FOR WANT OF A NAIL. RAMIFICATIONS OF A SINGLE GENE DELETION, DYSTROPHIN, IN THE BRAIN OF THE MOUSE

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TABLE OF CONTENTS

1. ABSTRACT

Lack of expression of a single gene, dystrophin, causes the severe, progressive muscle wasting and mental deficits characteristic of Duchenne muscular dystrophy. In this work, we investigated the impact of dystrophin deletion on expression of other genes in the brain cortex, hippocampus and cerebellum using the murine homologue, the *mdx* mouse, and RT-PCR.

Expression of the brain glucose transporters GLUT1 and GLUT2 was found to be decreased, as were some subunits of the GABA$_A$ and nicotinic acetylcholine receptors. Genes involved in bioenergetic homeostasis, such as the mitochondrial creatine kinase and the gamma subunit of ATP synthase were also found to be abnormally expressed, while expression of the structural proteins beta-dystrobrevin and rapsyn was also significantly affected.

We relate these findings to known functional deficits and discuss the possible mechanisms behind the altered gene expression.

2. INTRODUCTION

The *mdx* mouse is a murine homologue of the X-linked human disorder, Duchenne muscular dystrophy (DMD), the severe manifestation of Xp21 dystrophy. Characterised by complete absence of the 427 kDa protein dystrophin, the disease causes severe and progressive muscle wasting, inevitably culminating in death at an average age of 21 y, most typically from cardiorespiratory failure. In addition to the well described muscle wasting, DMD is also characterised by a "non-progressive" mental deficit, usually defined as a reduction in mental function of
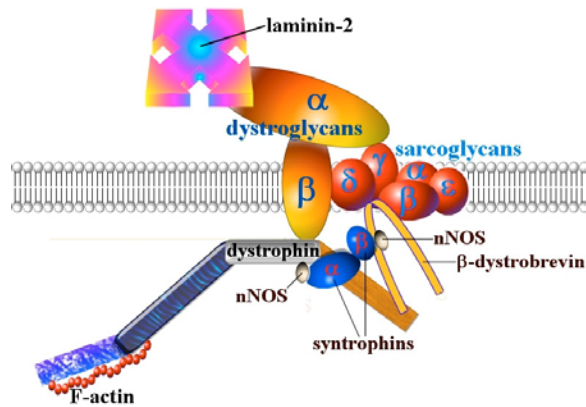
Figure 1. Scheme showing the relationship of dystrophin to other proteins in the dystrophin-associated protein complex. nNOS, neuronal nitric oxide synthase.

one standard deviation or 15 IQ points (reviewed in 1).

The gene for dystrophin is the largest known human gene (~2.5 Mb); it encodes a 14 kb mRNA with 79 exons. The large intronic regions present in the gene may be at least partly responsible for the high rate of mutation in this gene, and thus the relatively high frequency (1 in ~3500 live male births) of the disease (2).

The gene contains a number of discrete promoters. Dp427, the full-length transcript, is transcribed from at least three different promoters depending on the site of expression. The 'M' promoter is active in skeletal, smooth and cardiac muscle; the 'C' promoter in pyramidal cells of the neocortex and hippocampus, and the 'P' promoter in cerebellar Purkinje cells (3-6). These isoforms differ in only the first ~15 amino acids and have an identical domain structure. A number of smaller dystrophin isoforms are also transcribed from intragenic promoters. The most common of these is Dp71 (7), a 71 kDa protein transcribed from a promoter between exons 62 and 63 of the dystrophin gene (8). Other isoforms that have been isolated include Dp260, expressed in the retina (9), Dp140, expressed in the brain and kidney (10), and Dp116, expressed in Schwann cells of peripheral nerve (11). These different isoforms and truncated forms could have different functions in different tissues, all of which may be impaired in DMD (12).

In muscle, dystrophin is known to bind to a large membrane-bound complex of glycoproteins sometimes referred to as the dystrophin-associated protein complex (DPC; figure 1; (13)). This complex links the actin cytoskeleton with the extracellular matrix, and is required for muscle stability: defects in many of the proteins in the DPC result in related muscular dystrophies (14-16). The DPC can be divided into sub-complexes including the sarcoglycan complex (alpha, beta, gamma, delta and epsilon), which is defective in limb-girdle muscular dystrophies, the dystroglycan complex (alpha and beta), which links the DPC to the extracellular matrix via laminin-2, and the cytoplasmic complex, which involves various proteins that bind directly to dystrophin.

