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Contribution of olivofloccular circuitry developmental defects to atypical gaze in autism

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Abstract

Individuals with autism demonstrate atypical gaze, impairments in smooth pursuit, altered movement perception and deficits in facial perception. The olivofloccular neuronal circuit is a major contributor to eye movement control. This study of the cerebellum in 12 autistic and 10 control subjects revealed dysplastic changes in the flocculus of eight autistic (67%) and two control (20%) subjects. Defects of the oculomotor system, including avoidance of eye contact and poor or no eye contact, were reported in 88% of autistic subjects with postmortem-detected floccular dysplasia. Focal disorganization of the flocculus cytoarchitecture with deficit, altered morphology, and spatial disorientation of Purkinje cells (PCs); deficit and abnormalities of granule, basket, stellate and unipolar brush cells; and structural defects and abnormal orientation of Bergmann glia are indicators of profound disruption of flocculus circuitry in a dysplastic area. The average volume of PCs was 26% less in the dysplastic region than in the unaffected region of the flocculus ($p < 0.01$) in autistic subjects. Moreover, the average volume of PCs in the entire cerebellum was 25% less in the autistic subjects than in the control subjects ($p < 0.001$). Findings from this study and a parallel study of the inferior olive (IO) suggest that focal floccular dysplasia combined with IO neurons and PC developmental defects may contribute to oculomotor system dysfunction and atypical gaze in autistic subjects.

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Keywords

Autism; Atypical gaze; Cerebellum; Flocculus; Dysplasia

1. Introduction

Autism is a neurodevelopmental disorder characterized by deficits in social interactions and verbal and non-verbal communication, and restricted, repetitive behaviors (American Psychiatric Association, 2000). Among the most striking features of the social impairments in autism are deficits in coordinating visual attention with others. Making eye contact is one of the most fundamental aspects of human social behavior (Dawson et al., 2002). Face-to-face interactions and mutual gaze establish the first social relations and may be the driving force behind infants' interest in human faces long before the acquirement of vocal language (Gliga and Csibra, 2007; Hoehl et al., 2009). In triadic person-object-person interactions, eye gaze indicates another person's focus of attention and guides learning in the first months after birth (Striano and Reid, 2006). Numerous studies reveal abnormalities in processing information from the eyes (Johnson et al., 2005), with numerous implications for autistic subjects' social development (review by Hoehl et al., 2009). However, active avoidance of eye contact, poor eye contact or lack of eye contact reported very early in infancy appears to be the primary abnormality that is most likely caused by developmental defects within infant olivofloccular neuronal networks.

1.1. Atypical gaze in autism

Eye gaze-processing impairments appear early in the development of children with autism (Dawson et al., 1998; Mundy et al., 1986). Even high-functioning individuals with autism exhibit deficits in performing tasks involving mental inferences from viewing expression in the eyes (Baron-Cohen et al., 2001). Gaze-processing deficits result from impairment in using gaze to understand the intentions and mental states of other people (Baron-Cohen, 1995, 1999, 2001; Leekam et al., 1998, 2000). Children with autism demonstrate "atypical" gaze and frequently have stereotypies including eye-pressing and light-gazing. Several studies have revealed impairments in smooth visual pursuit in autism (Rosenhall et al., 1988; Scharre and Creedon, 1992; Takarae et al., 2004). Abnormalities of the oculomotor system, including atypical optokinetic nystagmus, gaze avoidance and stereotypic behaviors related to eyes, were reported in 91% of 34 children diagnosed with autism (Scharre and Creedon, 1992). A study of 60 high-functioning individuals with autism revealed pursuit eye movement deficits (Takarae et al., 2004). The subjects had a reduced closed-loop pursuit gain when tracking both oscillating and ramp targets. More apparent deficits after mid-adolescence suggest reduced maturational development of the pursuit system in autism. Bilateral disturbances in the ability to use internally generated extraretinal signals for closed-loop pursuit suggest defects in the frontostriatal or cerebellar circuitry. An fMRI study using saccadic and pursuit eye movement paradigms revealed reduced activation in cortical eye fields and cerebellar hemispheres in autistic subjects (Takarae et al., 2007).

1.2. The role of flocculus in eye movement control

Experimental studies indicate that the cerebellar flocculus is the part of the oculomotor system involved in both the olivo-cerebellar circuit and the vestibuloocular reflex arc. The flocculus exerts a specific inhibitory modulation of both excitatory and inhibitory branches of the vestibulo-ocular reflex pathways to the extraocular muscles. This modulation is mediated through inhibitory projections of floccular Purkinje cells (PCs) to the vestibular nuclei neurons, described as flocculus target neurons (reviewed by Du Lac et al., 1995; Sato and Kawasaki, 1991). The flocculus is also a region of vestibular afferent signal

convergence, through the input of mossy fibers from the vestibular nuclei, and of visual signals received via the climbing fibers of the inferior olive (IO) (Sato and Kawasaki, 1991).

A substantial number of PCs in the flocculus of the monkey receive converging visual inputs from functionally distinct portions of the retina and are involved in oculomotor control during slow eye movements. The flocculus provides the oculomotor system with eye position information during fixation and with velocity information during smooth pursuit and participates in the control of oculomotor functions (Noda and Suzuki, 1979; Zee et al., 1981). Velocity plays a stronger role (64%) than position (36%) in determining firing rate modulation during circular pursuit (Leung et al., 2000). The majority of visual PCs (85%) are identified as horizontal gaze-velocity neurons. The presence of position and velocity signals in the flocculus and paraflocculus suggests that these two cerebellar regions generate the position and velocity signal that are used to control eye motion (Leung et al., 2000). Moreover, PCs in the flocculus help to coordinate eye and head movements during active gaze shifts by modulating the vestibulo-ocular reflex (Belton and McCrea, 1999).

1.3. Olivo-cerebellar system

From a movement perspective, the olivo-cerebellar system has been considered to be involved in motor learning (Hesslow and Yeo, 2002; Highstein et al., 2005; Ito, 2001) and in the timing of motor execution (Lang et al., 1999). The olivo-cerebellar system is involved in both somatomotor and oculomotor cerebellar control. Electrophysiological studies have revealed that neurons in the dorsal cap of Kooy and the adjacent ventrolateral outgrowth of the IO control eye movements via their climbing fiber projections to the cerebellar flocculus, whereas neurons in the principle olive are involved in control of limb and digit movements via their climbing fiber projections to the lateral part of the cerebellar hemisphere (reviewed by Ito, 1984 and Voogd and Bigaré, 1980).

