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Neurons in the fusiform gyrus are fewer and smaller in autism

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Abnormalities in face perception are a core feature of social disabilities in autism. Recent functional magnetic resonance imaging studies showed that patients with autism could perform face perception tasks. However, the fusiform gyrus (FG) and other cortical regions supporting face processing in controls are hypoactive in patients with autism. The neurobiological basis of this phenomenon is unknown. Here, we tested the hypothesis that the FG shows neuropathological alterations in autism, namely alterations in neuron density, total neuron number and mean perikaryal volume. We investigated the FG (analysing separately layers II, III, IV, V and VI), in seven post-mortem brains from patients with autism and 10 controls for volume, neuron density, total neuron number and mean perikaryal volume with high-precision design-based stereology. To determine whether these results were specific for the FG, the same analyses were also performed in the primary visual cortex and in the cortical grey matter as a whole. Compared to controls, patients with autism showed significant reductions in neuron densities in layer III, total neuron numbers in layers III, V and VI, and mean perikaryal volumes of neurons in layers V and VI in the FG. None of these alterations were found in the primary visual cortex or in the whole cerebral cortex. Although based on a relatively small sample of post-mortem brains from patients with autism and controls, the results of the present study may provide important insight about the cellular basis of abnormalities in face perception in autism.

Keywords: fusiform gyrus; design-based stereology; autism

Abbreviations: AMG = amygdala; CGM = cortical grey matter; FFA = fusiform face area; FG = fusiform gyrus; fMRI = functional magnetic resonance imaging; IFG = inferior frontal gyrus; IOG = inferior occipital gyrus; KS = Kolmogorov–Smirnov; OFC = orbitofrontal cortex; STG = superior temporal gyrus

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Introduction

Autism is a neurodevelopmental disorder with an estimated heritability of >90% (DiCicco-Bloom *et al.*, 2006). It is defined by the presence of social deficits, language abnormalities and stereotyped and repetitive behaviours (American Psychiatric Association, 1994), which are thought to be specific to autism (Bodfish *et al.*, 2000). A key feature of normal social functioning in humans is the processing of faces, which allows people to identify individuals and enables them with the capacity to understand the mental state of others (Baron-Cohen *et al.*, 1994). Although not included in the current diagnostic criteria, patients with autism have marked deficits in face processing (Grelotti *et al.*, 2002). As such, alterations of this crucial skill for social interaction may represent a central feature of social disabilities in autism (Schultz *et al.*, 2000). Imaging studies have provided evidence for a role of temporal lobe structures in face processing. It is well recognized from functional magnetic resonance imaging (fMRI) studies that the fusiform gyrus (FG) is consistently active when normal humans view faces (Kanwisher *et al.*, 1999). Patients with autism can perform face perception tasks (Schultz, 2005)

Table 1 Clinical characteristics of all cases included in this study

No	Age (years)	S	H	PMI (h)	BW (g)	Fix (days)	Th (μm)	Cause of death
AI	4	M	l	30	1160	4560	200	Drowning
A2	5	F	l	13	1390	1568	200	Car accident
A3	8	M	r	22	1570	196	200	Sarcoma
A4	11	F	l	13	1460	311	200	Seizure prior to drowning
A5	13	M	l	8	1470	75	200	Seizures
A6	21	F	r	50	1108	136	200	Obstructive pulmonary disease
A7	23	M	r	14	1610	505	200	Drowning
CI	4	M	l	3	1380	67	500	Myocardial infarct
C2	4	F	r	21	1222	233	200	Lymphocytic myocarditis
C3	7	F	r	74	1350	1290	500	Status asthmaticus
C4	14	M	r	20	1464	1067	200	Electrocution
C5	23	M	r	6	1520	95	200	Ruptured spleen
C6	25	M	r	14	1388	89	500	Cardiac tamponade
C7	48	M	l	24	1622	215	200	Atherosclerotic heart disease
C8	52	M	r	13	1450	158	200	Atherosclerotic cardiovascular disease
C9	59	M	l	24	1412	266	200	Cardiac arrest
CI0	65	M	l	19	1430	85	500	Bronchpneumonia

A = patient with autism; C = control; S = sex; M = male; F = female; H = hemisphere; l = left; r = right; PMI = post-mortem interval (time between death and autopsy); BW = brain weight; Fix = fixation time; Th = section thickness.

but there is strong evidence that the FG, as well as other cortical regions supporting face processing in controls, is hypoactive in patients with autism (Kanwisher *et al.*, 1999; Pierce *et al.*, 2001, 2004; Bolte *et al.*, 2006). However, the neurobiological basis of this phenomenon remains unknown (Palmen *et al.*, 2004*a*; Van Kooten *et al.*, 2005; DiCicco-Bloom *et al.*, 2006)