The DPC is thought to reinforce the plasma membrane, protecting it from mechanical stress under contraction (17), however, evidence is emerging that the complex may also be involved in signal transduction and/or synaptic formation. Dystrophin immunoreactivity has been observed in close proximity to acetylcholine receptors (AChRs) at neuromuscular junctions (18), and agrin, a nerve-derived protein involved in acetylcholine receptor clustering, has been shown to bind alpha-dystroglycan, a member of the DPC (19). Furthermore, beta-dystroglycan binds to rapsyn, a protein intimately linked to AChR clustering at the neuromuscular junction. The DPC is also associated with a number of signal transduction cascades (20), and thus has crucial non-structural roles in the cell which may also be perturbed in DMD.

The C57Bl/10-ScSn-*mdx* (*mdx*) mouse is a murine model of DMD; it results from point mutation in exon 23 of the murine DMD gene that introduces a premature stop codon (21), causing an absence of the 427 kDa forms (M-, C-, and P-dystrophin), but not of the short forms of dystrophin (22). A variant of the *mdx* mouse, the C57Bl/6Ros-*mdx^3Cv* mouse (*mdx^3Cv*) is deficient not only in full-length (427 kDa) dystrophin isoforms, but also in the C-terminal isoforms, including Dp71, due to a mutation that creates an alternate splice-acceptor site and results in aberrant splicing of most dystrophin isoform-encoding mRNAs (23).

Interestingly, limb muscles of *mdx* mice do not suffer from the chronic, severe muscle wasting present in DMD but instead undergo rapid cycles of necrosis and regeneration; capacity for regeneration reaches a maximum level at 4-8 weeks of age (24). Limb muscles show only mild fibrosis later in life (25), and there is no obvious motor disability detectable before 12 months of age (22), although the diaphragm has a more severe pathology that is similar to that in DMD (26). It has been suggested that this lack of severe muscle wasting in *mdx* mice may be due to increased expression of utrophin, an autosomal homologue of dystrophin (27, 28), and indeed *mdx* mice with a transgene that increases expression of utrophin do not show the adverse effects of dystrophin deficiency (29, 30).

Like DMD patients, the *mdx* mouse also displays mental defects. *Mdx* mice aged 4-6 months exhibit a deficit in passive avoidance learning (31), and display impaired retention of information at long delays (32), suggesting that dystrophin may be involved in long-term memory processes. Task acquisition and procedural memory are unaffected however (32), and *mdx* and *mdx^3Cv* mice have normal levels of hippocampal long-term potentiation and spatial discrimination (33, 34).

In *mdx* mouse muscle, absence of Dp427 has been shown to lead to altered expression of up to 90 other proteins (35), including those involved in metabolism and energy production, growth and differentiation, calcium homeostasis and protein degradation, cellular organization and biogenesis, extracellular matrix, inflammation and macrophage activation.

Table 1. List of primers used for real-time RT-PCR

Gene	Primers
GLUT1	Fwd: CAGCCCTGCTACAGTGTATCCT Rev: AGCTTCTTCAGCACACTCTTGG
GLUT3	Fwd: CCTTGGCTTAACCATCATCATTCCA Rev: AAGCGCTGCAGGATCTCTGTAG
GABA$_A$ receptor alpha 1 subunit	Fwd: CCCGTTCAGTGGTTGTAGCA Rev: CTCTGTTGAGCCAGAAGGAGAC
GABA$_A$ receptor alpha 2 subunit	Fwd: ACAGTCCAAGCCGAATGTCC Rev: AACGGAGTCAGAAGCATTGTAAGT
GABA$_A$ receptor gamma 2 subunit	Fwd: TCGCTCTACCCAGGCTTCAC Rev: TACCCTTCCAGCAGGTTGTT
5-HT$_{3A}$ receptor	Fwd: CTTGCTGCCCAGTATCTTCC Rev: GGTCTCAGCGAGGCTTATCAC
Mitochondrial creatine kinase 1	Fwd: CCTTCTCCCGTCTGCTGTCT Rev: CATATCCATTATGCCGCTCTT
ATP synthase gamma subunit	Fwd: CCCAAACTCAAAGGCTCAA Rev: GTTTCCCAGTAATCTCCGTAAT
Rapsyn	Fwd: CTCGAAAGCTACCTGAACCTG Rev: CATCGTTGTTGTGGGCATAG
beta-Dystrobrevin	Fwd: AAGCGAGCCAGGATGATTGA Rev: GGCGACCATGAAATTGAGTAGG
NAChR alpha-3	Fwd: GGGTGGAGTTCATGCGAGTC Rev: GCGGCGAATGTATAGCGAGTA

These findings have been mirrored in human DMD muscle, where the expression of at least 130 proteins has been shown to be perturbed by absence of dystrophin and related isoforms (36, 37).

