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Nicotinic receptor abnormalities in the cerebellar cortex in autism

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Summary

Autism is a common developmental disorder associated with structural and inferred neurochemical abnormalities of the brain. Cerebellar abnormalities frequently have been identified, based on neuroimaging or neuropathology. Recently, the cholinergic neurotransmitter system has been implicated on the basis of nicotinic receptor loss in the cerebral cortex. Cerebellar cholinergic activities were therefore investigated in autopsy tissue from a series of autistic individuals. The presynaptic cholinergic enzyme, choline acetyltransferase, together with nicotinic and muscarinic receptor subtypes were compared in the cerebellum from age-matched mentally retarded autistic (eight), normal control (10) and non-autistic mentally retarded individuals (11). The nicotinic receptor binding the agonist epibatidine (the high affinity receptor subtype, consisting primarily of α3 and α4, together with β2 receptor subunits) was significantly reduced by 40–50% in the granule cell, Purkinje and molecular layers in the autistic compared with the normal group ($P < 0.05$). There was an opposite increase (3-fold) in the nicotinic receptor binding α-bungarotoxin (to the α7 subunit) which reached significance in the granule cell layer ($P < 0.05$). These receptor changes were paralleled by a significant reduction ($P < 0.05$) and non-significant increase, respectively, of α4 and α7 receptor subunit immunoreactivity measured using western blotting. Immunohistochemically loss of α_4 reactivity was apparent from Purkinje and the other cell layers, with increased α7 reactivity in the granule cell layer. There were no significant changes in choline acetyltransferase activity, or in muscarinic M1 and M2 receptor subtypes in autism. In the non-autistic mentally retarded group, the only significant abnormality was a reduction in epibatidine binding in the granule cell and Purkinje layers. In two autistic cases examined histologically, Purkinje cell loss was observed in multiple lobules throughout the vermis and hemispheres. This was more severe in one case with epilepsy, which also showed vermis folial malformation. The case with less severe Purkinje cell loss also showed cerebellar white matter thinning and demyelination. These findings indicate a loss of the cerebellar nicotinic α4 receptor subunit in autism which may relate to the loss of Purkinje cells, and a compensatory increase in the α7 subunit. It remains to be determined how these receptor abnormalities are involved in neurodevelopment in autism and what is the relationship to mental function. Since nicotinic receptor agonists enhance attentional function and also induce an elevation in the high affinity receptor, nicotinic therapy in autism may be worth considering.

Keywords: autism; cerebellum; cholinergic system; nicotinic receptors; muscarinic receptors

Abbreviations: ChAT = choline acetyltransferase; nAChR = nicotinic acetylcholine receptor; α-BT = α-bungarotoxin

Introduction

Autism is a developmental disorder characterized by social deficits, communication abnormalities, repetitive and stereotyped behaviours and impairments in planning and attention. It is associated with a variable degree of mental retardation in about three-quarters of the cases (Lockyer and Rutter, 1969) and with epilepsy in at least one-quarter of the cases (Rutter, 1970). Several biological factors are thought to be involved in autism, including, most importantly, genetic (with a number

of different gene loci implicated), but also complications during pregnancy or birth, metabolic conditions and viral infections (Baron-Cohen and Bolton, 1993).

Several brain regions have been implicated in the development of autism. Both neuropathological and neuroimaging studies have demonstrated cerebellar abnormalities. Neuropathologically, reductions in Purkinje cell numbers in cerebellar vermis and hemispheres have been reported in nearly all mentally retarded autistic cases (Ritvo *et al*., 1986; Bailey *et al*., 1998; Kemper and Bauman, 1998). In one study of a single 16-year-old female with autism (Guerin *et al*., 1996), no cerebellar abnormalities were found, although the clinical diagnosis of this case may have been doubtful.

MRI neuroimaging studies have been performed on both mentally retarded and high functioning autistic individuals, but the results are not clear-cut. Hypoplasia has been reported in cerebellar vermal lobules VI–VII (Courchesne *et al*., 1988; Hashimoto *et al*., 1993, 1995; Saitoh *et al*., 1995; Ciesielski *et al*., 1997), I–IV and VIII–X (Hashimoto *et al*., 1993, 1995; Levitt *et al*., 1999). In particular, the study of Hashimoto *et al*. (1995), including nearly 100 autistics and 100 controls, found reductions in all three vermis regions (I–V, VI–VII and VIII–X). Carper and Courchesne (2000) noted a correlation between increased frontal lobe and such cerebellar abnormalities.

Other MRI studies have reported normal size or even hyperplasia of cerebellar vermal lobules and hemispheres in autistic individuals (Garber and Ritvo, 1992; Holttum *et al*., 1992; Kleiman *et al*., 1992; Piven *et al*., 1992, 1997; Abell *et al*., 1999; Manes *et al*., 1999; Hardan *et al*., 2001). Courchesne *et al*. (1994*a*) identified subgroups with hypoplasia and hyperplasia, as did Saitoh and Courchesne (1998). Several factors have been proposed to explain these discrepancies, including statistical problems with small sample size (Baer and Ahern, 1993), heterogeneous autistic samples consisting of both hypoplastic and hyperplastic subtypes (Courchesne *et al*., 1994*a*), and inadequate matching for IQ (intelligence quotient) and gender (Filipek, 1995). It has also been suggested that cerebellar hypoplasia may not be a specific neuroanatomical marker for autism. First, a strong positive association between IQ and size of cerebellar vermal lobules VI–VII has been demonstrated in several studies of both autistic and non-autistic control individuals (Piven *et al*., 1992, 1997; Ciesielski *et al*., 1994; Levitt *et al*., 1999). Secondly, hypoplasia of cerebellar vermal lobules VI–VII has been demonstrated in several neurogenetic conditions without autistic behaviour (Schaefer *et al*., 1996).

Several neurotransmitters have been implicated in autism, including serotonin, dopamine, noradrenaline and some neuropeptides (Tsai, 1999). Investigations have involved primarily measurements in blood or CSF and response to pharmaceutical agents. Asymmetry of serotonin synthesis in autism, based on α-[^{11}C]methyl-L-tryptophan PET, has been observed in brain circuitry involving the cerebellum, ventrolateral thalamic nucleus and frontal regions (Chugani *et al*., 1997). The cholinergic system recently has been implicated on the basis of nicotinic and muscarinic receptor abnormalities detected in autopsy cerebral cortex (Perry *et al*., 2001).