It has been proposed that the failure to make direct eye contact may explain the observed hypoactivation of the FG in face perception tasks in autism (Dalton *et al.*, 2005). Imaging studies have reported unchanged (Pierce *et al.*, 2001) or increased (Waiter *et al.*, 2004) volumes of the FG in patients with autism compared to controls, or asymmetry abnormalities of the FG in autism (i.e. larger on the left side in patients with autism) (Herbert *et al.*, 2002). It has also been suggested that an innate impairment of specialized neural systems may explain the reported functional abnormalities of the FG in autism (Sasson, 2006). Based on this evidence, we hypothesized that the FG would show neuropathological alterations at the cellular level, i.e. in neuron density, total neuron number and mean perikaryal volume in autism compared to controls. We tested this hypothesis by investigating these parameters in the FG of seven post-mortem brains from patients with autism and 10 matched controls using high-precision design-based stereology. To determine whether these results were specific for the FG in autism, we performed the same analyses on the primary visual cortex and the whole cortical grey matter (CGM) as well. It should be mentioned that a subset of the post-mortem brains investigated here (i.e. six brains from patients with autism and six from controls) were recently also investigated for possible alterations in the modular organization of cellular microdomains (minicolumns) in the pre-frontal cortex (area 9), primary motor cortex (area 4), primary sensory cortex (area S1) and primary visual cortex (area 17) (Casanova *et al.*, 2006).

Materials and Methods

Brain specimens

Post-mortem brains (one hemisphere per case) from seven patients with autism (four males, three females; mean age 12.1 ± 2.8 years; mean $\pm$ SEM) and 10 matched controls (eight males, two females; mean age 30.1 ± 7.5 years) were analysed. Clinical data and the origin of the brains are shown in Tables 1 and S1 (in Supplementary data). The patients with autism did not differ from the controls with respect to mean age [two-tailed Student's *t*-test; $t_{(15)} = 1.917$ (15 degrees of freedom)], $P = 0.07$), mean brain weight [$t_{(15)} = 0.3913$, $P = 0.70$], mean interval between death and autopsy [$t_{(15)} = 0.0423$, $P = 0.97$] and mean fixation time [$t_{(15)} = 1.296$, $P = 0.21$]. All patients with autism met the Diagnostic Statistical Manual, fourth revision (DSM-IV) (American Psychiatric Association, 1994) and Autism Diagnostic Interview (Lord *et al.*, 1994) criteria of autism, and none of them exhibited any chromosomal abnormalities. In all of the cases, autopsy was performed after informed consent had been obtained from a relative. The use of these autopsy cases for scientific investigations was approved by the relevant Institutional Review Boards. Except for the tissue provided by the Morphologic Brain Research Unit, University of Wuerzburg, Wuerzburg, Germany (UWMBRU), allocation of tissue was officially approved by the Tissue Advisory Board (TAB) of the US-Autism Tissue Program (ATP). Clinical records were available for all cases.

Tissue processing

In all cases, the brains were divided mediosagittally. Either the left or the right hemisphere was available for each case (Table 1). After immersion-fixation in 10% formalin for at least 3 months, the selected hemispheres were embedded in celloidin and cut into complete series of 200 μm thick coronal sections as previously

described (Heinsen *et al.*, 2000) (all steps were performed at the New York State Institute for Basic Research in Developmental Disabilities, Staten Island, NY, USA). Every third section was shipped to UWMBRU. The hemispheres provided by UWMBRU were cut at a thickness of 500 μm, and every other section was selected (these differences did not influence the outcome of the study). All selected sections were stained at UWMBRU with gallocyanin, mounted and coverslipped as previously described (Heinsen and Heinsen, 1991).

Brain regions

The FG, the primary visual cortex (Brodmann's area 17) (Brodmann, 1909) and the CGM were identified on all sections showing these regions, according to anatomical landmarks and cytoarchitectonic criteria (Figs 1 and 2). The fusiform face area (FFA) within the FG could not be identified separately because neither gross anatomical landmarks nor cytoarchitectonic criteria have been established in the literature to identify the FFA within the FG in human post-mortem brains. However, potential cytoarchitectonic differences in volumes of cell layers, neuron densities, total neuron numbers and mean perikaryal volumes between patients with autism and controls can be assessed by measuring these variables within the FG that encompasses the possible range of the FFA within a comparable part of the FG in each brain section showing the FG. The FG is located in the temporal lobe, lateral to the parahippocampal gyrus. Its medial boundary was defined by the collateral sulcus and its lateral boundary by the temporo-occipital sulcus, which runs anterior to posterior from the temporal pole to the occipital gyrus. The superior boundary was characterized by a straight line between the cortical ribbon and the apex of each sulcus (McDonald *et al.*, 2000; Pierce *et al.*, 2001; see also Mai *et al.* at: http://braininfo.rprc.washington.edu/) (Fig. 2). Area 17 is located in the occipital lobe along the walls of the calcarine sulcus and adjacent portions of the cuneus and lingual gyrus (Carpenter, 1985). It is defined histologically by a broad layer IV divided into three sub-layers and numerous very small pyramidal cells in layers II and III. The abrupt disappearance of the stripe of Gennari allows for the precise delineation of the borders of area 17 (Braak, 1980). The CGM is characterized by its layered structure well visible with classical cellular stains, such as gallocyanin or cresyl violet (Fig. 1) (Paxinos, 2004). The boundaries of the FG and area 17 were identified using an Olympus SZX9 stereomicroscope (Olympus; Tokyo, Japan) and were marked on the backside of the glass slides with a felt-tip pen. Identification and delineation of boundaries was performed in a blind manner by I.A.J.v.K. (FG), S.J.M.C.P. (CGM) and P.v.C. (area 17) until all regions per hemisphere were analysed, and were independently cross-evaluated (and, if necessary, slightly modified) by C.S., H.H. and P.R.H.