Neuropathological studies indicate that the oculomotor circuit is often developmentally affected in individuals with autism. Our study of serial sections of the brain revealed multiregional dysregulation of cerebellum development in nine of 13 (69%) subjects with autism. The presence of cerebellar flocculonodular dysplasia in six autistic subjects (46%), focal dysplasia in the vermis of one subject, heterotopia in one case and focal cerebellar hypoplasia in one subject reflected a high susceptibility of the cerebellum to developmental defects (Wegiel et al., 2010). The presence of olivary dysplasia in three of five subjects with autism and of ectopic neurons related to the olivary complex in two cases reported by Bailey et al. (1998) suggests that both components of the oculomotor circuitry are prone to developmental defects.

The aim of this study was to detect defects of the olivocerebellar circuitry that may be responsible for the altered oculomotor activity, atypical gaze, impairments in smooth pursuit, altered movement perception and deficits in facial perception observed in autism. The main target of this study is the flocculus, known to be involved in oculomotor circuitry.

2. Results

Postmortem study of the brain of 12 autistic subjects and 10 control subjects revealed dysplasia of the flocculus (Fig. 1a and b) in eight autistic (67%) and two control (20%) subjects (Table 1). Clinical records revealed poor, very poor or no eye contact in seven of the eight subjects (88%) with floccular dysplasia (Table 2). Clinical records also revealed difficulties in establishing and maintaining eye contact in two autistic subjects without signs of floccular dysplasia in the examined cerebellar sections; however, histopathological evaluation was limited because of the loss of part of the flocculus during brain removal. In one case with floccular dysplasia, clinical records were not available. The other factor

limiting correlations between floccular pathology and eye contact deficiency is examination of only one cerebellar hemisphere. The partial loss of flocculus tissue noted in three other cases with floccular dysplasia results in downgrading of the percentage of dysplastic area. Therefore, only the presence of floccular dysplasia in eight cases (seven with clinical records of eye contact deficit, and one without records) is unbiased. The limited clinical records of the control subjects do not contain any reference to altered behavior or eye contact deficit. Therefore, the impact on eye contact of the very small and moderate-size floccular dysplasia observed in the two control subjects cannot be determined. Dysplasia was also found in the nodulus, but abnormalities were equally common in subjects with autism (9/12; 75%) and controls (8/10; 80%), and the affected area was smaller than in the flocculus (Fig. 1c).

2.1. Quantitative characteristics of the flocculus and PCs

The average volume of the flocculus was greater in the subjects with autism (226 mm^3) than in the controls (179 mm^3 ; $p < 0.002$) (Table 1). The average volume of the dysplastic area in eight subjects with autism was greater (7.2 mm^3) than in two controls (4.4 mm^3). Significant inter-individual differences in the volume of the dysplastic area were observed among eight subjects with autism (from 0.2 to 17.3 mm^3) and in two controls (1.6 and 7.3 mm^3). Dysplastic changes modified proportions between the major flocculus cytoarchitectonic subdivisions. There was a much smaller contribution of the molecular layer (29%) in the dysplastic than in the unaffected flocculus (40%), but a larger-volume contribution of the granule cell layer (58%) in the dysplastic flocculus than in the unaffected area (46%). However, contributions of the white matter were almost the same in the dysplastic (13%) and the unaffected (14%) area.

Application of the Nucleator revealed that the volume of the PCs in eight subjects with autism was reduced in the dysplastic area by 24% ($6240 \mu\text{m}^3$) in comparison to the non-dysplastic area ($8230 \mu\text{m}^3$) ($p < 0.01$). In two control subjects with dysplastic changes, the difference between the volume of PCs in the dysplastic ($7477 \mu\text{m}^3$) and the non-dysplastic ($9367 \mu\text{m}^3$) areas was 20% ($p < 0.01$). A broader pattern of cerebellar developmental alterations is reflected in the 26% smaller volume of the PCs' soma in the entire cerebellum in seven subjects with autism ($8382 \mu\text{m}^3$) than in seven control subjects ($11,268 \mu\text{m}^3$) ($p < 0.001$) (celloidin protocol only).

2.2. Topography and morphology of floccular dysplasia in autism

Dysplastic changes were observed in the most rostral portion of the flocculus (Fig. 1a and b). There was profound distortion of the granule, molecular and PC layers in the dysplastic portion of the flocculus. Granule cells did not form a layer, but instead, islands and a labyrinth of thin, irregular-shaped cellular septa. The granule layer was mixed with an altered molecular layer and small islands of white matter. PCs were sparse and were dispersed in an abnormal granule cell and molecular layer, and in the white matter. Dysplastic changes were also detected in the most rostral portion of the nodulus, but the size of dysplasia was smaller than in the flocculus (Fig. 1c).

In the unaffected portion of the flocculus, immunocyto-chemistry using anti-calbindin mouse mAb D-28 revealed that the PCs' dendritic tree (Fig. 2a) was the major factor determining the volume and structure of the molecular layer. Parallel bundles of PCs' axons are an integral component of the cytoarchitecture of granule cell layer and white matter (Fig. 2b). The dysplastic area was characterized by the loss of cortical lamination, and the loss of neurons' spatial orientation and their morphological characteristics limiting or making impossible subclassification of such large neurons as Purkinje cells, and Golgi and Lugaro

neurons (Fig. 2c–f). Striking deficits in PCs in dysplastic flocculus coexist with defects of axons and deficits of dendritic arborization (Fig. 2e).

Immunostaining with anti-calbindin (Fig. 2) and especially with anti-parvalbumin antibodies (Fig. 3) revealed not only severe dendritic abnormalities but also severe alterations of the PC axons in the dysplastic areas. Almost all axons showed alternative thickening and thinning, giving the axons a bead-like morphology. Moreover, immunostaining with anti-parvalbumin antibody revealed almost a complete lack of basket and stellate neurons in the developmentally distorted molecular layer in the dysplastic flocculus (Fig. 3b).

In control subjects, the study of distribution of calretinin-positive unipolar brush cells (UBCs) revealed a typically low number of UBCs in all lobules except the flocculus, with an increased number of UBCs (Fig. 4a–d). However, in the dysplastic area of the flocculus of autistic subjects, the number of calretinin-positive UBCs was reduced. In many islands of loosely arranged granule cells, UBCs were absent. The presence of calretinin-positive fibers several times thicker than normal suggests developmental abnormalities or dystrophic alterations (Fig. 4f).