The cholinergic innervation of the cerebellum is complex, as judged from non-human brain anatomy. The predominant cholinergic input to the cerebellum originates from the vestibular nuclei (Barmack *et al*., 1992). However, other projections may exist. In gerbil brainstem, cholinergic cerebellar projections originate in the midline medullar periventricular grey, the C3 adrenergic area raphe obscurus nucleus, and in a variety of the reticular formation nuclei, together with various sensory nuclei (Lan *et al*., 1995). In cat brain, cholinergic tegmental cerebellar projecting neurones occur in the pedunculopontine, lateral dorsal tegmental as well as locus coeruleus nuclei (Cirelli *et al*., 1998). In rat, a subset of cerebellar mossy fibres, rich in choline acetyltransferase (ChAT), originates from the medial vestibular nucleus and innervates unipolar brush cells (Jaarsma *et al*., 1996) and granule cells (Jaarsma *et al*., 1997). In the latter report, beaded cholinergic (ChAT-positive) fibres were also observed originating from the pedunculopontine tegmental and lateral paragigantocellular nucleus. In one study of cat brain, a subpopulation of intrinsic Golgi cells was reported to be ChAT positive in the granule cell layer (Illing *et al*., 1990).

In human cerebellum, ChAT neurochemical activity is highest in fetal brain, declining extensively post-natally and up to adulthood (Court *et al*., 1993). These changes are the reverse of those for the hydrolytic enzyme acetylcholinesterase which tends to increase post-natally in the molecular layer. Both muscarinic and nicotinic receptors have been localized in the cerebellar cortex. The granule cell layer of the rat vestibulocerebellum contains nicotinic binding sites (Jaarsma *et al*., 1996). Based on autoradiographic analysis in human cerebellum, nicotine binding has been detected in molecular and granule cell but not Purkinje layers, and α-bungarotoxin (α-BT) was distributed fairly evenly between the different layers (Court *et al*., 1995). In rat cerebellum, nicotinic acetylcholine receptor (nAChR) α4 subunit immunoreactivity has been identified in cell bodies in the molecular, granule and Purkinje cell layers and in axon terminals forming synapses with Purkinje cells (Nakayama *et al*., 1997). α7 nAChR subunit immunoreactivity is apparent in rat Purkinje cells and granule cell dendrites in glomeruli, but not granule cell somata (Caruncho *et al*., 1997).

Paralleling developmental changes in ChAT, cerebellar cholinergic receptors decline post-natally. Based on autoradiographic analyses of ligand binding, muscarinic M2 and nicotinic high and low affinity receptors were found to be higher in fetal than adult human cerebellum (Court *et al*., 1995). mRNAs for α4, α5, α7, β2 and β4 but not α3 nAChR subunits are also higher in pre-natal than adult human cerebellum (Hellström-Lindahl *et al*., 1998; Hellström-Lindahl and Court, 2000). In rat brain, M1, M3 and M4 muscarinic receptor subtypes decrease from juvenile to adult (Tice *et al*., 1996).

Table 1 *Patient details*

Clinical diagnosis	*n*	Age (years)	PM delay (h)	Gender M/F
Control	10	27.90 ± 6.19	36.60 ± 29.54	M (6) F (3)
Autism	8	24.63 ± 5.32	25.61 ± 19.85	M (7) F (1)
Down's syndrome	3	40.00 ± 7.00	53.05 ± 58.15	M (2) F (1)
Mental retardation (other)	8	31.13 ± 6.85	33.81 ± 16.12	M (5) F (3)

Age and PM delay are given as the mean ± SD; not all cases were available for all analyses.

Because of previous findings in autism of cholinergic receptor abnormalities in cerebral cortex and pathological abnormalities generally reported in the cerebellum, cholinergic biomarkers were examined in the cerebellum of autistic and age-matched non-autistic individuals. Markers, including the presynaptic enzyme ChAT, muscarinic M1 and M2 receptors, and both high and low affinity nicotinic receptors, which are stable up to 72 h post-mortem (Rodriguez-Puertas *et al*., 1996; Court *et al*., 1997, 1998; Perry *et al*., 1998), were measured in autopsy brain tissue.

Methods

Cases

Autopsy tissue was examined from eight adults with autism, meeting DSM-IV criteria [which include delay or abnormal functioning in at least one of (i) social interaction; (ii) language; and (iii) symbolic or imaginative play]. Mental retardation was present in all except one case, for which this information was lacking. Further clinical details, including those elicited from the parents using the revised Autism Diagnostic Interview, were available for five of the eight autistic cases. In these cases, age of onset of symptoms ranged from 3 to 48 months; verbal language was never achieved in four cases and first noted at 10 years for one case; Autism Diagnostic Interview communication non-verbal was 12–21 months, social 11–25 months, behaviour 3–10 months, and early development 2–4 or 5 months; performance and verbal IQ ranged from <20 up to 40; early development histories included juvenile diabeties, legally blind with some vision and neurological trauma at birth; family histories included one case with a father with language delay, and another case with the father with four siblings with autism. Epilepsy occurred in five out of the total of eight cases.

Comparisons were made with 10 age-matched controls, and also with eight individuals affected by other congenital cerebral disorders involving intellectual impairment (associated with birth trauma, viral encephalopathy, congenital cerebellar degeneration or congenital epilepsy) and three adults with Down's syndrome (aged 32–45 years). There was no history of mental retardation in the control group, and causes of death included traffic accident (four), asphyxia (laryngeal injury), drug overdose, myocardial infarction or left ventricular failure, cardiomyopathy, arteriosclerotic cardiovascular disease and pneumonia. Causes of death amongst the autistic cases included cardiac arrest, pneumonia, asphyxia, renal failure (two), pyloric ulcer, epileptic convulsion and severe burns. Patient details are provided in Table 1. There were no significant differences between the groups in mean age or autopsy delay.

Frozen cerebellar tissue from control, autistic, non-autistic mentally retarded and Down's syndrome cases was obtained from the Newcastle Brain Bank (UK), the Autism Research Foundation, the Harvard Brain Tissue Resource Center (USA) and University of Miami Brain and Tissue Bank for Development Disorders (USA), with six of the eight autistic cases from the Harvard Center. For the Harvard Brain Bank material, written consent, approved by McLean Hospital IRB, was obtained for each case from the legal next of kin, authorizing donation of tissue to the Bank for use in research. For the Newcastle series, permissions for post-mortem and brain donation were obtained by consent from the next of kin, in accordance with the regulations of the North Tyneside Health Authorities Joint Ethics Committee.

For the Newcastle series, vertical slices through the left mediolateral cerebellar hemisphere were snap-frozen at autopsy in liquid Arcton (cooled in liquid nitrogen) and then stored at –70°C. Cortical tissue from the superior or inferior semilunar lobule (VII) was sub-dissected at –20°C. Tissue from the Harvard Brain Tissue Resource Center was sub-dissected at –20°C from whole hemispheres (three right and two left) originally frozen intact. Samples were analysed for: ChAT biochemical activity (specific for cholinergic neurones); muscarinic M1 receptor binding (assessed using [^{3}H]pirenzepine); muscarinic M2 receptor binding ([^{3}H]AFDX-384); and low and high affinity nicotinic receptors measured using [^{125}I]α-BT and [^{3}H]epibatidine, respectively.