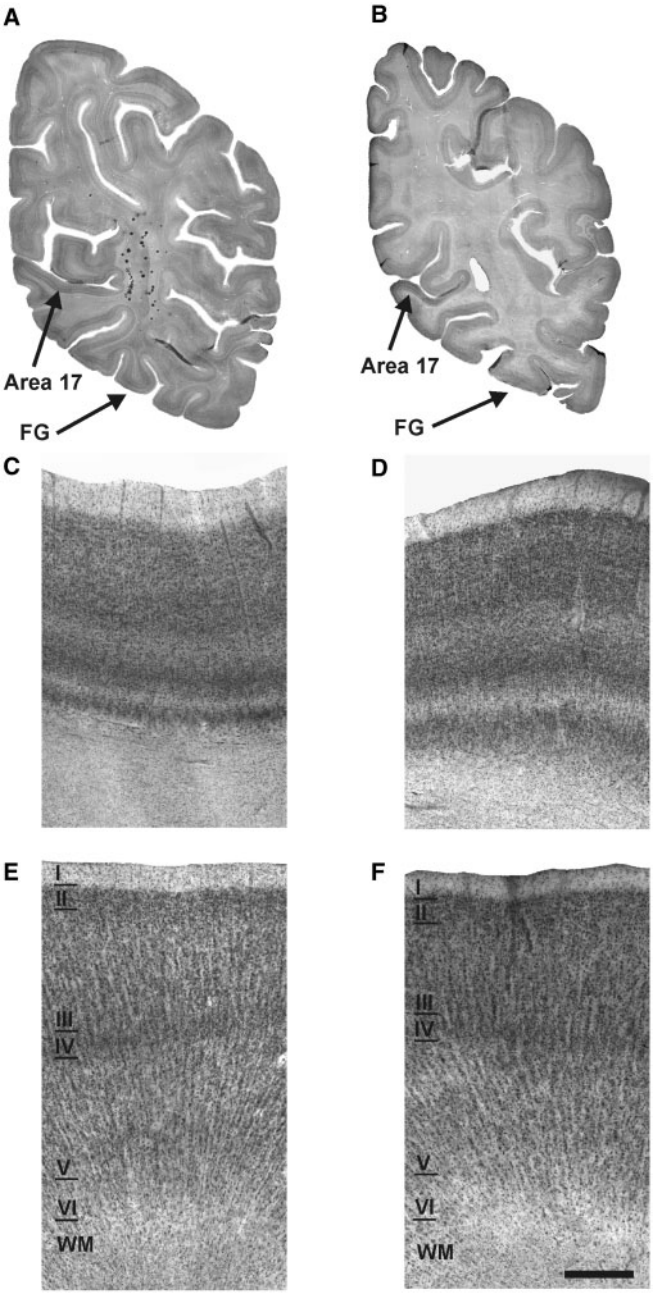

Fig. 1 Representative photomicrographs of 200 μm thick coronal sections of the brain hemispheres from a control patient (**A, C, E**) and a patient with autism (**B, D, F**), showing either the entire hemisphere (**A, B**) or area 17 (**C, D**) and the fusiform gyrus (FG) (**E, F**). Scale bar = 15 mm in **A** and **B**, and 400 μm in **C** to **F**.

Stereological analysis

Stereological analyses were performed with a computerized stereology workstation, consisting of a modified light microscope (Olympus BX50 with PlanApo objective 1.25 × [numerical aperture (N.A.) = 0.04] and UPlanApo objective 20 × [(oil; N.A. = 0.8); Olympus], motorized specimen stage for automatic sampling (Ludl Electronics; Hawthorne, NY, USA), CCD colour video camera (HV-C20AMP; Hitachi, Tokyo, Japan) and stereology software (StereoInvestigator; MBF Bioscience, Williston, VT, USA).

