal detection (see Supplementary Materials and methods).

No signal was detected in samples from mice similarly administered sense (indicated as ‘S’) morpholino (n = 5 mice). Intracerebroventricular administration of sense or antisense morpholino induced no dystrophin expression in the gastrocnemius muscle of mdx mice (Fig. 6B). These results suggest that intracerebroventricular administration of antisense morpholino oligonucleotide selectively rescues dystrophin expression in the brain.

Total RNA was isolated from mouse brains administered morpholino (mice were ~65-days old), and nested PCR was carried out using primers that amplify the sequence encoding exons 20–26. Several bands smaller in size than native dystrophin mRNA (indicated as ‘22–23–24’) were evident only in the antisense morpholino-treated mdx mouse amygdala, cortex and hippocampus. These bands were not present in the sense morpholino-treated mdx mouse cortex, amygdala or in WT mouse cortex (Fig. 6C). As reported for morpholino-treated muscle (Alter et al., 2006), the largest band was ~200 bp shorter than the native band, and probably corresponds to a fragment encoding the sequence lacking the 213-bp region encoding exon 23 (‘22–24’). The band shorter than the 22–24 band is likely to be a fragment lacking the sequence encoding exons 22 and 23 (‘21–24’) (Alter et al., 2006). These data suggest that the antisense morpholino oligonucleotide induces exon skipping in dystrophin mRNA, thereby recovering expression of truncated forms of dystrophin.

Immunohistochemical analysis of the BLA of WT and morpholino-treated mdx mice is shown in Fig. 6D–G. As mentioned above, dystrophin immunoreactivity was abundantly detected in the BLA of WT mice (Fig. 6D). In mdx mice treated with antisense morpholino, less dense punctate staining compared with WT mice was observed, as shown in Fig. 6E. Figure 6F shows an image from different slices in the same mdx mouse treated with antisense morpholino, focused on two cells in which somatic staining was recovered to the level in WT mice (although such cells were sparse compared with WT mice). Punctate staining was
not detected in the BLA of mdx mice treated with sense morpholino (Fig. 6G, mdx + S).

Next, mdx mice were administered morpholino oligonucleotide to examine whether expression of a truncated form of dystrophin reduces the restraint-induced freezing behaviour observed in mdx mice. The freezing response was reduced on PD65 and PD80 in conjuction with expression of truncated dystrophin (Fig. 6H, see also Supplementary Videos 2 and 3). The freezing response was not reduced on PD55, when expression of truncated dystrophin was only trace. These results suggest that the behavioural abnormalities in mdx mice, at least restraint-induced freezing, can be partially rescued by antisense morpholino oligonucleotide-induced expression of truncated dystrophin in the brain.

Discussion

The results presented here suggest that a deficit of brain dystrophin has an impact on unconditioned and conditioned defensive behaviour in mice. Previous studies suggest that particular memory functions are altered in mdx mice. Our present results provide new information that these mice have a severe alteration in particular aspects of emotion in addition to memory functions.

Among emotional defensive behaviours, fear-motivated unconditioned and conditioned defensive responses were altered in mdx mice, while anxiety-motivated responses, as assessed by elevated plus maze, were not changed. The amygdala and hippocampus modulate the brain aversion system to alter the extent of the
unconditioned freezing response (Pentkowski et al., 2006; Ruiz-Martinez et al., 2006), and the amygdala is important for association learning, which is necessary for conditioned fear memory (LeDoux, 2000). In terms of abnormal GABAergic synaptic activity in the amygdala, the alteration of fear-motivated unconditioned and conditioned defensive responses in mdx mice is consistent with these previous findings, because local GABAergic inhibition is one of the crucial factors regulating amygdala neuronal circuit activity (Sah et al., 2003). Indeed, attenuation of GABAergic inhibition in the BLA is known to be correlated with enhancement of conditioned fear memory (Rodriguez Manzanares et al., 2005). On the other hand, the absence of an alteration in anxiety behaviour is consistent with a previous report showing that the performance of mdx mice in the light/dark box, another test assessing the anxiety state of mice, is similar to those of control mice (Vaillend et al., 1995).

Abnormal emotional behaviour has also been reported of mice in which collybistin, another scaffolding protein in GABAergic postsynapses, is deleted (Papadopoulos et al., 2007). Collybistin has been implicated in the plasma membrane targeting of gephyrin at glycineergic and GABAergic synapses (Kins et al., 2000). These mice show a decrease in miniature IPSC frequency in hippocampal CA1 pyramidal neurons, and impairment of long-term potentiation in CA3-CA1 synapses in the hippocampus, being accompanied by a reduction in the number of gephyrin- and GABA_A receptor subunit-immunoreactive puncta in the hippocampus and amygdala. This report and our present results suggest that GABAergic postsynaptic scaffolds are important factors that determine emotional features in mice. In this context, the absence of an alteration in anxiety, as assessed by the elevated plus maze in the mdx mice reported here, is in contrast to the severe alterations in performance in the elevated plus maze seen in collybistin-deficient mice (Papadopoulos et al., 2007). Differences in the behavioural phenotypes between collybistin-deficient mice and mdx mice imply that each GABAergic scaffold protein plays a role in specific emotional behaviour, probably according to its impact upon synaptic activity and the subtype of GABAergic synapses in which it is distributed.