Severe alterations of the number, distribution and morphology of all types of neurons in the dysplastic portion of the flocculus were associated with total loss of typical vertical Bergmann glia fibers in the molecular layer (Fig. 5a and b). GFAP-positive cells were dispersed within the developmentally modified molecular layer, but the lack of typical topography, orientation and morphology of GFAP-positive glia makes impossible the differentiation of Bergman glia and cortical astrocytes. Another sign of dysplastic flocculus was abnormally large clusters of GFAP-positive glia (Fig. 5c).

In the dysplasia-free portions of the flocculus, a few serotonin transporter (ST51)-immunopositive axons traverse the granule cells and molecular layer (Fig. 6a). In contrast to the moderate serotonergic innervation in the unaffected part of the flocculus, the dysplastic area was characterized by a several-fold increase in the number of ST-51-positive fibers. The largest serotonergic bundles of fibers were observed on the periphery of the dysplastic areas, frequently mixed with loosely arranged cells with the morphology of granule cells (Fig. 6b). The characteristic feature of the disproportionally dense network of serotonergic fibers within the dysplastic, loosely arranged granule cell layer was a large number of varicosities (Fig. 6c).

3. Discussion

3.1. Cerebellar pathology in autism

Neuroimaging and neuropathological studies indicate that cerebellar abnormalities are one of the most consistent findings in autism (Bauman and Kemper, 1996; Courchesne et al., 2001; Kemper and Bauman, 1993; Ritvo et al., 1986; Whitney et al., 2008, 2009). MRI studies have shown smaller vermal lobules VI and VII, which are likely due to neuronal hypoplasia (Bauman and Kemper, 1996; Courchesne et al., 1989, 2001; Hashimoto et al., 1995; Kaufmann et al., 2003). Neuropathological studies revealed that the cerebellum is frequently affected by such developmental abnormalities as defects of migration with heterotopias, focal nodulus and flocculus dysplasia, other forms of vermis and cerebellar folia hypoplasia, and sporadically by focal polymicrogyria (Wegiel et al., 2010, 2012). A review by Palmen et al. (2004) noted a reduced number of PCs in 72% of the reported autism cases. Both qualitative (Kemper and Bauman, 1993) and quantitative studies (Bailey et al., 1998; Fatemi et al., 2002) revealed a regional decrease in the number of PCs and the prenatal loss of PCs (Whitney et al., 2008, 2009). The reduction by 24% in PC size in adult autistic subjects compared to the age-matched control subjects was considered by Fatemi et

al. (2002) a marker of PC atrophy. Reduction in the volume of PCs by 31% (in comparison to age matched control), of the dentate nucleus by 25% and of the IO by 14% in 4- to 8-year-old autistic subjects ($p < 0.001$) demonstrates that different components of brainstem-cerebellar circuits are affected by developmental defects and that the range of deficits is brain region-specific (Wegiel et al., submitted for publication). This broad spectrum of structural changes appears to define a high vulnerability of the cerebellum to developmental alterations, which may contribute to the autism phenotype. As reported here, the dysplastic changes in the flocculus appear to be a peculiar form of cerebellar focal developmental defect, resulting in profound local alterations of the cytoarchitecture and a disorganization of the neuronal circuits involved in eye movements.

3.2. The role of altered Bergmann glia in spatial disorganization in the dysplastic flocculus

The profound spatial disorganization of the granule cell, molecular and PC layer in the dysplastic part of the flocculus indicates that the mechanisms controlling cortical folding, neuronal migration, spatial arrangement and connectivity during flocculus development are altered in the rostral flocculus portion of the majority of individuals with autism. Neuronal migration in different domains of the cerebellar cortex is regulated by domain-specific factors. Granule cell migration from the external granular layer to the internal granule layer is guided by surface-mediated interactions with Bergmann glial fibers, which traverse the developing molecular layer (Rakic, 1971, 1981). Migration to a final destination within the granule layer is modulated in response to local environmental cues (Komuro and Rakic, 1998). The lack of parallel Bergmann fibers and the spatial disorientation of their processes in the molecular layer of the dysplastic flocculus indicate that they were not able to serve as a guide for migrating granule cells. The result is a profound defect of granule cell arrangement. This argument is supported by experimental studies of the role of Bergmann glia in cerebellar cortex development. They indicate that developmental deficits of the Bergmann glia, alterations of their distribution and the abnormal length of their processes affect cerebellar architecture and that the severity of Bergmann glia defects correlates with misplacement of cortical cells and alterations of cortical layering (Kaartinen et al., 2001; Qu and Smith, 2005; Yue et al., 2005). In rats X-radiated neonatally (Ferguson, 1996; Li et al., 2006) and rats treated with methylazoxymethanol (Lafarga et al., 1998) or cisplatin (Pisu et al., 2005), alterations of the Bergmann radial fibers result in abnormal migration and ectopic displacement of granule cells in the molecular layer.

Lack of cerebellar cortex folding within the flocculus dysplastic area might also be a sign of a regional foliation defect. The crucial event of the initial formation of cerebellar fissures is the formation of anchoring centers at the base of developing cortical fissures (Sudarov and Joyner, 2007). In the anchoring centers, a basic role is assumed by the Bergmann glial fibers that direct migration of granule cells at the base of the fissure. PCs express and release sonic hedgehog (Shh), which regulates the number of folia via regulation of granule cell proliferation in the external granule layer (Corrales et al., 2004, 2006). Engrailed2 regulates the morphogenetic clock, which normally controls the timing of genetic and cellular events that direct formation of the anchoring centers (Sudarov and Joyner, 2007). Rat studies indicate that cerebellar cortical anomalies may also occur during postnatal life. The first signs of pathology—retraction of Bergmann radial glia, distortion of their processes and lack of endfeet—are detected in 40% of rats at 10 days of life, in the stage of active granule cell proliferation and migration (Necchi et al., 2000).

3.3. UBC abnormalities in dysplastic flocculus

The flocculus belongs to cerebellar structures, with the highest numerical density of UBCs (Diño et al., 1999). The UBC-rich region extends to the nodulus and in animals to the paraflocculus. This topography is consistent with data indicating that the flocculus extends

beyond its anatomical borders to a “flocculus-like” region of the ventral paraflocculus (Tan et al., 1995). Experimental studies indicate that the flocculus and nodulus play antagonistic roles in learning and timing of eye movements (Koekkoek et al., 1997). UBCs are involved in the control of movements dependent on vestibular, proprioceptive and ocular stimuli, including vestibuloocular reflexes (VORs) controlling eye movements.