Volumes of brain regions were analysed using the Cavalieri's principle (Cavalieri, 1966; Schmitz and Hof, 2005), by determining the projection area of a given brain region on each selected section showing this region, summing up the data from all sections and multiplying this value with the interval of selecting sections for staining with gallocyanin (2 or 3; see above) and the average actual section thickness after tissue processing [determined with the stereology workstation (in case of the 200 μm thick sections) or a calibrated fine adjustment knob of an Olympus BH

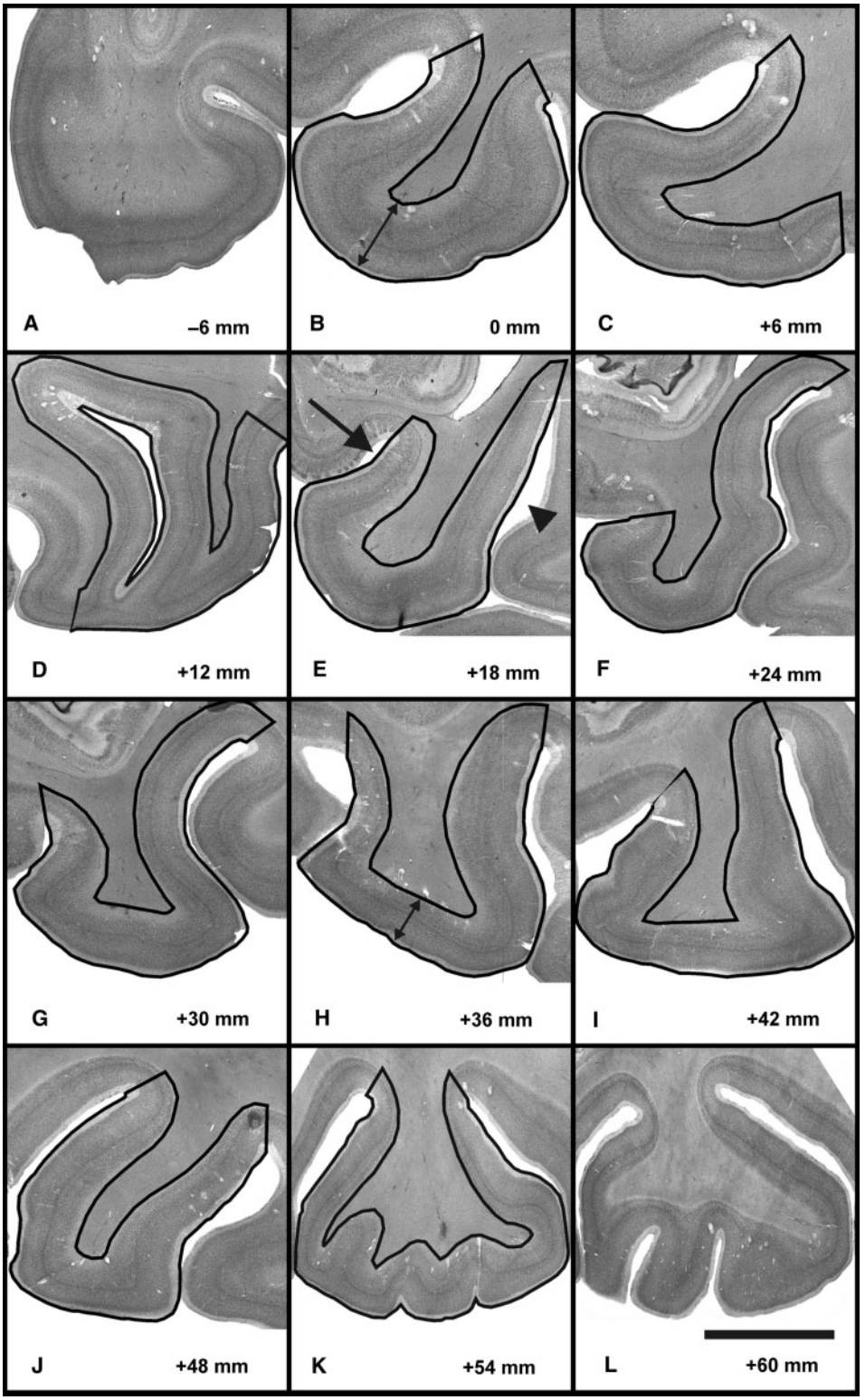

Fig. 2 Representative photomicrographs of 200 μm thick coronal sections throughout the fusiform gyrus (FG) in the post-mortem brain from a patient with autism, showing the delineation procedure of the entire FG (**B** to **K**). The next sections in the series rostral to the FG (**A**) and caudal to the FG (**L**) are also shown. The arrow in **E** indicates the collateral sulcus and the arrowhead the temporo-occipital sulcus. Note the tangential cut of the FG in **B** (arrow) indicating the rostral pole of the FG compared to the centre of the FG (**H**) in which the cortical grey matter was found to be much thinner (arrow in **H**). Scale bar = 15 mm.