Neurohistology

For the two Newcastle Bank autistic and three age-matched control cases, the vermis and the right cerebellar hemisphere were fixed in formalin and tissue blocks were embedded in paraffin. Sections of 6 μm were stained with haematoxylin and eosin, cresyl–fast violet and Luxol–fast blue for histological assessment.

Receptor autoradiography

Frozen tissue blocks were sub-dissected at –20°C and mounted onto aluminium chucks. Cryostat sections of 10 μm were cut at –12°C and thaw-mounted onto Vectabonded

slides. Sections were air dried at room temperature for 2 h prior to storage at –70°C until required. Adjacent sections were used to compare total with non-specific binding, and determinations for each case were made in duplicate and singly, respectively, for all except [^{125}I]α-BT binding (triplicate and duplicate, respectively).

Muscarinic M1 receptor binding was measured using 2 nM [^{3}H]pirenzepine in 22 mM HEPES buffer pH 7.5 at room temperature, and non-specific binding assessed in the presence of 1 μM atropine (Perry *et al*., 1998). After incubation for 1 h, sections were washed for a total of 9 min in three changes of ice-cold buffer, dipped in water and dried rapidly under a stream of air at room temperature. For muscarinic M2 receptor binding, sections were incubated in 7 nM 2,3-dipropylamine [^{3}H]AFDX-384 and 10 mM sodium potassium phosphate buffer pH 7.4 at room temperature for 1 h (Crook *et al*., 1999). Non-specific binding was assessed in the presence of 1 μM atropine. Sections were washed for 2 min in two changes of buffer at room temperature, dipped in water and dried as above

For [^{3}H]epibatidine binding to the high affinity nicotinic receptor, tissue sections were pre-incubated in 50 mM Tris–HCl buffer pH 7.4 containing 8 mM $CaCl_2$ at room temperature for 20 min, to remove endogenous ligands (Court *et al*., 1997). Sections were then incubated in the presence of 1 nM (±)[^{3}H]epibatidine for 3 h at room temperature. Non-specific binding was assessed in the presence of 1 μM cytisine. After incubation, tissue sections were washed for a total of 90 s in three changes of ice-cold buffer, dipped in water and dried. For [^{125}I]α-BT binding to the low affinity nicotinic receptor, tissue sections were pre-incubated in 50 nM Tris–HCl buffer pH 7.4 containing 1 mg/ml BSA (bovine serum albumin) for 30 min at room temperature (Court *et al*., 1997). Sections were incubated in the presence of 1.2 nM [^{125}I]α-BT for 2 h at room temperature. Non-specific binding was assessed in the presence of 2.5 mM nicotine during both the pre-incubation and incubation periods. After incubation, sections were washed for 30 min with six changes of ice-cold buffer, dipped in water and dried.

Dried sections were exposed to Hyperfilm [^{3}H] with high and low tritium standards for 4 months for pirenzepine, 6 weeks for AFDX-384 and 3 months for epibatidine, and with ^{125}I standards for 1 week for α-BT. After development, images were quantified in the different layers of the cerebellar cortex using a Lynx densitometry system (Applied Imaging International, Newcastle upon Tyne, UK). Receptor binding data are expressed per mg tissue equivalent.

Nicotinic receptor subunit immunochemistry

Nicotinic receptor subunits α4 and α7 were quantified using western blotting. Polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, Calif., USA) included sc-1772 against the C-terminus of the nAChR α4 subunit; and sc-1447 against a human epitope mapping at the C-terminus of the α7 subunit. The secondary antibodies used were HRP (horseradish peroxidase)-conjugated anti-goat (Chemicon International Ltd, Harrow, UK). Boehringer Mannheim chemiluminescence blotting substrate was from Boehringer Roche, UK. Membrane pellets prepared as previously described were subjected to SDS–PAGE (sodium dodecyl-sulphate–polyacrylamide gel electrophoresis) (Martin-Ruiz *et al*., 1999). Proteins were transferred by electroblotting onto PVDF (polyvinyldifluorene) membranes and evaluated by western blotting between two and five times. Following exposure to Hyperfilm enhanced chemiluminescence (ECL; Amersham Biosciences, Little Chalfont, UK), the integrated optical density of each band and gel background was measured using the Image-Pro Plus densitometry system (Media Cybernetics, Silver Spring, MD, USA).

Immunohistochemistry for nAChR α4 and α7 subunits was carried out on 10 μm formalin-fixed, paraffin-embedded sections of cerebellar hemisphere and vermis from three controls and two autistic patients. Sections were deparaffinized and rehydrated, following which endogenous peroxidase was quenched by incubating in 0.9% hydrogen peroxide for 30 min. Antigen unmasking was carried out by microwaving the sections in 0.01 M citrate buffer, pH 6 for 10 min. Non-specific binding was blocked with normal serum from the same species in which the secondary antibody was raised, diluted 1:60 in PBS (phosphate-buffered saline) pH 7.4. Primary antibodies were diluted in PBS containing 0.1% BSA and applied to the sections for 1 h at room temperature or overnight at 4°C. Visualization of antigen–antibody reactions was carried out using the Vectastain Elite Kit (Vector Labs Inc., Burlingame, CA, USA) according to the manufacturer's protocol with 3,3′-diaminobenzidine hydrochloride as chromogen. Sections were counterstained with haematoxylin before dehydrating, clearing and mounting in DPX (p-xylene-bis(*N*-pyridinium bromide). Sections from which primary antibody was omitted and adsorbed controls showed no immunoreactivity. Digital images of selected sections were captured using a JVC 3-chip CCD true colour camera mounted on a Zeiss Axioplan 2 brightfield photomicroscope and Neotech Image Grabber Software.

Choline acetyltransferase

Representative tissue samples across the width of the cerebellar cortex were removed from the surface of the frozen blocks using a micro-scalpel. Ten percent homogenates were prepared in 0.32 M sucrose containing 0.5% Triton X-100 at 4°C. Duplicate samples were incubated at 37°C for 20 min with [^{14}C]acetyl-coenzyme A using an adaptation (Perry *et al*., 1977) of the method of Fonnum (1969). Enzyme activities are expressed in terms of unit protein, assayed by the method of Lowry *et al*. (1951).