Dysplastic flocculus reveals severe deficits of calretinin-positive UBCs in autistic subjects. One may hypothesize that these developmental defects contribute to a disruption of both the structure and the function of the UBC/granule cell/ PC circuits. The UBCs are excitatory interneurons located in the granule cell layer (Diño et al., 2000; Ito, 2006; Mugnaini and Floris, 1994; Nunzi et al., 2001). The majority of UBCs have a single dendritic process terminating with a compact arrangement of short dendrites forming a brush-like structure. These cells receive glutamatergic input on the brush from an individual mossy fiber (Diño et al., 2000). Mossy fiber synapses facilitate fast and peculiarly slow currents mediated by α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and *N*-methyl-D-aspartate (NMDA) receptors (Mugnaini and Floris, 1994; Rossi et al., 1995). The UBCs identified by calretinin immunoreactivity express the glutamate receptor subunits GluR2/3, NMDAR1 and mGluR2/3. They provide a powerful network of feed-forward excitation within the cerebellar granular layer, which may amplify vestibular signals and synchronize activity in clusters of functionally related granule cells, and patches of PCs innervated by vertical projections of granule cells (Nunzi et al., 2001). Patches of synchronously active PCs reflect the activity of underlying granule cell clusters that exert a strong excitatory drive by means of the axonal branch, which terminates on the most distal portions of the PC dendritic tree (Cohen and Yarom, 1998; Gundappa-Sulur et al., 1999; Llinás, 1982). In dysplastic flocculus of autistic subjects, UBCs’ deficits may result in the loss of synchronized excitatory drive to clusters of granule cells and disruption of granule cells’ drive for patches of PCs.

3.4. Deficit of inhibitory interneurons: basket and stellate cells

PCs’ function is also dependent upon their innervation by the GABAergic parvalbumin-positive basket cells and stellate cells in the molecular layer. Basket cells are located in the lower one-third of the molecular layer, and their axons contribute to the plexus surrounding PCs’ soma (Sultan and Bower, 1998). Stellate cells, located in the upper two-thirds of the molecular layer, innervate PC dendrites (Palay and Chan-Palay, 1974). These two types of interneurons receive excitatory input from granule cell parallel fibers and inhibitory input from the Lugaro cells residing in the granule cell layer (Laine and Axelrad, 1998) and other basket and stellate cells (Palay and Chan-Palay, 1974). Inhibitory innervation by basket and stellate cells plays an important role in PC function; however, the reduction of PC numbers observed in the cerebellum of individuals with autism (Whitney et al., 2008) does not affect the density of basket or stellate cells detected by immunohistochemistry for parvalbumin (Whitney et al., 2009). The deficit of basket and stellate neurons observed in the dysplastic flocculus suggests focal defects of the inhibitory system in the olivofloccular circuitry in the majority of individuals with autism.

3.5. Abnormal serotonergic innervation of dysplastic flocculus

Serotonergic hyper-innervation in focal cortical dysplasia indicates that developmental brain epileptogenic lesions are associated both with abnormal cytoarchitecture and altered neuronal circuitry, including focal alterations of the serotonergic fibers (Trottier et al., 1996). One may assume that focal serotonergic hyper-innervation in the dysplastic flocculus is a response to defective development of a portion of the flocculus and expresses the sprouting of serotonergic fibers to build connections with missing PCs and basket, stellate and granule cells.

The cerebellar cortex receives serotonergic innervation in the form of a plexus of fine varicose fibers that do not face any postsynaptic element. Serotonin is released through volume transmission and acts as a paracrine agent (Bishop et al., 1993; Trouillas and Fuxe, 1993). The striking increase in the number of varicose fibers in the dysplastic flocculus appears to be a sign of enhanced release of serotonin in this cerebellar structure already severely affected by developmental abnormalities. The serotonin level in the cerebellar cortex is specifically and positively correlated to the level of motor activity (Mendlin et al., 1996). Because serotonin selectively excites Lugaro cells, one may expect a modification of Golgi cell inhibitory input on large populations of granule cells, and indirectly on PCs (Dieudonne and Dumoulin, 2000). The oculomotor activity abnormalities observed in autism could be the collective product of severe changes of flocculus circuits and of exposure of this dysfunctional system to excessive amounts of serotonin.

The presence of strong serotonin transporter immunoreactivity (a) within bundles of fibers passing by the flocculus and nodulus in the cerebellar pedunculi and providing serotonergic innervation to all cerebellar subdivisions, (b) in bundles of these axons encompassing the nodulus and flocculus and (c) in bundles of these axons entering in the dysplastic flocculus suggests serotonergic hyper-innervation in the flocculo-nodular region in autism. The increased number of serotonin axons in the medial forebrain bundles, ansa lenticularis, stria terminalis, temporal superior gyrus, amygdala and globus pallidus in young autistic subjects (Azmitia et al., 2011) suggests that cerebellar regional serotonergic hyper-innervation is a component of more complex developmental alterations in autism.

3.6. Complex nature of olivofloccular circuit abnormalities

Flocculus dysplasia was found in eight of 12 subjects (67%) with idiopathic autism. A similar prevalence of flocculus dysplasia (6/8; 75%) was also detected in a cohort with autism caused by chromosome 15 duplication (Wegiel et al., 2012). These findings suggest that flocculus alterations are associated with autism of a different etiology.

Clinical records revealed that in the examined autistic subjects, eye contact deficits ranged across a broad spectrum. However, the strength of clinicopathological correlations is limited by partial losses of the flocculus in some cases. Moreover, postmortem examination of one cerebellar hemisphere does not preclude the presence of developmental defects in another hemisphere. In spite of these methodological deficiencies, the active avoidance of eye contact and the poor or no eye contact reported in 88% of subjects with floccular dysplasia in the examined hemisphere support the hypothesis that flocculus dysplasia in the range of 0.1–7.6% of the flocculus volume may contribute to eye contact deficits in autism.

The lack of records regarding eye contact in the control subjects with a moderate or very small dysplastic area in the flocculus precludes interpretation of the findings in this group. An intact cerebellum is a prerequisite for optimal ocular motor performance. However, cerebellum-driven motor learning promotes adaptation and compensation for lesions. Therefore, some focal cerebellar lesions do not cause eye movement alterations that strictly correspond to the defect (Kheradmand and Zee, 2011). An example of this compensatory effect is the response to flocculus-paraflocculus lesions by a significant correction of smooth pursuit deficits by PCs in the dorsal vermis and cerebellar hemispheres (Ohki et al., 2009; Xiong et al., 2010).