In the brain, however, the effect of the absence of dystrophin on gene expression remains unexplored. Here, we examined age-related changes in expression of a number of genes in the hippocampus, cerebral cortex and cerebellum of the *mdx* mouse compared to control and interpreted our findings in relation to known perturbations in brain biochemistry and function.

3. METHODS

3.1. Materials

Primers for RT-PCR were obtained from Geneworks (Thebarton, South Australia, http://www.geneworks.com.au) and are shown in table 1. D-[1-^{13}C]glucose (99.9%) and sodium [^{13}C]formate were obtained from Cambridge Isotope Laboratories Inc., MA. 2-Deoxy-D-[1-^{14}C]glucose (2DOG) was obtained from Amersham Pharmacia Biotech UK, Ltd.

3.2. Mice

All experiments were conducted in accordance with the guidelines of the Australian National Health and Medical Research Council and were approved by the institutional animal ethics committee. Mice (C57Bl/10-ScSn-*mdx* and C57Bl/10-ScSn (control)) were maintained on a 12 h light/dark cycle and fed *ad libitum*.

3.3. Real-time PCR analysis of differential gene expression

Old (>1 y) control (n = 6) and *mdx* (n = 6), and young (<4 months) control (n = 6) and *mdx* (n = 4) mice were sacrificed by cervical dislocation and their brains rapidly excised. Brain tissue was dissected into cerebral cortex, cerebellum and hippocampus.

RNA was extracted from brain tissue with 1 mL of TRI-reagent and 0.5 mL of zirconia beads and vigorously homogenized for 20 s in a Savant Fastprep. After addition of 200 uL of chloroform this mixture was spun at 10 000g for 20 min (4 °C). To the aqueous layer, 500 μL of isopropanol was introduced. After standing at room temperature for 5 min, the sample was subjected to another identical centrifugation step. The resultant pellet was washed in ice-cold 70% (v/v) ethanol (RNAse free), air dried and resuspended in water. cDNA was synthesized using the Invitrogen MMLV-RT 2 step reverse transcriptase kit. Approximately 2 μg of RNA was added to a reaction containing 1x RT buffer, 0.5 mM dNTP, 0.1 μg of random primers, 0.1 mM dithiothreitol ((R,R)-1,4-dimercapto-2,3-butanediol), 20 U RNase inhibitor and 100 U of MMLV-reverse transcriptase. The samples were incubated for 1 h at 37 °C and then subjected to an inactivation step at 92 °C for 5 min. The cDNA was diluted with water and used as a template for real-time polymerase chain reactions. Primers used are shown in table 1. The PCR reaction contained 1x SYBR green reaction mix, 1x ROX dye, 2 μM of each primer and 8 μL of template in a 20 μL reaction volume. Amplification was carried out on an ABI 7700 with incubation times of 50 °C / 2 min, 95 °C / 10 min followed by 40-60 cycles of 95 °C / 15 s and 60 °C / 1 min. Specificity of the amplification was checked by melting curve analysis and the mRNA content was measured relative to the housekeeping gene hypoxanthine phosphoribosyl transferase using the $2^{-\Delta\Delta Ct}$ method (38). Results are expressed relative to the expression of that gene in the cerebral cortex of young control mice.

3.4. Uptake of 2-deoxyglucose by brain tissue prisms

Young (~ 3 months of age) and aged (~ 8 months of age) *mdx* and control mice were killed by cervical dislocation. The brains were rapidly removed and the cerebral cortices dissected. Cortical, and cerebellar tissue prisms were made using a McIlwain tissue chopper (Mickle Laboratory Engineering Co, Ltd, Gomshall, UK) by chopping tissue in the parasaggital plane at a slice thickness of 100 μm, rotating 45° and slicing again. The prisms were washed and resuspended in ice-cold modified Krebs-Hensleit buffer (124 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM $NaHCO_3$) with 5 mM glucose (0.1 g/mL suspension). 100 μL (10 mg tissue) was added to 10 mL of buffer. The suspension was then incubated in a water bath at 37 °C for 15 min and after addition of 1 μCi of 2-deoxy[1-^{14}C]glucose, incubated for another 10 min. The tissue prisms were then separated by vacuum filtration (20 to 25 psi negative pressure) through Whatman No. 1 filters (2.5 cm diameter) and rapidly washed twice with 10 mL of fresh buffer, maintained at room temperature. The filters were placed directly into standard scintillation vials, extracted overnight in 1 mL distilled water and, after addition of 7 mL Optifluor, (Canberra Packard), the radioactivity was determined by scintillation counting.