Radiochemicals

[^{3}H]Pirenzepine (85.6 Ci/mmol), [^{3}H]AFDX-384 (106.5 Ci/mmol), (±)[^{3}H]epibatidine (33.8 Ci/mmol), (3-

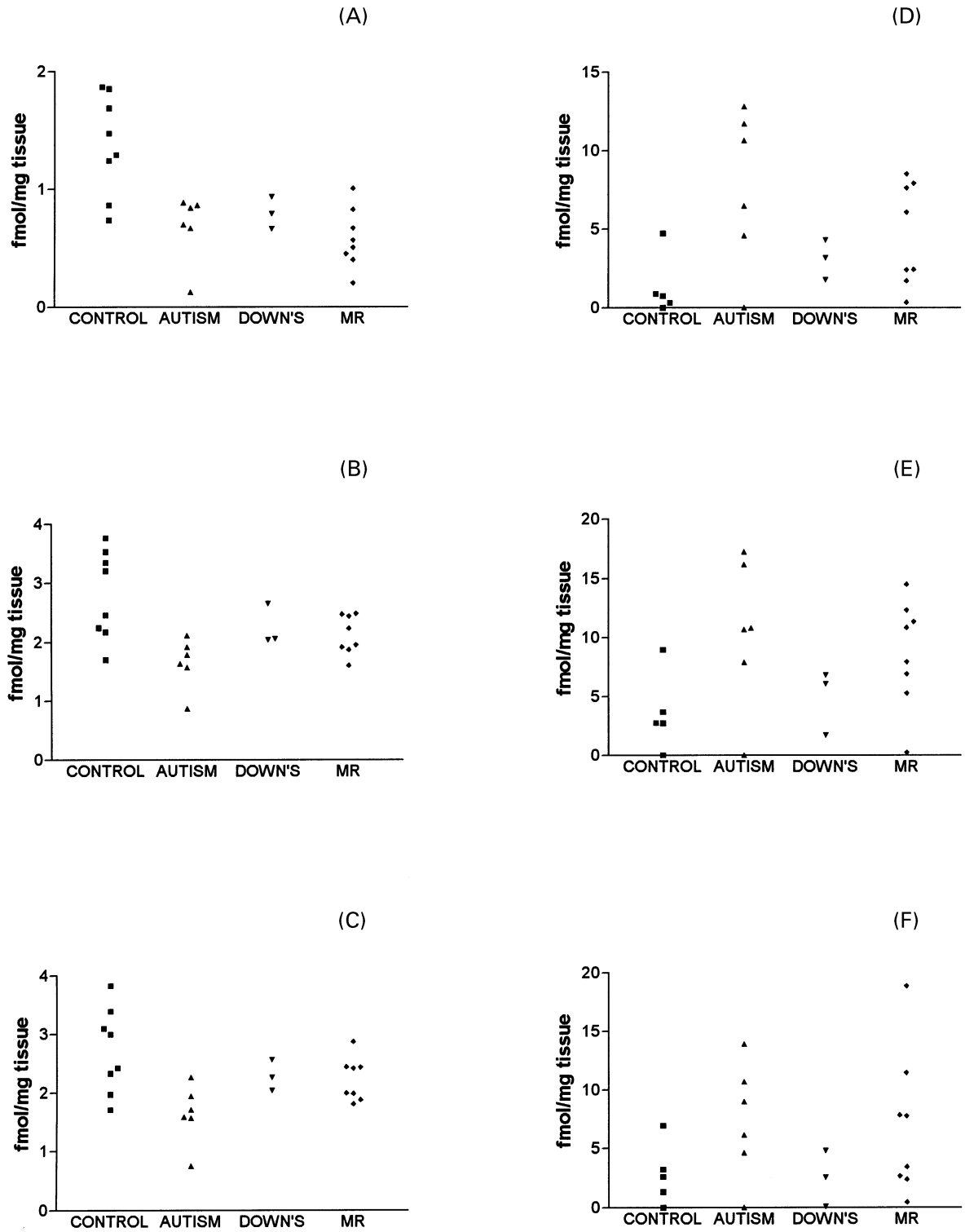

Fig. 1 (**A–C**) Epibatidine and (**D–F**) α-bungarotoxin binding in the cerebellum. **A** and **D** = granule cell layer; **B** and **E** = Purkinje cell layer; **C** and **F** = molecular layer. MR = mental retardation (non-autism).

[^{125}I]iodotyrosyl)-α-BT (150 Ci/mmol) and [^{14}C]acetyl-coenzyme A (58.9 mCi/mmol) were purchased from New England Nuclear (NEN) Life Science Products, Inc. (Boston, Mass., USA).

Statistical analysis

Values for cerebellar nicotinic and muscarinic binding were analysed by two-way ANOVA (analysis of variance; general linear model, Minitab Release 12) with factors: group and