Table 2 Details of the stereological analysis procedures

	Hem	CGM	Area 17	FG I-VI	FG II	FG III	FG IV	FG V	FG VI
S	20.2	20.2	10.2	10.8	10.8	10.8	10.8	10.8	10.8
Obj. 1	1.25×	1.25×	1.25×	1.25×	1.25×	1.25×	1.25×	1.25×	1.25×
sla-*x*, sla-*y* (μm)	2000	2000	–	–	–	–	–	–	–
ΣP	9989	5645	–	–	–	–	–	–	–
Obj. 2	–	20×	20×	–	20×	20×	20×	20×	20×
sln-*x*, sln-*y* (μm)	–	6500	1100	–	700	900	700	900	650
a (μm^2)	–	6400	6241	–	4900	4900	4900	4900	6400
h (μm)	–	20	20	–	20	20	20	20	20
d (μm)	–	10	10	–	10	10	10	10	10
ΣOD	–	506	585	–	211	289	203	281	218
ΣN	–	2250	2195	–	582	634	581	696	456
t_1 (μm)	172	172	172	–	172	172	172	172	172
t_2 (μm)	472	472	472	–	472	472	472	472	472
$CE_{pred}(n)$	–	0.021	0.021	–	0.041	0.040	0.041	0.038	0.047

Hem = entire hemisphere; CGM = cortical grey matter; FG = fusiform gyrus; I, II, III, IV, V and VI, cortex layers I, II, III, IV, V and VI. S = average number of analysed sections; Obj. 1 = objective used for delineating the regions of interest and point counting; sla-*x* and sla-*y* = distance between the points used for volume estimates in mutually orthogonal directions *x* and *y*; ΣP = average number of points counted; Obj. 2 = objective used for counting neurons and estimating perikaryal volume; sln-*x* and sln-*y*, distance between the unbiased virtual counting spaces used for counting neurons in mutually orthogonal directions *x* and *y*; a and *h* = base and height of the unbiased virtual counting spaces; d = depth within the section at which the unbiased virtual counting spaces were placed; ΣOD = average number of unbiased virtual counting spaces used; Σ*N* = average number of neurons counted; t_1 = measured actual average section thickness of the sections cut at 200 μm after histological processing; t_2 = measured actual average section thickness of the sections cut at 500 μm after histological processing; $CE_{pred}(n)$ = average predicted coefficient of error of the estimated total neuron numbers using the prediction method described by Schmitz and Hof (2000).

microscope and a PlanApo objective (40×; N.A. = 1.0) as described (Heinsen *et al.*, 1994) (in the case of the 500 μm thick sections)]. The projection areas of the entire hemisphere and the CGM were determined with point counting (Gundersen and Jensen, 1987; Schmitz and Hof, 2005). In contrast, the projection areas of the FG and area 17 [combined analysis of all layers (FG and area 17), followed by separate analyses of layers II, III, IV, V and VI (FG)], were determined by tracing their boundaries on each selected section on video images displayed on the monitor of the stereology workstation (see Fig. S1 in Supplementary data). No specific descriptions of the cytoarchitecture of the CGM in the FG have been provided in the literature. We therefore used the general criteria as provided by, for example, Braak (1980) and Kandel *et al.* (2000) to discriminate cortical layers using the advanced differentiability of laminar boundaries in 200 μm thick and 500 μm thick gallocyanin-stained sections from human post-mortem brains (see also Heinsen *et al.*, 2000); layers II and IV comprise mainly small spherical (granule) neurons, layer III contains mainly pyramidal-shaped neurons and those laying deep in layer III are larger compared to those located more superficially. Layer V includes pyramidal-shaped neurons that are larger than those in layer III, and layer VI is a heterogeneous layer of neurons blending into the white matter and forming the deep limit of the cortex (Fig. S1 in Supplementary Data).

Total neuron numbers were estimated with the Optical Fractionator (West *et al.*, 1991; Schmitz and Hof, 2005). All neurons whose nucleus top came into focus within unbiased virtual counting spaces distributed in a systematic-random fashion throughout the delineated regions were counted, and their perikaryal volume was measured with the Nucleator (Gundersen, 1988; Schmitz and Hof, 2005) (see Supplementary data about the use of the Nucleator to estimate mean perikaryal volumes on coronal sections). Neurons were differentiated from glial and endothelial cells by histological criteria. Neurons showed a large cytoplasm, and a prominent nucleolus within a pale nucleus. Glial cells were identified by the absence of cytoplasmic staining, intense staining of the nucleus with dispersed chromatin and lack of a nucleolus (see Fig. S2 in Supplementary data).

Then, total neuron numbers were calculated from the numbers of counted neurons and the corresponding sampling probability, as well as the mean perikaryal volume of all analysed neurons. All details of the counting procedure (including information about the sampling parameters) for all investigated brain regions are summarized in Table 2. Select cases were analysed for total neuron numbers with the same parameters by three independent researchers (I.A.J.v.K., S.J.M.C.P. and P.v.C.). In all cases, the inter-rater variability was <5%, reflecting the benefits of the high-precision design-based stereology approach used here (see also Schmitz and Hof, 2005). However, a comprehensive inter-rater/intra-rater analysis was not performed.