3.5. Cerebral metabolism of D-[1-^{13}C]glucose

Young (< 6 months of age) *mdx* and control mice (N = 11 for each group) were injected in the tail vein with 20 mg D-[1-^{13}C]glucose (40 % w/v). After 15 min, mice were killed by cervical dislocation and the head snap frozen in liquid nitrogen. Brains were removed from the cranial vault whilst frozen, extracted in ice-cold perchloric acid (6% w/v) and centrifuged (4500 rpm) at 4 ºC. The resulting pellet was retained for protein estimation by the Lowry method (39) and the supernatant neutralised to pH 7.2 with NaOH. After removal of $NaClO_4$ by centrifugation, lyophilised supernatants were stored at -20 ºC until required for NMR analysis.

Samples were resuspended in 0.65 mL D_2O containing 2 mM sodium [^{13}C]formate as an internal intensity and chemical shift reference (δ 171.8). {^{1}H-decoupled} ^{13}C spectra (typically 14,000-18,000 transients, duty cycle 4 s, 83,300 data points) were acquired at 9.4 T, on a Bruker DRX-400 WB spectrometer using a 5 mm dual ^{1}H/^{13}C probe. Fully relaxed ^{1}H and {^{13}C-decoupled}^{1}H spectra (duty cycle 30 s, WURST-40 (40) with a 112-step phase cycle (41), decoupling during acquisition) were obtained at 600.13 MHz on a Bruker DRX-600 spectrometer with a broadband inverse xyz-gradient probe. Assignments were aided by reference to standard spectra, coupling constant analysis, and by the acquisition of heteronuclear single quantum coherence and heteronuclear multiple-bond correlation spectra with gradient selection, and a broadband inverse xyz-gradient probe at 600.13 MHz.

Following zero filling to 128K data points and 3 Hz exponential line-broadening, {^{1}H-decoupled} and ^{13}C spectra were transformed and peak areas were determined by integration using standard Bruker software (XWINNMR, Version 3.1). Peak areas were adjusted for nuclear Overhauser effect, saturation and natural abundance effects and quantified by reference to the area of the internal standard resonance of [^{13}C]formate. Metabolite pool sizes (lactate, alanine, GABA, glutamate, glutamine and aspartate) were determined by integration of resonances in fully relaxed 600 MHz {^{13}C-decoupled}^{1}H spectra using [^{13}C]formate as the internal intensity reference. ^{13}C Data are expressed as absolute concentrations (net flux; after adjusting for natural abundance contributions) or as fractional enrichments (% of total metabolite pool which has been ^{13}C labelled) as described previously (42, 43).

4. RESULTS

4.1. Gene expression in *mdx* vs control brain
4.1.1. Glucose transporter

Relative gene expression changes in cortex, cerebellum and hippocampus from young and old *mdx* mice are shown in figure 2. Expression of the major brain glucose transporter GLUT1 was significantly lower in both young (P = 0.033) and old (P = 0.037) *mdx* mouse hippocampus compared to age-matched controls (figure 2A). A similar result was seen for the neuronal glucose transporter GLUT3, where expression was significantly decreased in old *mdx* (P = 0.019) *vs* age-matched control hippocampus (figure 2B).

4.1.2. Neurotransmitter receptors

Expression of some subunits of the GABA$_A$ receptor was also affected in *mdx* mice. In young *mdx* mice both cortical (P = 0.034) and hippocampal (P = 0.05) expression of the GABA$_A$ alpha1 subunit was decreased (figure 2C) compared to age-matched controls, as was cortical expression of the GABA$_A$ alpha2 subunit (P = 0.021). By contrast, there were no significant differences in expression of the GABA$_A$ gamma2 subunit in *mdx vs* controls at any age (figure 2D). Similarly, expression of the 5HT$_{3A}$ receptor (figure 2E) was not affected by lack of dystrophin in the tissues examined.

Decreased expression of the nicotinic acetylcholine receptor (nAChR) alpha3 subunit was seen in the cortex (P = 0.044) and hippocampus (P = 0.034) of young, but not old *mdx* mice compared to age-matched controls (figure 2F).

4.1.3. Bioenergetics

The expression of mitochondrial creatine kinase was not significantly different in young *mdx* mice vs controls (figure 2G) but was sharply decreased in the cortex in old *mdx* mice (P = 0.009; figure 2G). Expression of the gamma subunit of the ATP synthase was significantly decreased in young *mdx* cerebellum (P = 0.033) and in the hippocampus (P = 0.05) compared to age-matched control.