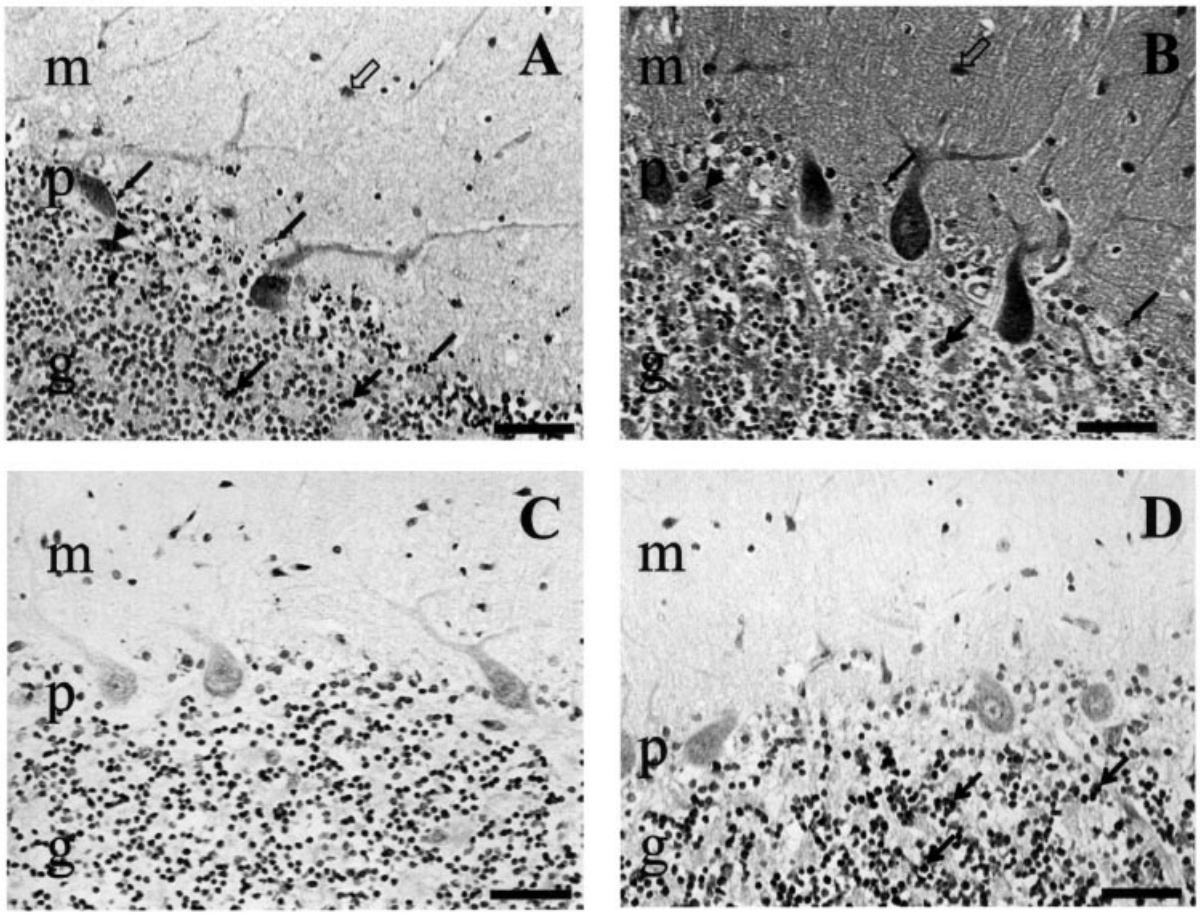

Fig. 2 nAChR subunit immunohistochemistry in the cerebellar cortex. nAChR (**A** and **C**) α4 and (**B** and **D**) α7 subunits in (**C** and **D**) autistic compared with (**A** and **B**) control cases. Scale bars = 50 μm. m = molecular layer; p = Purkinje cell layer; g = granule cell layer. Unfilled arrows indicate immunoreactive stellate cells in the molecular layer. Filled arrows indicate granular immunoreactivity in the Purkinje cell layer. Open arrows show immunoreactive granule cells, and arrowheads point to immunoreactive Golgi cells. A decrease in α4 and an increase in α7 immunoreactivity was noted in granule cells of autistic cases. α4 and α7 neuropil immunoreactivity was decreased throughout the autistic cerebellar cortex, and α4 and α7 immunoreactivity in Purkinje cells was diminished.

Table 2 *Choline acetyltransferase activity*

Clinical diagnosis	ChAT activity (nmol/h/mg protein) mean ± SD
Control	5.43 ± 3.12 (8)
Autism	4.07 ± 3.18 (7)
Down's syndrome	3.00 ± 0.78 (3)
Mental retardation (other)	3.01 ± 1.17 (8)

Case numbers are in parentheses.

cortical layers. If a statistically significant difference was demonstrated between groups and/or cortical layers, but there was no significant interaction between the factors, the data were analysed as suggested by Kinnear and Gray (1994), by one-way ANOVA followed by Fisher's *post hoc* analysis rate with significance set at $P < 0.05$. The density of bands on western blots for autistic and control cases was compared using the two-tailed *t* test.

Results

Neurochemistry

Epibatidine binding in the normal young adult cerebellum was higher in the Purkinje and molecular layers than in the granule cell layer (Figs 1 and 2, and Table 3) although α-BT binding was similar in the different cortical layers (Figs 1 and 2, and Table 4). M1 receptor binding was, as expected, extremely low in the cerebellum, and M2 binding was similar to cerebral cortex, with highest binding in the Purkinje, intermediate binding in the molecular, and lowest binding in the granule cell layers.

There was no alteration in cerebellar ChAT in autism or the other groups examined (Table 2). There was a significant reduction of up to 50% in epibatidine binding in the autism

Table 3 *Epibatidine binding*

Clinical diagnosis	Granule cell layer	Purkinje layer	Molecular layer
Control	1.38 ± 0.43 (8)	2.81 ± 0.75 (8)	2.72 ± 0.73 (8)
Autism*	0.68 ± 0.29 (6)	1.66 ± 0.43 (6)	1.64 ± 0.51 (6)
Down's syndrome	0.80 ± 0.14 (3)	2.26 ± 0.35 (3)	2.29 ± 0.27 (3)
Mental retardation (other)	0.58 ± 0.25 (8)*	2.13 ± 0.33 (8)*	2.23 ± 0.37 (8)

Specific binding of 1 nM (±)[^{3}H]epibatidine in fmol/mg tissue equivalents is expressed as the mean ± SD; number of cases in parentheses. Data were analysed for comparisons between clinical groups and cerebellar cortical layers by two-way ANOVA. There was a significant difference between groups and cortical layers [$F(3,63) = 15.94$, $P = 0.000$, and $F(2,63) = 58.42$, $P = 0.000$, respectively]. *Significant differences (derived from *post hoc* analysis using Fisher's pair-wise comparison) from normal controls ($P < 0.05$). In the control group, the granule cell layer binding was significantly lower than that in other layers ($P < 0.05$).

Table 4 α-*Bungarotoxin binding*

Clinical diagnosis	Granule cell layer	Purkinje layer	Molecular layer
Control	1.34 ± 1.94 (5)	3.64 ± 3.29 (5)	2.83 ± 2.63 (5)
Autism*	7.71 ± 4.93 (6)	10.48 ± 6.24 (6)	7.40 ± 4.90 (6)
Down's syndrome	4.65 ± 1.27 (3)	7.65 ± 2.74 (3)	5.92 ± 2.34 (3)
Mental retardation (other)	4.63 ± 3.25 (8)	8.70 ± 4.57 (8)	6.85 ± 6.08 (8)

Specific binding of 1 nM [^{125}I]α-BT in fmol/mg tissue equivalents is expressed as the mean ± SD; number of cases in parentheses. Data were analysed for comparisons between clinical groups and cerebellar cortical layers by two-way ANOVA. There was a significant difference between groups [$F(3,54) = 6.22$, $P = 0.001$]. *The autistic group was significantly increased compared with control (*post hoc* analysis, $P < 0.05$).

Table 5 *Muscarinic M1 (pirenzepine) receptor binding*

Clinical diagnosis	Cerebellar cortex
Control	0.51 ± 0.30 (8)
Autism	1.54 ± 1.78 (6)
Down's syndrome	0.75 ± 0.26 (2)
Mental retardation (other)	0.46 ± 0.32 (8)

Binding in fmol/mg tissue equivalents is expressed as the mean ± SD; number of cases in parentheses.

compared with the normal control group in all layers (Table 3). In the other disease groups, this nicotinic receptor was significantly reduced in granule cell and Purkinje layers (Table 3). In contrast, α-BT binding tended to be elevated in the autism compared with the control group, with a significant 3-fold increase in the granule cell layer (Table 4). This receptor increase was not apparent to the same extent in the other mental retardation groups (Table 4). There were no significant changes in M1 or M2 muscarinic receptor binding in autism compared with normal control and other groups (Tables 5 and 6), although there was a trend towards increased M1 in autism.