Statistical analysis

For both patients with autism and controls, mean and SEM were calculated for all investigated variables. Then, Kolmogorov–Smirnov (KS) tests were performed to assess whether the values from each investigated variable came from a Gaussian distribution (these analyses were performed separately for the patients with autism and the controls). Only in four out of 58 datasets (6.9%) (two groups and 29 investigated variables per group) it was found that the data did not pass the KS normality test [patients with autism: density in the CGM ($P = 0.007$) and neuron density in layer V of the FG ($P = 0.028$); controls: volume of area 17 ($P = 0.047$) and mean perikaryal volume of the neurons in layer III of the FG ($P = 0.045$)]. All other datasets passed the KS normality

test with $P > 0.1$. Furthermore, *F*-tests were performed to compare the variances of all investigated variables between patients with autism and controls. For none of the 29 investigated variables, the variances were significantly different between the two groups (i.e. $P > 0.05$). Accordingly, comparisons between patients with autism and controls could be performed with parametric statistics using generalized linear model multivariate analysis (MANOVA), with diagnosis as fixed factor and the patients' age, sex, hemisphere, post-mortem interval, brain weight and fixation time as covariates (see Supplementary data for details about reasons not to consider the history of seizures of some of the patients with autism in the statistical analysis). For each investigated variable, all investigated brain regions were tested simultaneously. *Post hoc* tests in the analyses of covariance were performed with linear regression analysis (patients' age and fixation time) or two-way analysis of variance (hemisphere). In all analyses, an effect was considered statistically significant if its associated *P*-value was <0.05. To test the hypothesis that the results of this study were independent of the higher mean age of the controls than the mean age of the patients with autism, the statistical analysis was repeated by disregarding the control cases (i) C10, (ii) C9 and C10 and (iii) C8 to C10. Calculations were performed using SPSS (Version 12.0.1 for Windows; SPSS, Chicago, IL, USA) and GraphPad Prism (Version 4.0 for Windows, GraphPad software, San Diego, CA, USA).

Photography

Photomicrographs shown in Figs 1, 2 and S1 (Supplementary data) were produced by digital photography using the stereology workstation described above. On average, ~100 images were captured for the composite in each Fig. 1A and B, 25 images for the composite in each Fig. 1C–F, 70 images for the composite in Fig. 2 and 60 images for the composite in Fig. S1A. These images were made into one montage using the Virtual Slice module of the StereoInvestigator software. Photomicrographs shown in Figs 3A–K and S2 (in Supplementary data) were produced by digital photography using an Olympus DP 70 digital camera attached to an Olympus AX 70 microscope and cell^{P} software (version 2.3; Soft Imaging System, Münster, Germany). The final figures were constructed using Corel Photo-Paint v.11 and Corel Draw v.11 (Corel, Ottawa, Canada). Only minor adjustments of contrast and brightness were made, without altering the appearance of the original materials.

Results

The FG (and separate layers II, III, IV, V and VI), area 17 and the CGM were identified on all sections showing these regions according to Figs 1, 2 and S1 (Supplementary data). The mean volumes of the investigated brain regions did not significantly differ between the patients with autism and the controls (Fig. S3 and Table S2 in Supplementary data).

Compared to the controls, the patients with autism showed a significantly reduced mean neuron density in layer III of the FG [−13.1%; $F(1) = 19.321$ (one degree of freedom), $P = 0.002$] (Fig. S4 in Supplementary data). Furthermore, the patients with autism had a significantly reduced mean total neuron number in layers III [−23.7%; $F(1) = 6.356$, $P = 0.033$], V [−14.3%; $F(1) = 6.446$, $P = 0.032$] and VI [−10.6%; $F(1) = 5.518$, $P = 0.043$] of the FG compared to the controls (Figs 3 and 4). In layer III, the reduced mean total neuron number reflected a combined reduction in the mean volume of this layer [−12.7% (patients with autism versus controls)] as well as the mean neuronal density within this layer (−13.1%). In contrast, the reduced mean total neuron number in layers V and VI reflected a reduced mean volume of these layers