4.1.4. Structural proteins

Expression of beta-dystrobrevin was significantly lower in young *mdx* cortex (P = 0.021) and also lower in hippocampus from old *mdx* mice (P = 0.049) compared to age-matched controls (figure 2H). Expression of the neurotransmitter anchoring protein rapsyn was significantly higher in cortex from young *mdx* mice (P = 0.034) and old *mdx* cerebellum (P = 0.021) compared to age-matched controls (figure 2I).
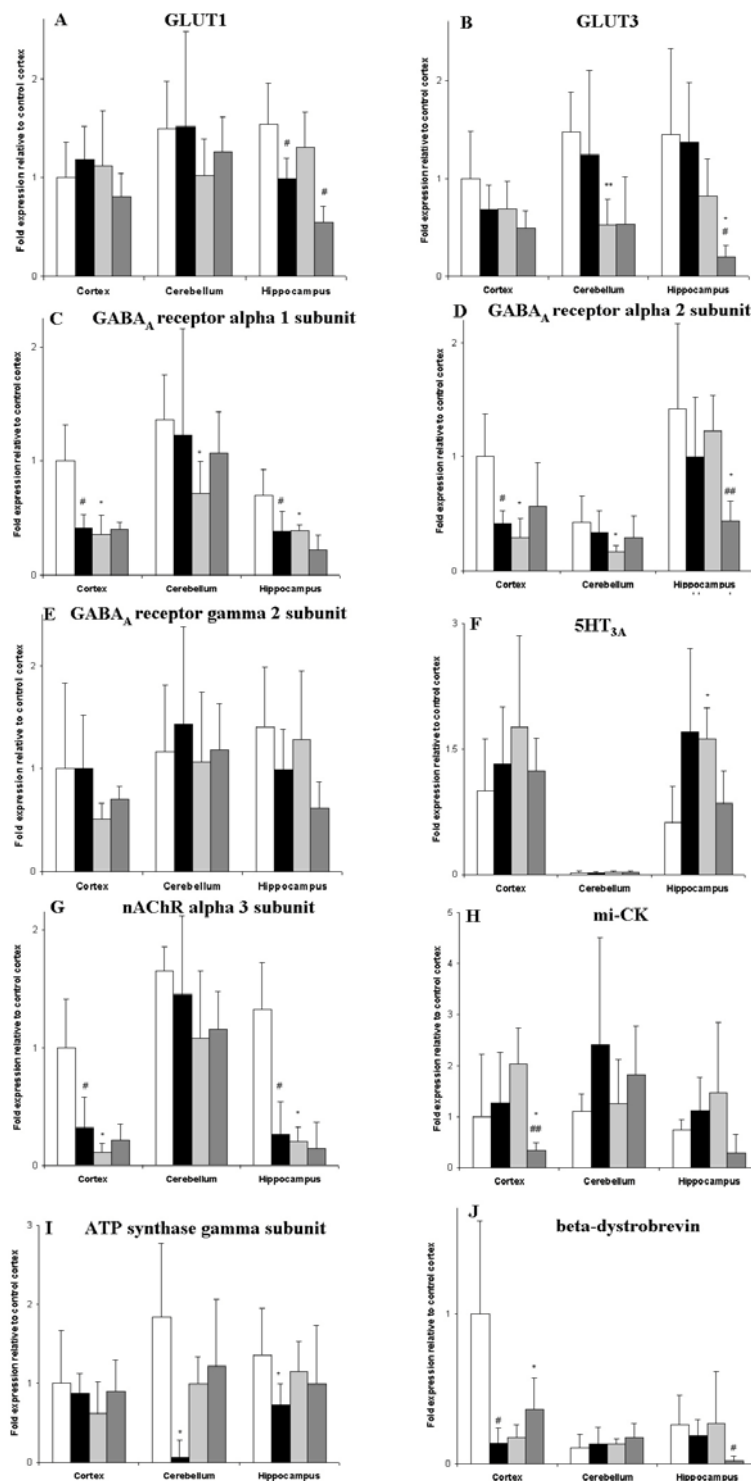
Figure 2. Changes in relative gene expression determined by RT-PCR. White bars, young controls, N = 6; black bars, young *mdx* (N = 4); light grey bars, old controls (N = 6); dark grey bars, old *mdx* (N = 6). Data are shown as means and error bars represent standard deviations. Significant differences between control and *mdx* mice (#, P < 0.05; ##, P < 0.01), significant differences between young and old mice of the same strain (*, P < 0.05; **, P < 0.01). Statistics were calculated using Mann-Whitney U test (alpha = 0.05).