Immunochemistry

Since nicotinic receptor abnormalities were evident from ligand binding, receptor subunit immunoreactivities were also investigated. Based on western blotting, there were selective subunit changes, α4 being significantly reduced and α7 increased, though not significantly (Table 7).

Immunohistochemically, α4 nAChR immunoreactivity was present throughout the control cerebellar cortex (Fig. 3A). Diffuse immunoreactivity was apparent in the neuropil of the molecular layer and granule cell layers. Stellate cells of the molecular layer, occasional large cell bodies—possibly Golgi cells—in the granule cell layer and a small proportion of granule cell soma were α4 immunoreactive. α4 immunoreactivity in Purkinje cells extended into branching apical dendrites. Strong coarse granular α4 immunoreactivity was present in the neuropil surrounding the Purkinje cells and also in and between neurones of the deep cerebellar (fastigial and dentate) nuclei. In both autistic cases, α4 immunoreactivity was decreased in all layers of certain areas of the cerebellar cortex and this was particularly noticeable in the culmen and declive of the cerebellar vermis (Fig. 3C). In these lobes, α4 immunoreactivity was lost from the neuropil of the molecular and granule cell layers. Stellate and granule cell soma appeared to be decreased and granular immunoreactivity was markedly reduced in the granule cell and Purkinje cell layers. α4 immunoreactivity in the deep cerebellar nuclei appeared unaffected.

Diffuse α7 nAChR subunit immunoreactivity was present throughout the layers of the cerebellar cortex and the deep white matter of control sections (Fig. 3B). Astrocytes and astrocytic fibres in the deep white matter and molecular layer were immunoreactive for α7, as were stellate cells in the molecular layer, Golgi cells in the granule cell layer and a small subset of granule cells. Purkinje cells showed variable

Table 6 *Muscarinic M2 (AFDX-384) receptor binding*

Clinical diagnosis	Granule cell layer	Purkinje layer	Molecular layer
Control	2.73 ± 2.31 (8)	6.85 ± 3.07 (8)	4.19 ± 2.17 (8)
Autism	3.16 ± 1.31 (5)	6.03 ± 1.24 (6)	4.07 ± 1.65 (6)
Down's syndrome	2.63 ± 0.48 (3)	7.72 ± 1.23 (3)	6.02 ± 0.92 (3)
Mental retardation (other)	1.90 ± 1.08 (8)	5.88 ± 2.12 (8)	4.17 ± 2.02 (8)

Binding in fmol/mg tissue equivalents expressed as mean ± SD; number of cases in parentheses. While there were no significant differences between clinical groups, in the control group binding in the Purkinje layer was higher than in the other layers ($P < 0.05$) and binding in the molecular layer was higher than in the granule cell layer ($P < 0.05$).

Table 7 *Nicotinic receptor subunit immunochemistry*

Clinical diagnosis	α4 subunit	α7 subunit
Control	100.00 ± 27.12 (5)	100.00 ± 52.82 (5)
Autism	67.29 ± 10.86 (5)*	312.29 ± 376.73 (5)

Mean ± SD integrated optical density for anti-α4 and anti-α7 antibody immunoreactivity signals. Values are expressed as a percentage of average value of control cases. *$P < 0.05$, compared with the control group.

α7 immunoreactivity sometimes extending into apical dendrites. Granular immunoreactivity was present in the neuropil surrounding the Purkinje cells and between α7 immunoreactive neurones in the deep cerebellar nuclei. Foci of α7 immunoreactivity were noted occasionally along the edges of blood vessels. In the autistic cases, there was a reduction in diffuse α7 immunoreactivity throughout the layers of the cerebellar cortex which was more evident in peripheral than central areas (Fig. 3D). Purkinje cells and stellate cells also showed decreased immunoreactivity. In contrast, granule cells in certain areas of the autistic sections showed an increase in α7 immunoreactivity. α7 immunoreactive astrocytes and astrocytic fibres were present in the autistic cases, and focal areas of α7 immunoreactivity were present along the outer layer of blood vessels as in the controls. As with α4 immunoreactivity, α7 immunoreactivity in the deep cerebellar nuclei appeared unaffected.

Neurohistology

One autistic case, in which epilepsy was present, showed severe Purkinje cell loss on folial crowns of central and culmen lobules of the cerebellar vermis (lobules II–V). The loss was present to a lesser degree on the folial crown of the declive (lobule VI). All these affected areas also showed partial loss of granule cells, focal thinning of the molecular layer, Bergmann gliosis at the Purkinje cell layer, and molecular layer glial clusters. The depth of the folia in these lobules showed a patchy loss of Purkinje cells, and some of the surviving Purkinje cells showed signs of acute ischaemia. Tuber and pyramis (lobules VII and VIII) showed patchy Purkinje cell loss only, affecting both superficial and deep folia. The uvular–nodular junction (lobules IX and X) showed a narrowed molecular layer, possibly of ischaemic origin, and distorted folia, possibly of developmental origin, characterized by focal absence of granule and Purkinje cells, and vascular prominence. In the cerebellar hemispheres, extensive Purkinje cell loss, and thinning and irregularity of the molecular layer was present in both the crown and depth of folia of the lateral part of the inferior semilunar lobule, with focal Purkinje cell loss only elsewhere. In the other case, with no evidence of epilepsy, focal Purkinje cell loss and ischaemic changes (eosinophilia) were present in the central lobule, culmen and declive (lobules II–VI) of the cerebellar vermis. Both these features were less prominent in the other lobules. Moderate granule cell loss was evident in regions adjacent to the pericentral white matter of the culmen, declive and uvular–nodular junction (lobules IX and X). White matter showed focal demyelination and unusual thinning. The superior side of the cerebellar hemisphere showed less Purkinje cell loss and only scattered ischaemic neurones.

In the hippocampal CA1 subfield, which was examined in relation to the question of whether neurone loss in the cerebellum is related to epilepsy, the pyramidal cell population was unaffected in both cases.