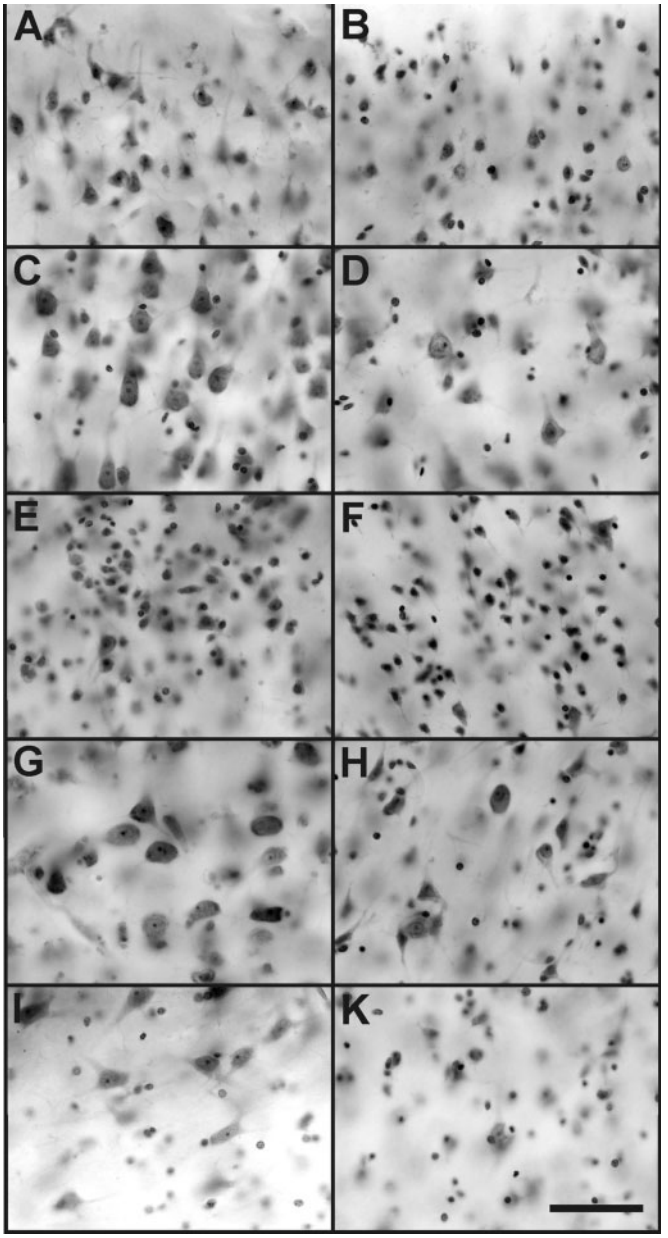

Fig. 3 Representative photomicrographs of 200 μm thick coronal sections showing layers II (**A, B**), III (**C, D**), IV (**E, F**), V (**G, H**) and VI (**I, K**) of the fusiform gyrus in the brains from a control patient (**A, C, E, G, I**) and a patient with autism (**B, D, F, H, K**). These photomicrographs are representative of the magnification at which the stereological estimates were performed. Note the reduced numbers of neurons in layers III, V and VI in the brain from the patient with autism compared to the control. Scale bar = 50 μm.