Previous studies suggest that a deficit of dystrophin impairs a subset of GABAergic synapses in the brain. In addition to the above-mentioned marked reduction in the number of GABA_A receptor α1 and α2 subunit clusters in mdx mice (Knuessel et al., 1999), inhibitory inputs to cerebellar Purkinje cells and long-term depression of the synaptic responses in these cells are reduced in mdx mice (Anderson et al., 2003a, b). Destabilization of GABA_A receptors at postsynaptic sites by a deficit of dystrophin is considered to be the mechanism underlying the reduction in the number of receptor clusters (Knuessel et al., 1999). Consistently, our results obtained in the BLA also suggested that the number of clusters of α2 subunit and GABAergic synaptic transmission were reduced in mdx mice.

A plausible interpretation for the marked reduction in the frequency of NE-induced IPSCs in mdx mice is as follows. A reduction in the number of α2 subunit-containing GABA_A receptors would result in attenuation of synaptic responses mediated by this receptor. In the rat BLA, somatostatin-containing non-pyramidal neurons, which respond to NE with action potential firing in rat frontal cortex (Kawaguchi and Shindou, 1998), make synaptic contacts with the distal dendrites of pyramidal cells (Muller et al., 2007). In our patch clamp recordings from somata, the synaptic currents generated in distal dendrites are attenuated after being conducted along the long dendrite. Therefore, in the case that the amplitude of current changes in distal dendritic synapses is attenuated because of a reduction in the number of α2 subunit-containing GABA_A receptors in mdx mice, the current changes detected in somata cannot be distinguished from baseline noise (about ±5 pA in our experiments), which results in a reduction in the frequency of NE-induced IPSCs recorded from somata. On the other hand, the frequency and amplitude of baseline (without NE) IPSCs were not altered in NE-insensitive mdx neurons compared with WT neurons (Table 1). One plausible explanation for this absence of an alteration is that the baseline IPSCs and NE-induced IPSCs are generated in different synapses; for example, baseline IPSCs are generated in synapses between interneurons other than NE-sensitive interneurons and these synapses are not affected by the lack of dystrophin. Consistently, spontaneous bursts of action potentials in interneurons, which generate spontaneous (baseline) IPSCs in pyramidal neurons, are never observed in BLA regular spiking interneurons (Rainnie et al., 2006), a subset of which was shown to be NE sensitive in the present study.

Previous reports suggest that NE is released in the amygdala in response to aversive stimuli such as restraint (Tanaka et al., 1983) and footshock (Galvez et al., 1996). In mdx mice, therefore, there is a possibility that the attenuation of BLA NE-induced GABAergic synaptic transmission perturbs the control of excitability of BLA pyramidal neurons in response to aversive stimuli (Braga et al., 2004), which inappropriately modulates the brain aversion system in unconditioned defensive behaviour and neuronal circuits for conditioned fear. As mentioned above, somatostatin-containing non-pyramidal neurons make synaptic contacts with the distal dendrites of pyramidal cells (Muller et al., 2007); these synapses are often in close proximity to asymmetrical (excitatory) synapses (Muller et al., 2007). Because synchronous activation of these excitatory and inhibitory synapses attenuates local depolarization elicited by the excitatory synapses, the spatial relationship of somatostatin-containing inhibitory synapses and excitatory synapses may be an important factor in the perturbation of excitability of BLA pyramidal neurons induced by a deficit of dystrophin.

Although the results of the present study show alterations in BLA GABAergic synapses, impairment in the BLA is not necessarily the only mechanism underlying the alteration of defensive behaviour in mdx mice, because dystrophin is also expressed in the CA1 field of the hippocampus. The CA1 field and BLA are connected with each other and their interaction is known to be important for contextual fear memory (Maren and Fanselow, 1995) and hippocampal synaptic plasticity (Nakao et al., 2004). Therefore, the impact of a lack of dystrophin in the CA1 region and in an interaction between the CA1 field and the BLA should be investigated for further understanding of the neuronal mechanism underlying abnormal defensive behaviour in mdx mice. Regarding the impact on the CA1, Graciotti et al. recently suggested that the frequencies of miniature spontaneous IPSCs, not evoked IPSCs, in hippocampal CA1 pyramidal neurons in mdx mice...
were increased and they propose that the increment is due to presynaptic effects by a dystrophin-deficit (Graciotti et al., 2008).

Another piece of information provided in the present study is that a morpholino oligonucleotide that induces expression of truncated dystrophin is able to ameliorate dystrophin deficit-induced alterations in brain function. By comparing DMD patients with spinal muscular atrophy patients, Billard et al. (1992) suggested that the mental deficiency in DMD is not secondary to the musculoskeletal handicap. From the point of view that dystrophin deficit in the brain has a direct influence on brain function, the results of our morpholino experiments are in line with this previous report. In the present study, restoration of truncated dystrophin expression to about 30% of the level expressed by WT mice resulted in partial amelioration of the behavioural phenotype in mdx mouse brain. It is reported that dystrophin levels as low as 30% of normal are sufficient to avoid muscular dystrophy in humans (Neri et al., 2007). Therefore, it is not unreasonable that 30% recovery of dystrophin expression ameliorates the behavioural phenotype. However, as the amelioration of abnormal freezing response was only partial with the expression level we achieved, further improvement in the efficacy of dystrophin expression is necessary for application of morpholino oligonucleotides to therapy for the central nervous system symptoms in human DMD.

**Supplementary data**

Supplementary data are available at *Brain* online.

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