3.6.1. Different role of flocculus and nodulus—A study of 147 normal infants revealed nodular and floccular dysplasia (heterotaxia) in 14% of cases (Rorke et al., 1968); however, detection of dysplasia in a small portion of the nodulus in 75% of autistic and 80% of the control subjects in our study suggests that nodular dysplasia is a common developmental defect of the nodulus.

The role of the flocculus and nodulus is significantly different. Lesion studies reveal that the injury of the flocculus/paraflocculus impairs (a) smooth tracking of a moving target, either when the head is still (smooth pursuit) or passively moving (Belton and McCrea, 1999, 2000; Zee et al., 1981); (b) gaze-holding with the eyes drifting centripetally after eccentric eye movement, resulting in a gaze-evoked nystagmus (Kheradmand and Zee, 2011; Zee et al., 1981) and (c) downbeat nystagmus, in which eyes drift up and are brought back to the fixation target by a corrective downward saccade (Kheradmand and Zee, 2011).

The main role of the interaction of the nodulus with the ventral uvula is to contribute to the control of the rotational and translational vestibular ocular reflex (Zee et al., 2002). They act upon the “low-frequency” (sustained) component of the vestibular ocular reflex via projections to a “velocity storage” mechanism within the vestibular nuclei (Kheradmand and Zee, 2011). Lesions in the nodulus/uvula alter (a) the velocity-storage mechanisms for the horizontal vestibular ocular reflexes and spatial orientation (Cohen et al., 2002; Waespe et al., 1985) and (b) the smooth pursuit and optokinetic nystagmus. However, their exact contribution to these visual-following reflexes is unclear (Kheradmand and Zee, 2011).

3.6.2. Role of inferior olive in olivofloccular circuit—Olivary dysplasia in three of five subjects with autism, ectopic neurons related to the olivary complex in two subjects with autism (Bailey et al., 1998), and reduced size of the IO neurons by 14% ($p<0.001$) (Wegiel et al., submitted for publication) suggest that IO developmental defects contribute to olivo-floccular circuit developmental defects. The flocculus is the target of the IO climbing fibers (Bastianelli, 2003; Diño et al., 1999). Experimental studies support the hypothesis that IO pathology affects PCs and that maturation of cerebellar connectivity and motor activity require a transition from multiple climbing fibers’ innervation to a single climbing fiber innervation to each PC (Chen et al., 1995). Experimental lesions of IO indicate that climbing fibers control PCs’ activity (Benedetti et al., 1984; McCormick et al., 1985). Climbing fibers transmit to PCs error signals that are detected by IO neurons as the difference between the intended and the executed movements (Thach et al., 1992). The cerebellar cortex is composed of distinct compartments, known as microzones (Ito, 1984; Oscarsson, 1979). PCs in each microzone receive common excitatory inputs from climbing fibers of distinct subgroup of IO neurons in the brainstem (Chen et al., 1995). However, the profound disorganization of the PC layer and the loss of PCs’ basic morphological features indicate total disruption of microzones’ compartmentalization within the dysplastic flocculus.

One may assume that dysplasia-related flocculus cytoarchitecture and connectivity defects are clinically expressed because they are combined with developmental defects in the IO, the second component of the olivocerebellar circuit, and with developmental alterations of PCs within the non-dysplastic part of the flocculus, vermis and cerebellar hemispheres. This hypothesis is supported by results of a study of the global pattern of neuronal growth defects (Wegiel et al., submitted for publication) demonstrating (a) a deficit in neuron volume in the IO by 14% in autistic subjects 4 to 8 years of age ($p<0.000$), and an acceleration of growth in subjects older than 8 years old, resulting in increase of the neuron volume to 9% above control ($p<0.000$) and (b) a 31% deficit of PCs’ volume in autistic subjects 4–8 years of age, and a 17% deficit in autistic subjects older than 8 years of age. Moreover, the cerebellar dentate nucleus reveals a 25% deficit in neuron volume in autistic subjects 4–8 years of age and a 13% deficit in autistic subjects older than 8 years old.

These data suggest that in autistic subjects, the complex of (a) developmental defects of the IO, (b) profound defects of PCs; basket, stellate, unipolar and granule neurons; and Bergmann glia in dysplastic area, (c) dysplasia in the nodulus, (d) reduced size of PCs in non-dysplastic portion of the flocculus and (e) reduced size of PCs in the entire cerebellum

contribute to a peculiar pattern of oculomotor system developmental alterations resulting in autistic subjects' gaze abnormalities including poor eye contact.

4. Experimental procedures

Brain hemispheric samples of 12 subjects with autism from 4 to 66 years of age, and 10 control subjects from 4 to 64 years of age were examined (Table 3). Average brain weight recorded during autopsy was 1504 g in the autistic group and 1374 in the control group ($p < 0.04$). The average interval between death and tissue fixation was 23 h in the autistic group and 16 h in the control group ($p < 0.08$). One brain hemisphere from each subject was fixed in 10% buffered formalin for on average 1005 days in the autistic group and 318 days in the control group ($p < 0.04$). The brain hemisphere was dissected into 30-mm-thick frontal slabs and was dehydrated in a graded series of ethyl alcohol for on average 32 days in the autistic group and 36 days in the control group ($p < 0.08$). Dehydration resulted in a comparable loss of weight of the hemispheric tissue sample by 46% in the autistic group and 48% in the control group ($p < 0.17$). Tissue slabs were embedded in celloidin as described (Wegiel et al., 2010) or in polyethylene glycol (PEG) as described (Wegiel et al., 2012). Seven brain hemispheres in the autistic group and seven in the control group were embedded in celloidin, whereas five brain hemispheres of the autistic group and three of the control subjects were embedded in PEG. Combining the material preserved in the celloidin and PEG protocols was necessary to have a sufficient number of cerebellar tissue samples with the flocculus and nodulus region preserved for estimation of the prevalence of dysplastic changes and the link between pathology and clinical manifestations of the oculomotor system abnormalities. The cerebellum and brainstem were cut on a sagittal plane. The results of neuropathological studies of these cohorts were reported by Wegiel et al. (2010, 2012). The global characteristics of neuronal growth abnormalities in the brain of autistic and control subjects are summarized in the report by Wegiel et al. (submitted for publication).

Tissue acquisition was based on individual tissue transfer agreements with several tissue banks, including (a) the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, (b) the Harvard Brain Tissue Resource Center and (c) the Brain and Tissue Bank for Developmental Disabilities and Aging of the New York State Institute for Basic Research in Developmental Disabilities (IBR). The Institutional Review Board at IBR approved the study. Tissue samples and case clinical records were identified with a codified brain bank number.