Discussion

Abnormalities in nicotinic receptor binding

In keeping with previous neurochemical data obtained in the cerebral cortex of autistic cases (Perry *et al*., 2001), in the cerebellum there was no change in ChAT or M2 receptor binding but a significant reduction in high affinity nicotinic receptor binding. As discussed in relation to cortical findings (Perry *et al*., 2001), the loss of this nAChR in the cerebellum may reflect synaptic abnormalities common to several developmental disorders including autism. Although tobacco use is associated with elevated high affinity receptor sites in most areas including cerebellum (Court *et al*., 1998), it is unlikely that the reduction in binding in autism is accounted for by differential tobacco use between the groups, and also the receptor is unchanged in basal forebrain in autistic cases (Perry *et al*., 2001).

In contrast to the parietal cortex in which M1 muscarinic receptor binding was significantly and moderately reduced, in

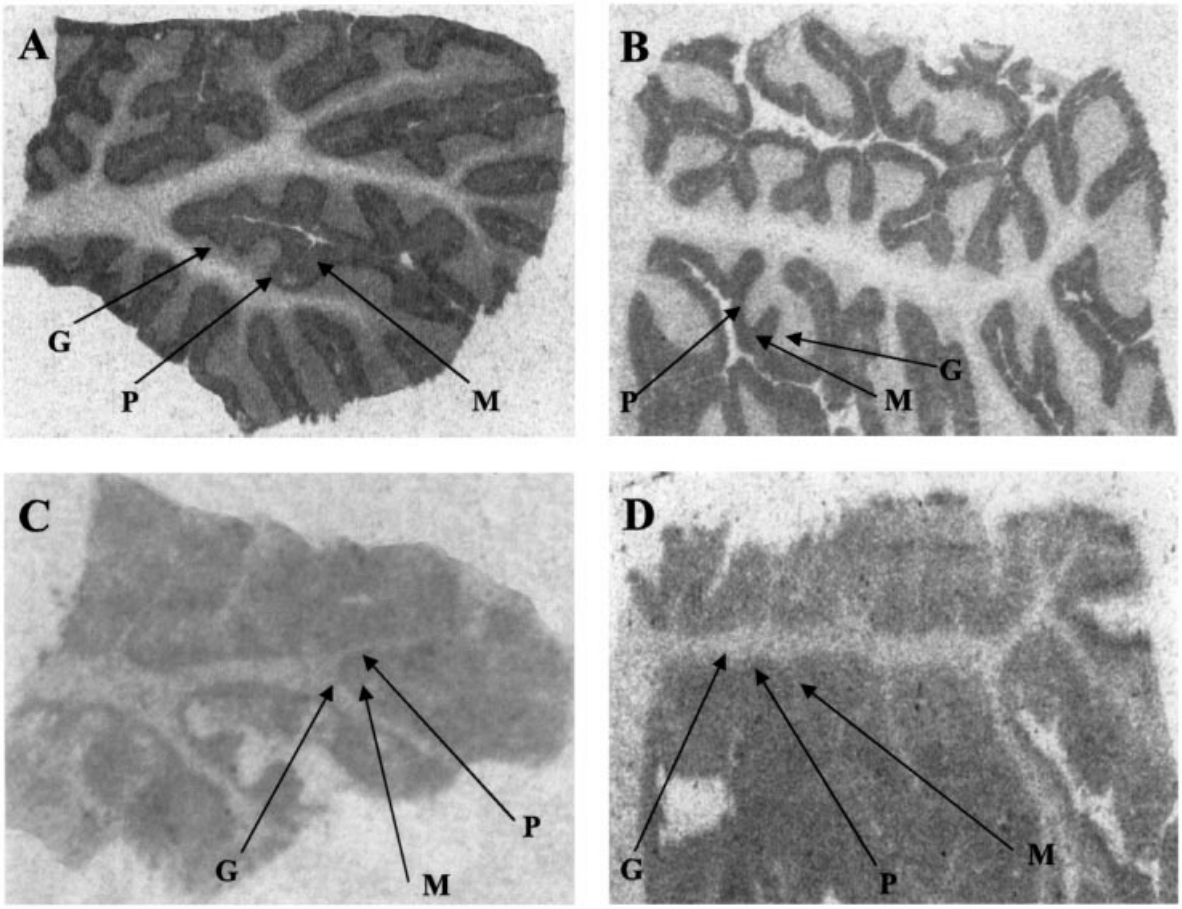

Fig. 3 Autoradiographs of (**A** and **B**) epibatidine binding and (**C** and **D**) α-bungarotoxin binding, demonstrating the loss of epibatidine and increase in α-bungarotoxin binding in (**B** and **D**) autistic compared with (**A** and **C**) control cases. M, P and G are the molecular, Purkinje and granule cell layers, respectively.

the cerebellum there was a non-significant elevation in M1 binding. The M1 subtype, however, is expressed to only a very small extent in cerebellar compared with cerebral cortex. Since this receptor is expressed to a greater extent neonatally in areas including the cerebellum (Zhu *et al*., 1998), it is possible that its decline post-natally is attenuated in autism.

α-BT binding was elevated in the autism cases, but not in the other mental retardation group. This abnormality is of interest since the gene encoding this subunit is located close to q11–15 on chromosome 15 (Chini *et al*., 1994) near to the portion of this chromosome associated with abnormalities in autism (Lamb *et al*., 2000).

Nicotinic receptor immunoreactivities

The nicotinic receptor binding abnormalities were paralleled by changes in the respective subunits α4 and α7 assessed by western blotting of frozen tissue extracts. The changes in α4 immunoreactivity and [^{3}H]epibatidine binding were similar (a 50 and 30% reduction, respectively), as were the increases in α7 immunoreactivity (3-fold) and α-BT binding (3–5-fold). This suggests that the abnormality in both nicotinic receptors may be related primarily to functional membrane-bound receptor proteins as opposed to cytoplasmic receptor pro-forms.

There was a reduction in both α4 and α7 immunoreactivity in the Purkinje cells in the two cases examined. In both of these cases, a loss of Purkinje cells was evident histologically. Whether the α4 receptor subunit reduction is a cause or consequence of the Purkinje cell loss remains to be determined. The apparent increase in α7 immunoreactivity in the granule layer may reflect compensatory upregulation during development.

Abnormalities in cerebellar neurohistology

In the present study, Purkinje cell loss was found in the cerebellar vermis (lobules II–VI, and lobules IX and X) and the hemisphere of the two mentally retarded autistic cases examined histologically. It was more severe in the case with a history of epilepsy, indicating that epilepsy could account for some though not all of the Purkinje cell loss. The pyramidal

cell population in the hippocampal CA1 subfield, which frequently shows neurone loss in epilepsy (Honavar and Meldrum, 1997), was unaffected in either of the two cases. These findings are consistent with previous neuropathological studies which demonstrated Purkinje cell loss in cerebellar vermis and hemisphere of the majority of mentally retarded autistic cases examined (Ritvo *et al*., 1986; Bailey *et al*., 1998; Kemper and Bauman, 1998). However, it is unclear whether the Purkinje cell loss found in this and previous studies might underlie the autistic behaviour or rather be associated with mental retardation. Most reports have included mentally retarded autistic cases; however, reduced numbers of Purkinje neurones have also been reported in isolated high functioning cases (Ritvo *et al*., 1986; Kemper and Bauman, 1998).