4.2. Age-related changes in gene expression
4.2.1. Glucose transporter

Expression of the neuronal glucose transporter GLUT3 was significantly decreased by age, with expression in the cerebellum of young control mice being significantly higher than in old mice (P = 0.006). This finding was also mirrored in the hippocampus, with an age-related decrease in GLUT3 expression which reached statistical significance in *mdx* mice (P = 0.021) but not controls (P = 0.2; figure 2B). This contrasted with GLUT1 expression which did not vary with age in any section of the brain examined (figure 2A).

4.2.2. Neurotransmitter receptors

Expression of the GABA$_A$ alpha1 subunit significantly decreased with age in control cortex (P = 0.02), cerebellum (P = 0.01) and hippocampus (P = 0.047). Expression of the GABA$_A$ alpha2 subunit mirrored this, with expression decreasing significantly in the cortex (P = 0.01) and cerebellum (P = 0.025) but not the hippocampus (P = 0.63). By contrast, expression of the GABA$_A$ gamma2 subunit did not alter with age. The 5HT$_{3A}$ receptor was expressed significantly less in hippocampus in old mice (P = 0.011) and in the cortex, although this result did not reach statistical significance due to lack of statistical power in this estimation (control N = 3 only).

Similarly, age-related declines in expression were observed for the nAChR alpha3 subunit in both cortex (P = 0.011) and hippocampus (P = 0.049).

4.2.3. Bioenergetics

No age-related decline in expression of mitochondrial creatine kinase was seen in any section of the brain examined in control mice, but expression was significantly decreased in old *mdx* hippocampus (P = 0.028). No age-related declines were seen in expression of the gamma-subunit of the ATP synthase.

4.2.4. Structural proteins

Expression of beta-dystrobrevin decreased significantly with age in control cortex (P = 0.014). Expression of rapsyn was not affected by age in control mice, but was significantly greater in old *mdx* than in young *mdx* cerebellum.

4.3. 2-Deoxy-D-[1-^{14}C]glucose uptake

Uptake of 2DOG was significantly lower in both young (P = 0.046) and aged (P = 0.004) *mdx* mouse cortex, *vs* controls (N = 6, Mann-Whitney U test). Uptake of 2DOG was significantly higher in cortex of both aged control (P = 0.006) and old *mdx* mice (P = 0.025) compared to their younger counterparts. There was no significant difference between either young or aged *mdx* and control 2DOG uptake in the cerebellum (N = 4) but 2DOG uptake was significantly higher in aged than young cerebellum in both *mdx* and control brains (P = 0.021 in each case).

4.4. Cerebral metabolism of D-[1-^{13}C]glucose

There were no significant differences in total metabolite pool sizes (lactate, alanine, glutamate, glutamine, GABA and aspartate) in young *mdx* brains vs controls. Similarly, no significant difference in flux of ^{13}C was observed into any metabolite measured (Glu C2, C3, C4, Gln C4, GABA C2, Asp C2, C3, Lactate C3 and Ala C3). *Mdx* brains showed significantly higher amounts of residual (unmetabolised) [1-^{13}C]glucose compared to controls (P < 0.045). In line with these results, there was also no significant difference in fractional enrichment of any isotopomer measured. The total amount of ^{13}C label taken up by brains from each group was not significantly different (control, 4.05 ± 1.05; *mdx* 3.51 ± 1.09, N = 11).

5. DISCUSSION

Studies of the ramifications of single gene deletions have tended to focus on the gene product and its immediate roles. They are further complicated by genetic and biochemical redundancies (44) which make determining the effect of the gene deletion problematic. Indeed, redundancy is "alive and well" in the *mdx* mouse; in *mdx* muscle, the role of dystrophin is compensated by increased expression of the related protein utrophin (27, 28). In this work, we have found gene expression alterations which, although plainly related to clinical manifestations and deficits in brain function, are not (at present) immediately linkable to dystrophin itself through known direct biochemical pathways. This may be either due to the fact that the role of dystrophin in the brain is still largely unknown, or to indirect effects produced by deletion of dystrophin itself.

5.1. Glucose transporter expression and glucose use

We found significant decreases in expression of both GLUT1 (major, non insulin-sensitive brain glucose transporter, mainly found in capillaries with some expression in astrocytes and neurons (45)) and GLUT3 (non insulin-sensitive transporter, mainly found in neurons, with kinetic responses geared for increased turnover rates at low glucose concentrations, such as are found after neuronal activation (45)) glucose transporters in the hippocampus of both young and old *mdx* mice compared to controls.