4.1. ADI-R and clinical characteristics

The Autism Diagnostic Interview-Revised (ADI-R) was administered to each donor family in order to confirm the diagnosis of autism on a postmortem basis. Inclusion of a case was based on a summary of scores of four domains including qualitative abnormalities in reciprocal social interaction; qualitative abnormalities in reciprocal social interaction; restricted, repetitive and stereotyped patterns of behavior; and abnormality of development evident at or before 36 months of age. Ten subjects met the ADI-R criteria for autism (Table 4). In two other cases, inclusion was based on clinical diagnosis of autism. Four autistic subjects were diagnosed with epilepsy, and the death of one patient was seizure-related. Intellectual deficit, anxiety, aggression, self-injurious behavior and hyperactivity were reported in the majority of autistic subjects. Eye contact abnormalities (poor or absent eye contact, difficult to establish and maintain eye contact, lack of typical or reciprocal eye gaze) were reported in 9/12 cases (75%). In one case, clinical records were not available, and in the other two cases, clinical records were incomplete, and it is not known whether the subjects had eye contact deficit.

4.2. Quantitative methods

To detect quantitative changes, an image analyzer with a computer-controlled automatic stage installed on an Axiophot microscope (Zeiss) and a stereology software package (MicroBrightField, Williston, VT, USA) were used.

The volume of the unaffected flocculus and the dysplastic area was determined in 12 autistic and 10 control subjects using 13 or 14 serial sections with equal distance. In both parts of the flocculus, the volume of the molecular and granule layer and white matter was estimated at $48\times$ magnification (objective lens $1.25\times$) using Stereo-Investigator (MicroBrightField). The border between subdivisions was verified at $184\times$ magnification (objective lens $5\times$). In dysplastic areas, granule layer borders were usually fuzzy as a result of loose granule cell arrangement. In affected areas, only islands of high density were delineated as a granule layer.

The volumes of the PCs' soma and nuclei were estimated in the flocculus and in the entire cerebellar cortex at $1480\times$ magnification on screen (objective lens $40\times$) using Nucleator (MicroBrightField). Nucleator in the entire cerebellar cortex was applied using from 4 to 6 equidistant serial sections using grid size $1800\times 1800\text{ }\mu\text{m}$, test frame $180\times 180\times 30\text{ }\mu\text{m}$ and a mean number of counting frames of 664/case.

PCs were distinguished from other cerebellar neurons and from glial cells using histological features in CV staining including topography, spatial orientation, shape, size, prominent nucleus and nucleolus and distinct marginal and central chromatin. In the dysplastic area, misplacement, loss of spatial orientation and reduction of PCs' body size partially impeded identification, and cells with uncertain classification were omitted. Because $200\text{-}\mu\text{m}$ -thick celloidin sections are not penetrated with antibodies and sera, the PCs' morphometry was limited to CV-stained sections. To keep one standard, the dissector depth was limited to $30\text{ }\mu\text{m}$ below the top $5\text{-}\mu\text{m}$ guard zone regardless of section thickness ($200\text{ }\mu\text{m}$ in the celloidin protocol, and $50\text{ }\mu\text{m}$ in the PEG protocol).

Five rays of Nucleator were used to estimate cell nucleus and cell body volume. The center of the Nucleator was located in the nucleolus. In the first approach, five intersections of Nucleator radii with the nucleus border were marked, and in the second approach five intersections with cell soma border were marked. The nucleus and soma volume were estimated from the lengths of nuclear and cellular segments of Nucleator radii. In the non-affected part of the flocculus, 397 PCs, and in dysplastic area, 130 PCs were measured on average. The mean CE was 0.004 in the non-affected area and 0.01 in the dysplastic area. The mean number of PCs examined in the entire cerebellar cortex was 253 with $\text{CE}<0.002$.

4.3. Immunostaining

Several antibodies were employed to detect and characterize neuronal and glial developmental alterations in the flocculus (Table 5). Anti-calbindin mouse monoclonal antibody (mAb) D-28k was used to characterize the topography and morphology of the PC soma, dendritic tree, and axons. Rabbit polyclonal anti-parvalbumin antibody was employed to characterize the morphology of the PCs, basket cells in the internal one-third of the molecular layer, and the stellate cells in the external two-thirds of the molecular layer of the cerebellar cortex. UBCs in the granule cell layer were detected with an anti-calretinin mouse mAb Ab-1. Bergmann glia and astrocytes were immunostained with a goat anti-GFAP polyclonal antibody. Projections of the raphe nuclei serotonergic neurons were identified by using mouse mAb ST51-2 detecting serotonin transporter. Anti-mouse HRP/DAB detection kit (ab64259, Abcam Inc., Cambridge, MA) was used to enhance the immunoreactivity of the serotonin transporter-positive neuronal processes.

Serial free-floating 30- μ m-thick PEG sections stored in 70% ethyl alcohol were treated in ascending concentrations of ethanol. Endogenous peroxidase was blocked with 0.5% hydrogen peroxide in absolute methanol. To block unspecific binding, sections were incubated with 10% serum in phosphate buffer solution (PBS). The primary antibodies diluted in 10% serum in PBS (as shown in Table 5) were used for sections' overnight incubation at 4 °C. The sections were washed and treated for 30 min with biotinylated species-specific secondary antibodies diluted 1:300 and then with an extravidin peroxidase (1:200) for 1 h. The reaction product was visualized with diaminobenzidine (0.5 mg/mL with 1.5% hydrogen peroxide in PBS). After immunostaining, sections were lightly counterstained with hematoxylin. Specificity was controlled on adjacent sections using the same protocol but with primary antibody.

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Fig. 1.

Topography of dysplastic changes in the flocculus and nodulus. Dysplastic changes in the rostral portion of the flocculus of a 36-year-old subject with autism (a, b). Low magnification (a) illustrates the proportions between the size of the cerebellum, flocculus (large arrowhead) and dysplastic area (small arrowhead). (b) shows the loss of spatial organization of the granule and molecular layers in the dysplastic portion (small arrowhead) of the flocculus. (c) illustrates the topography and morphology of dysplastic changes (small arrowhead) in the rostral portion of the nodulus of a 7-year-old subject with autism. 200- μ m-thick cresyl violet-stained celloidin sections.

Fig. 2.