The loss of Purkinje cells combined with loss of granule cells and narrowing of the molecular layer and white matter in the cerebellar vermis and hemispheres of the two mentally retarded autistic cases in this study suggests that cerebellar hypoplasia would be demonstrable if MRI was available. Previous MRI studies of both autistic and non-autistic individuals provided evidence for strong positive association between IQ and the size of the cerebellar vermal lobules VI–VII (Piven *et al*., 1992, 1997; Ciesielski *et al*., 1994; Levitt *et al*., 1999). It has been reported that about three-quarters of autistic cases are known to be mentally retarded (Lockyer and Rutter, 1969). Courchesne *et al*. (1994*a*) have reanalysed MRI data of cerebellar vermal area measurements from several previously published studies, and have found that between 84 and 92% of autistic patients fall into the hypoplastic subtype and between 8 and 16% of patients fall into the hyperplastic subtype. Although direct evidence is not available, it is tempting to speculate that the autistic patients with cerebellar hypoplasia might represent the mentally retarded subgroup, and the autistic patients with cerebellar hyperplasia the high functioning subgroup.

Implications for cerebellar development

Neurogenesis in the cerebellum occurs at approximately the fifth week of gestation, a period which may represent a 'window' of vulnerability for autism (Courchesne, 1997). Pre-natal exposure of rats to valproic acid results in significantly fewer Purkinje cells in the embryonic cerebellar vermis (Ingram *et al*., 2000). From other studies in developing rat cerebellum, it is apparent that an increase in Purkinje cell α7 immunoreactivity coincides with major developmental synaptogenic events (Dominguez del Toro *et al*., 1997). It has also been suggested that the α7 subunit mediates developmental plasticity in the chick cerebellum (Kaneko *et al*., 1998). To investigate further the role of nicotinic receptors in cerebellar abnormalities in autism, it would be worth examining the status of the receptors in the brains of younger individuals.

Functional implications of a cerebellar cholinergic abnormality

The absence of a reduction in cerebellar ChAT suggests that presynaptic cholinergic activity is normal in autism. It cannot, however, necessarily be deduced from the overall normal ChAT biochemical activity in autism that there are not derangements in a subpopulation of this cholinergic innervation. Given the complexity of cholinergic innervation of the cerebellum, including, in addition to inputs from different brainstem nuclei, intrinsic neurones, immunohistochemical studies are required to resolve this issue. It is interesting that in the developmental disorder Rett syndrome, ChAT is reduced in the cerebellum as in other areas (Wenk *et al*., 1993). In another disorder involving cerebellar dysfunction, olivo-pontocerebellar atrophy, presynaptic cholinergic activities are also affected, shown by a 47% reduction in acetylcholinesterase (Kish *et al*., 1989). If it can therefore be assumed that presynaptic cholinergic structures are intact in autism, the nicotinic receptor changes evident in autism are likely to reflect abnormalities in cholinoceptive neurones or in non-cholinergic presynaptic structures.

Nicotinic receptors govern the release of other transmitters such as GABA (γ-aminobutyric acid) or glutamate. The presence of both α4 and α7 subunits on Purkinje cells and their dendrites in normal adult cerebellum is consistent with localization of nAChRs on this GABA-ergic neuronal population. Punctate immunoreactivity in both Purkinje and granule cell layers suggests that a proportion of the receptors in the cerebellar cortex are located presynaptically, modulating the release of, for example, glutamate from mossy fibres (Didier *et al*., 1995). The alterations in α4 and α7 subunits observed in autism are thus likely to result in alterations in such types of non-cholinergic neurotransmission.

In terms of the contribution of cholinergic cerebellar abnormalities to mental function, early reports of cerebellar abnormalities in autism (Courchesne *et al*., 1988) and of intellectual and behavioural abnormalities in patients with cerebellar damage (Botez *et al*., 1989) originally suggested a cognitive role for the cerebellum. Since then, many studies have confirmed that the cerebellum contributes to cognitive and other non-motor functions. There is thus increasing evidence that the cerebellum is involved in cognition (Allin *et al*., 2001), behaviour and emotion (Schmahmann and Sherman, 1998). Numerous multimodal cortical areas transmit information to the cerebellum via the corticopontocerebellar system, while the cerebellar corticonuclear projection facilitates deep cerebellar nuclear transmission to the thalamus (via the red nucleus) (Schmahmann, 1994). Courchesne *et al*. (1994*b*) suggested that cerebellar maldevelopment in autism may contribute to an inability to execute rapid attention shifts, and Harris *et al*. (1999) suggested that it might contribute to slowed orienting of attention. Riva and Giorgi (2000) reported that removal of vermal tumours in children is associated with behavioural disturbances ranging from irritability to behaviours reminis-

cent of autism. Deficits in spatial learning have been observed in mutant mice with cerebellar damage (Botez, 1993). In rats, early post-natal midline cerebellum lesions are associated with autistic-like symptoms including persevering behaviour, lack of attention to environmental distractors and disinhibition in anxiety and social discrimination tests (Bobee *et al*., 2000). Pierce and Courchesne (2001) demonstrated that behavioural defects based on such animal models are evident in young autistic patients, the degree of behavioural impairment being correlated with the extent of cerebellar structural abnormality. Townsend *et al*. (1999) reported abnormalities in visuospatial attention in both autistic patients and non-autistic patients with tumour- or stroke-related cerebellar damage, and later suggested (Townsend *et al*., 2001) that event-related electrophysiological response abnormalities reflect spatial attention deficits in autism that may involve cerebellar influence on frontal and spatial attention function.

The implication of cerebellar dysfunction in attentional deficits, and evidence that attention involves distinct cerebellar regions based on functional MRI (Allen *et al*., 1997), together with the present finding of nicotinic receptor abnormalities in autism, is of particular interest. The cholinergic system has long been implicated in attention (reviewed in Perry *et al*., 1999; Sarter and Bruno, 2000). In view of the specific role of the nicotinic receptor in attention (Mirza and Stolerman, 2000), it may be worth considering the effects of nicotine administration (e.g. as patches) on attentional performance in autism.

Conclusion

In conclusion, the present findings of nAChR α4 and α7 subunit abnormalities in the cerebellum suggest a role for these receptors in the neurodevelopmental pathological process in autism and in the aetiology of particular clinical symptoms.

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