Glucose transporter expression is known to be regulated by a number of factors, including oxygen, glucose and insulin levels (46, 47), as well as cell stresses such as hyperosmolarity (48). However, in *mdx* brain there is little evidence that levels of these effectors are altered, suggesting another mechanism through which glucose transporter expression is altered. The expression of glucose transporters GLUT1 and GLUT3 is known to be related to local cerebral glucose usage rates and to increase during development as the rates of glucose use increase (45), although the molecular mechanisms controlling this are not well understood.

Previous gene expression studies in muscle in *mdx* mice showed decreased expression of the insulin-sensitive glucose transporter GLUT4 in both skeletal muscle and diaphragm (35), and this is in keeping with several studies showing altered metabolic response to glucose in skeletal muscle in both mice (49) and humans (50) with Becker muscular dystrophy, a milder form of the disorder.

These anomalies in glucose metabolism have also been shown in the dystrophin-deficient brain. Using positron emission tomography and uptake of a 2-deoxyglucose ligand, Bresolin et al. reported decreased uptake in the cerebellum of boys with DMD compared to a control with Wernig-Hoffman disease (51). A more recent PET study (52) has also shown decreased glucose metabolism in children with DMD compared with an unaffected adult population, including the medial temporal structures and cerebellum bilaterally and the sensorimotor and lateral temporal cortex on the right side. In the *mdx* mouse, abnormal metabolism of [1-^{13}C]glucose has been reported in old *mdx* brain (43), with these mice showing increased relative use of glucose (a larger fraction of the glucose taken up into the brain is oxidized) compared to controls. Given the age-related changes in gene expression in the brain we have seen in this study, we were interested to see whether the same metabolic response was also observed in younger *mdx* mice, particularly given the emerging evidence showing age-related changes in the brains of *mdx* mice. Although glucose uptake (measured by the 2DOG uptake method) was lower in the cortex of young *mdx* brain compared to controls, as is also seen in the older mice, we found no significant difference in glucose metabolism in young *mdx* vs control mice. This suggests that the changes in glucose metabolism seen in older *mdx* mice are due more to changes in other factors (such as neurotransmitter expression and function, for example), than they are to changes in the expression of glucose transporters. Indeed, in hypoglycaemia, it is known that expression of glucose transporters is increased, but glucose use decreases, showing that uncoupling of the two effects occurs (53).

The decrease in GLUT1 and GLUT3 expression seen in the *mdx* mouse seems most likely related to decreased synaptic integrity, and hence activity, which would be expected to result in decreased glucose use (45) and hence decreased glucose transporter expression. A mechanism for a link between glucose transporter expression and synaptic activity has been proposed for hypothalamic (glucose sensing) neurons, involving glucokinase and the Na$^+$/K$^+$ ATPase (54). It is known that glucose transporter expression increases in developing brain parallel increases in Na$^+$/K$^+$ ATPase activity but the mechanism by which they may be linked remains unknown.

5.2. Neurotransmitter receptor expression

Both the alpha1 and alpha2 subunits of the GABA$_A$ receptor were expressed at significantly lower levels in the cortex of young *mdx* mice, and alpha1 subunit expression was decreased in the hippocampus of young *mdx* mice, and alpha2 in the hippocampus of old *mdx* mice. These findings are consistent with the reduction in immunoreactivity for these subunits observed in *mdx* hippocampus (4) but do not mirror the reduction in immunoreactivity seen in the cerebellum (4). It has previously been shown that mdx hippocampus does not display the increased basal transmission expected following addition of the GABA$_A$ antagonist bicuculline (55), application of bicuculline to *mdx* Purkinje cells did not produce the expected reduction in evoked post-synaptic potential (56) and that old *mdx* mice do not show the expected metabolic response following administration of the GABA$_A$ agonist muscimol (43), indicating that GABA$_A$ function is perturbed in these mice.

The GABA$_A$ receptor is a pentameric receptor potentially composable from at least 16 separately identified subunits (57) each of which confers different activity and specificity on the receptor. There are likely at least 10 major GABA$_A$ receptor subtypes in the human brain (57) and, currently, the functional consequences of the multiplicity of GABA$_A$ receptor subtypes is largely unknown (58). The major subtype of GABA$_A$ receptor in the brain is that containing alpha1, 2 and gamma2 subunits (59, 60). In two different strains of alpha1 subunit knockout mice, the alpha2 and alpha3 subunits are upregulated, preserving viability and behavioural characteristics (60, 61). In young *mdx* mice, however, the decrease in the alpha1 subunit is not compensated for by upregulation of the alpha2 subunit.