Calbindin immunostaining. Non-dysplastic (a, b) and dysplastic (c–f) portions of the flocculus of an autistic subject immunostained with anti-calbindin D-28k mouse mAb. (a) illustrates the morphology of PCs and their dendritic tree in the molecular (M) layer in the non-dysplastic portion of the flocculus, whereas low magnification (b) shows the morphology of the PCs' axons (arrowheads) penetrating the granule (G) layer and white matter (WM). In the dysplastic part of the flocculus of the same subject (c, d), irregular islands of loosely arranged granule (G) neurons are mixed with white matter-like areas (WM) accentuated by bundles of calbindin-positive PCs axons (small arrowheads). Almost all PCs display abnormal morphology of axons, with an alternative increase and reduction of

the axon diameter (bead-like axons) (d). (e) demonstrates dendritic arborization defects of calbindin-positive PCs. (f) shows the fuzzy border of the island of granule neurons with calbindin-positive, poorly differentiated large and moderate-size neurons. This dysplastic area illustrates the profound defects of cortex cytoarchitecture, with the loss of neurons' spatial orientation, structure and connectivity.

Fig. 3. Parvalbumin immunostaining. Non-affected (a, c, e) and dysplastic (b, d, f) parts of the flocculus of an autistic subject immunostained with rabbit pAb. (a) shows the soma and dendritic tree of PCs, basket cells (small arrowhead) in the internal one-third of the molecular layer, and stellate cells (large arrowhead) in the external two-thirds of the molecular layer in the non-affected portion of the flocculus. (b) illustrates the dysplastic area with a few large, parvalbumin-positive neurons (large arrowheads) located in the fuzzy border zone between poorly defined granule (G) cells islands and abnormal molecular (M) layer devoid of PCs' dendrites, stellate and basket neurons but enriched in ectopic granule cells. (c) and (e) show the non-affected part of the flocculus with normal axons of PCs (pairs

of small arrowheads) in the granule (G) cells layer and in the subcortical white matter. (d) and (f) demonstrate axonal abnormalities with alternative thick and thin segments (bead-like axons) (pairs of arrowheads) within dysplastic granule (G) cell islands and white matter (WM).

Fig. 4. Immunostaining of unipolar brush cells (UBCs). Calretinin immunostaining in lobule II (a, b), not affected (c, d) and dysplastic (e, f) parts of the flocculus in an autistic subject. (a) shows the typical low number of UBCs in the granule cell layer in lobule II. (c) demonstrates the typical high number of UBCs in the granule cell layer in the non-affected part of the flocculus. High magnification shows calretinin-positive UBCs with a single dendritic process terminating with a compact arrangement of short brush-like dendrites in lobule II (b) and clusters of UBCs in the non-dysplastic part of the flocculus (d). Anti-calretinin immunostaining demonstrates reduction and, in some areas, absence of UBCs

(large arrowhead; e) as well as striking thickening of calretinin-positive fibers (small arrowhead; f) in dysplastic flocculus.

Fig. 5. GFAP in Bergmann glia and astrocytes. Anti-GFAP immunostaining reveals numerous straight Bergmann fibers (arrowheads) in the molecular layer in the control subject (a), whereas dysplastic flocculus of an autistic subject is characterized by the total loss of the regular arrangement of Bergmann cell bodies in the borderline between the granule cell and molecular layer, and loss of parallel Bergmann fibers within the molecular layer. GFAP-positive glial cells (arrowheads) are dispersed within the molecular layer (b) or form clusters (c), but because of morphological alterations, distinguishing Bergmann glia from cortical astrocytes is impossible.

Fig. 6. Serotonin transporter. In an autistic subject, immunostaining with ST51 revealed a moderate amount of serotonin transporter-positive fibers in the molecular and granule cell layers in the non-dysplastic part of the flocculus (a). In dysplastic regions, granule cell islands are penetrated with numerous dense bundles of serotonergic fibers (arrowhead; b). Dysplastic islands with very low numerical density of granule cells reveal disproportional massive serotonergic innervation and very numerous varicosities of ST-51-positive axons (c).

Table 1

The volume of the flocculus dysplastic area.

No.	Group	Case/brain number	Flocculus volume (mm ³)		Dysplasia: % of total flocculus volume	
			Total	Dysplastic area		
1	A	IBR-425-02	221	0.2	0.1	
2	A	ANI3961	225	11.5	5.1	
3	A	HSB-4640	217	3.6	1.7	
4	A	AN01293	174	0.0	0.0	
5	A	B-5223	287	2.3	0.8	
6	A	AN09730	240	4.2	1.7	
7	A	AN08166	303	0.0	0.0	
8	A	NP06-54	179	0.0	0.0	
9	A	ANI6961	228	17.3	7.6	
10	A	AN08105	270	12.1	4.5	
11	A	AN09714	170	0.0	0.0	
12	A	ANI2698	198	6.5	3.3	
		Average	226	7.2	3.1	
		SE	13	2.1	0.9	
1	C	AN2456	177	0.0	0.0	
2	C	UMB-4898	148	0.0	0.0	
3	C	UMB-1708	224	0.0	0.0	
4	C	UMB-1670	176	0.0	0.0	
5	C	UMB-4722	155	7.3	4.7	
6	C	UMB-1846	154	0.0	0.0	
7	C	UMB-4543	170	0.0	0.0	
8	C	UMB-1576	196	1.6	0.8	
9	C	IBR-291-00	187	0.0	0.0	
10	C	ANI1184	203	0.0	0.0	
		Average	179	4.4	2.7	
		SE	8	2.9	2.0	
		P<	0.002			

The average dysplastic region volume and percentage were calculated only for cases with dysplasia.

Table 2

Psychiatric and neurological evaluation of autistic subjects including eye contact. I

Case/ Brain number	Psychiatric and neurological evaluation	Eye contact
IBR-425-02	Regression at age of 2 years. Tantrums. Self-injurious behavior when upset. Hyperactivity. Asthma	In general, poor eye contact, but on occasion, a brief eye contact with other people. (Flocc.D.: 0.1%)
AN13961	Hypotonia. Seizures (age of onset, 14 m)	No eye contact. (Flocc.D.: 5.1%)
HSB-4640	Regression at age of 18 m Asthma from age of 2.5 years to death	Gaze directed at the objects of interest but not at people. (Flocc.D.: 1.7%)
AN01293	Moderate intellectual deficit	No records. (Flocc.D.: 0.0%)
AN11206	Severe autism. Intellectual deficit. Temper tantrums. Self-injurious behavior. Hyperactivity. Sleep disorder. Hypotonia. Abnormal gait. Allergy	No eye contact at age of 1.5 to 4 years. Poor eye contact at age from 4 to 15 years. (Flocc.D.: 0.8%)
AN09730	Tourette's syndrome, obsessive-compulsive disorder, mania. Self-injurious behavior with head-banging and tantrums. Seizures (age of onset, 15 y)	No eye contact during childhood. As adult, patient looked at people, as looking at his reflection in people's eyes. (Flocc.D.: 1.7%)
AN08166	High-functioning autism. Pervasive developmental disorder. Intermittent explosive personality disorder. Rigidity, obsessiveness. Hypotonia. Tremor Seizures (age of onset, 22 y)	Difficulty establishing and maintaining eye contact. Poor pursuit and limitation of upgaze. (Flocc.D.: 0.0%)
NP06-54	No records	No records. (Flocc.D.: 0.0%)
AN16961	Functional regression at age of 16 month. Obsessive-compulsive disorder. Anxiety. Aggression. Severe self-injurious behavior. Severe intellectual deficit. Allergy	Lack of "typical" or reciprocal eye gaze, but in adulthood sporadically a brief eye contact was noticed. (Flocc.D.: 7.6%)
AN08105	Recurrent depression. Anxiety, irritability, aggression, self-injurious behavior. Hyperactivity. Moderate intellectual deficit (IQ 48). Allergy, asthma. Hypothyroidism	Very poor eye contact (avoiding eye contact and difficulty looking people in the face). Strabismus. (Flocc.D.: 4.5%)
AN09714	Intellectual deficit. Abnormal gait (walking like "drunk"), might be related to severe hypoplasia of cerebellar lobes 1-4 affecting approximately 25% of cerebellar cortex. (Case B-7090; Wegiel et al., 2010) Seizures (age of onset, 3 y)	The only record indicates that at early infancy, child was unable to make eye contact. However, there is no record of eye contact deficit in childhood or adulthood. (Flocc.D.: 0.0%)
AN12698	Mild intellectual deficit. Abnormal gait	Clinical documentation not available. (Flocc.D.: 3.3%)

Flocc. D., the percent of the flocculus volume occupied with dysplasia.

Table 3

Tissue samples of 12 autistic and 10 control subjects, brain weight, tissue processing and embedding, cause of death.

G	Case/brain number	Sex	Age (y)	PMI (h)	Brain weight (g)	H	Fix (d)	Deh. (d)	Weight loss (%)	Emb	Cause of death
A	IBR-425-02	M	4	30	1280	R	4560	28	49	Cell	Drowning
A	AN13961	M	7	25	1610	R	330	37	55	Cell	Drowning
A	HSB-4640	M	8	14	1740	R	196	23	47	PEG	Asthma
A	AN01293	M	9	4	1690	R	1006	25	44	PEG	SUDC
A	AN11206	M	15	48	1880	R	1.733	31	-	PEG	Cardiac arrhythmia
A	AN09730	M	22	25	1375	R	1034	39	38	Cell	SUDEP
A	AN08166	M	28	43	1580	R	155	22	43	PEG	Seizure-related
A	NP06-54	M	32	-	1260	L	1139	23	42	PEG	Brain tumor
A	AN16961	M	36	24	1480	R	721	37	44	Cell	Cardiac arrest
A	AN08105	M	56	3	1570	R	692	38	52	Cell	Cardiac arrest
A	AN09714	M	60	26	1210	R	398	39	42	Cell	Pancreatic cancer
A	AN12698	M	66	13	1380	R	99	37	53	Cell	Drowning
Average			23	1504		-	1005	32	46		
SE			4	60			352	2	2		
C	AN2456	F	4	17	1530	R	126	41	49	Cell	Broncho-pneumonia
C	UMB-4898	M	7	12	1240	R	130	41	51	Cell	Drowning
C	UMB-1708	F	8	20	1222	R	650	36	51	Cell	Rejection of cardiac transplant
C	UMB-1670	M	13	5	1420	R	-	27	45	PEG	Hanging (asphyxia)
C	UMB-4722	M	14	20	1464	R	1067	38	44	PEG	Multiple trauma
C	UMB-1846	F	20	9	1340	R	245	37	52	Cell	Multiple trauma
C	UMB-4543	M	29	13	1514	R	89	41	49	Cell	Multiple trauma
C	UMB-1576	M	32	24	1364	R	460	37	42	Cell	Asphyxia-car accident
C	IBR-291-00	M	32	14	1401	R	50	21	-	PEG	Heart failure
C	AN11184	M	64	28	1250	R	52	37	51	Cell	Heart failure
Average			16	1374		-	318	36	48	-	-
SE			2	35			116	2	1		
p<			0.08	0.04			0.04	0.08	0.17		

Group (G): Autism, A; Control, C.

PMI, postmortem interval; h, hours. H-hemisphere examined (R, right; L, left).
 Fixation (Fix): duration (in days, d) of fixation in 10% buffered formalin.
 Dehydration (Deh.): Duration (in days, d) of dehydration in ethyl alcohol.
 Weight loss: decrease (%) of hemispheric brain sample weight during dehydration.
 Embedding (Emb), Celloidin (Cell), polyethylene glycol (PEG).

Table 4

Results on autism diagnostic interview – revised (ADI-R).

No.	Case/brain number	Social deficits (10)	Communication deficits		Repetitive behavior (3)
			Verbal (8)	Non-verbal (7)	
1	IBR-425-02	14	NA	10	3
2	AN13961	29	NA	14	3
3	HSB-4640	19	14	8	4
4	AN01293	26	NA	12	5
5	AN11206	29	NA	14	7
6	AN09730	28	NA	14	6
7	AN08166	22	16	NA	5
8	NP06-54	-	-	-	-
9	AN16961	23	NA	10	6
10	AN08105	19	8	13	3
11	AN09714	26	8	14	4
12	AN12698	-	-	-	-

ADI-R cut-off score shown in parentheses.

Table 5

Antibodies used for characterization of cerebellar flocculus dysplasia.

Antibody	Target protein/cells	Host	Dilution	Pretreatment	Producer/vendor
Calbindin D-28k	Purkinje cells	Mouse monoclonal	1:1000	No	Swant; Bellinzona, Switzerland
Parvalbumin	Purkinje, basket and stellate cells	Rabbit polyclonal	1:1000	No	Swant; Bellinzona, Switzerland
Calretinin Ab-1 Clone 5A5	Unipolar brush cells	Mouse monoclonal	1:100	10 min microwaving in citric acid	Thermo Fisher Scientific; Fremont, CA
GFAP	Mature Bergmann glial cells	Goat polyclonal	1:500	No	Santa Cruz Biotechnology; Santa Cruz, CA
ST51-2 Ab45525	Serotonergic projections from raphe nuclei	Mouse monoclonal	1:300	No	Abeam Inc.; Cambridge, MA