It has been shown that dystrophin is not directly involved in anchoring or clustering the GABA$_A$ receptor in the synapse; rather the presence of dystrophin is required to maintain synaptic integrity (62). The expression of GABA$_A$ receptor components has been shown to relate to synaptic activity (63). It may therefore be that lack of dystrophin in the post-synaptic density impedes the activity of the synapse, this then leading to decreased expression of GABA$_A$ receptor components due to decreased GABAergic demand. Autopsy analysis of DMD brain has shown neuronal loss, reduced dendritic arborisation, heterotopias, Purkinje cell loss, perinuclear vacuolation and disordered architecture (reviewed in 1). These findings are consistent with decreased synaptic integrity and overall reduction in function.

A similar effect to that seen with the GABA$_A$ receptor could be occurring with the nAChR. Synaptic activity has also been shown to affect expression of subunits of the nicotinic acetylcholine receptor, including alpha 3 (64, 65). We found significantly decreased expression of the alpha3 subunit of the nAChR in both hippocampus and cortex in young *mdx* mice. These mice showed levels of alpha3 nAChR expression comparable to old mice. This is the first report of direct evidence for a nicotinic acetylcholine lesion in the brain due to the absence of dystrophin. It is supported by a recent finding that *mdx* mice exhibit a reduced response to nicotine enhancement of passive avoidance behaviour (66). Altered clustering of nAChR subunits has been reported in the superior cervical ganglion of mdx mice (67) and it has been shown that dystrophin stabilizes alpha3 but not alpha7 nAChR subunits in this tissue (68). Similarly, it has been known for some time, that dystrophin expression is important for normal clustering of nAChRs in the neuromuscular junction.

5.3. Brain bioenergetics

Our findings of decreased expression of mitochondrial creatine kinase and the gamma subunit of the

ATP synthase are not entirely unexpected given the known bioenergetic abnormalities seen in the brains of both *mdx* mice (69) and boys with DMD (70). These findings mirror those seen in muscle where the expression of mitochondrial creatine kinase, adenine nucleotide translocase and the gamma subunit of the ATP synthase are all altered from control levels (35). This has been shown in muscle from persons with Becker muscular dystrophy, to translate into altered bioenergetic recovery from exercise (50), similar to that seen in the *mdx* mouse (71). It has been suggested that dystrophin may be involved in the clustering of this complex of proteins; mitochondrial creatine kinase forms a complex with the adenine nucleotide translocase and the porin which allows creatine to cross the outer mitochondrial membrane forming adhesions between the inner and outer mitochondrial membrane and thus facilitating ATP synthesis. The formation of this complex has been shown to be decreased in the absence of dystrophin (72).

A lowered ability to form this complex, shown to be important in regulating ATP synthesis rates (73, 74) may be reflected in the altered bioenergetics seen in *mdx* brain and in the observed increased susceptibility to hypoxia (75, 76).

5.4. Structural protein expression

The structural protein, beta-dystrobrevin, is a dystrophin-associated protein expressed in the brain and other non-muscle tissues (77). In the brain, expression of beta-dystrobrevin is confined to neurons where it forms a specific DPC complex with dystrophin and syntrophin in the post-synaptic density (78). Lack of dystrophin has been shown not to disrupt interactions between beta-dystrobrevin and syntrophin and not significantly to affect the level of these proteins in immunoblots (78). It is possible, however, that there may be a feedback mechanism suppressing transcription of beta-dystrobrevin in the absence of dystrophin.

Rapsyn is a protein intimately involved in the clustering of nAChRs. It has been shown to bind the dystrophin-associated protein beta-dystroglycan *in vivo* (79). Rapsyn bound to beta-dystroglycan has been suggested to be involved in the signalling cascade that leads to nAChR clustering (79). It was not clear from our limited data whether there was any correspondence between rapsyn and nAChR subunit expression.

5.5. Summary

It is apparent that deletion of a single gene, dystrophin, has ramifications for expression of other genes in the brain. Genes whose expression is affected by the absence of dystrophin code for products, the alteration of which causes functional and clinical manifestations which are largely easily linkable to these gene products. By contrast, the mechanism by which the expression of these gene products is altered due to absence of dystrophin is not so immediately obvious. The similarities between gene expression alterations due to lack of dystrophin in the brain and in muscle suggest that the mechanisms may be similar in both tissues.

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