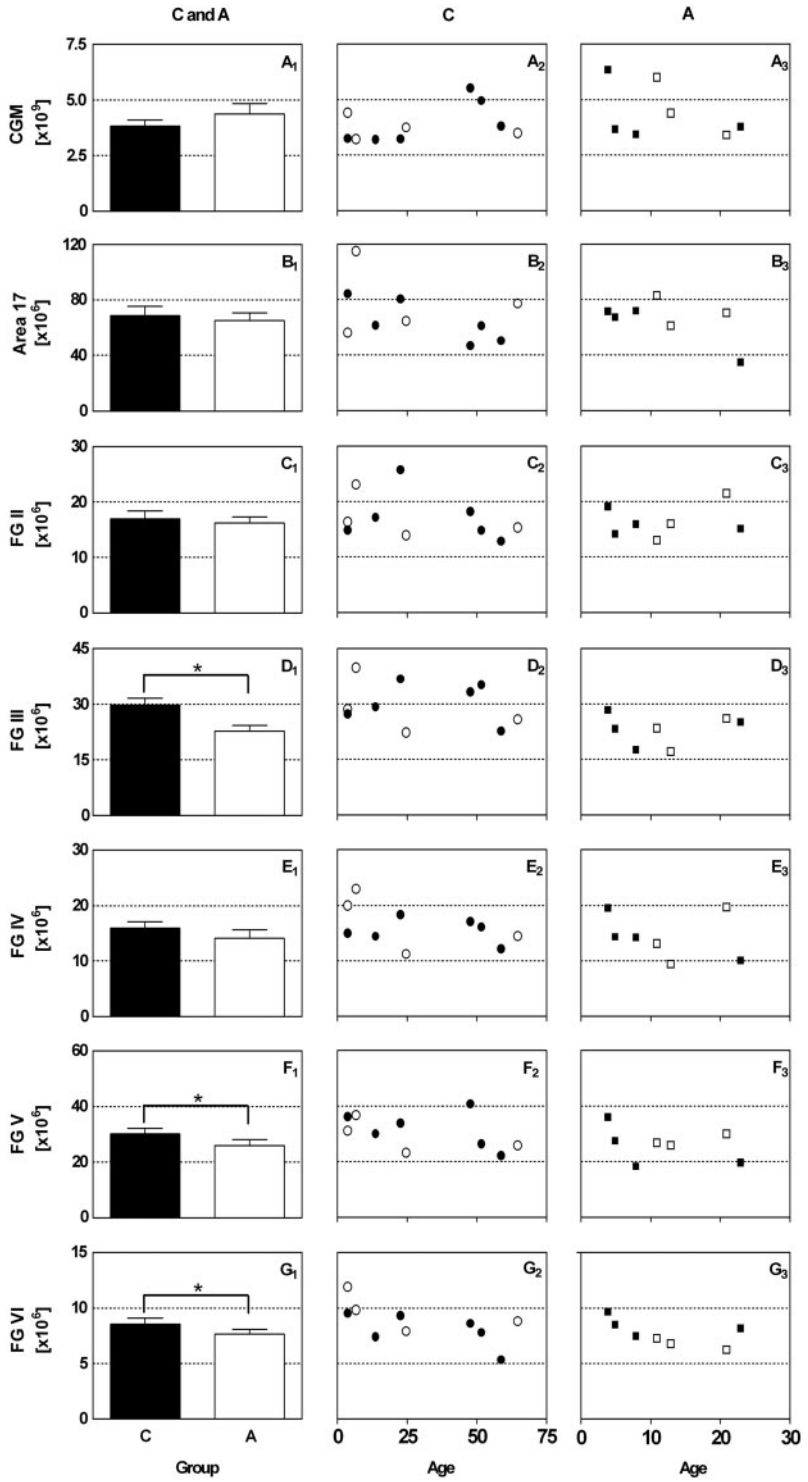

Fig. 4 Total neuron numbers in the cortical grey matter (CGM) (**A_1, A_2, A_3**), area 17 (**B_1, B_2, B_3**) and layers II, III, IV, V and VI of the fusiform gyrus (FG) (**C_1, C_2, C_3** to **G_1, G_2, G_3**, respectively), in post-mortem brains from seven patients with autism (A; open bars in A_1 to G_1, squares in A_3 to G_3) and 10 matched controls (C; closed bars in A_1 to G_1, dots in A_2 to G_2). In A_1 to G_1, data from patients with autism and controls are shown as mean and standard error of the mean. In A_2 to G_2, individual data from controls are shown as a function of the persons' age. Black dots represent data obtained on brains cut at 200 μm and open dots data obtained on brains cut at 500 μm. In A_3 to G_3, individual data from patients with autism are shown as a function of the patients' age. Black squares represent data obtained on brains from patients without history of seizures, and open squares data obtained on brains from patients with a history of seizures. $^*P < 0.05$ for the fixed factor diagnosis in general linear model multivariate analysis of variance (MANOVA).

[−16.8% (layer V) and −17.0% (layer VI), respectively], rather than a reduced mean neuronal density within these layers [actually the mean neuronal density was slightly increased in layer V (+2.7%) and layer VI (+8.0%) in the brains from the patients with autism compared to the controls].

In addition, the patients with autism showed a significantly reduced mean perikaryal volume of the neurons in layers V [−21.1%; $F(1) = 14.763$, $P = 0.004$] and VI [−13.4%; $F(1) = 8.853$, $P = 0.016$] of the FG (Figs 3 and 5) compared to the controls. There were no significant differences between the patients with autism and the controls with respect to neuron density, total neuron number and mean perikaryal volume in the whole CGM and in area 17 (Figs 4 and 5, as well as Fig. S4 in Supplementary data).

The statistical analysis showed a number of significant effects of the covariates on the investigated variables. With respect to the variables which showed significant differences between the patients with autism and the controls, the age of the subjects under study and the fixation time had a significant effect on the perikaryal volume in layer V of the FG [$F(1) = 6.910$, $P = 0.027$ (patients' age) and $F(1) = 5.446$, $P = 0.044$ (fixation time), respectively]. However, *post hoc* linear regression analysis only revealed a positive significant correlation between the controls' age and the perikaryal volume in layer V of the FG [$r^2 = 0.444$, $F(1,10) = 6.384$, $P = 0.035$]. Accordingly, there was no positive correlation between the age of the patients with autism and the perikaryal volume in layer V of the FG [$r^2 = 0.053$, $F(1,7) = 0.27$, $P = 0.620$]. Furthermore, the hemisphere had a significant effect on the mean perikaryal volume in layer VI of the FG [$F(1) = 5.147$, $P = 0.049$] (Fig. S5 in Supplementary data). Moreover, two-way ANOVA showed a significant difference only in the mean perikaryal volume in layer VI of the FG with respect to diagnosis ($P = 0.021$) but not with respect to hemisphere ($P = 0.073$) or the interaction between diagnosis and hemisphere ($P = 0.839$). In summary, it can be concluded that the alterations in mean perikaryal volumes found in the investigated regions in the brains from the patients with autism were not caused by the patients' age and sex, the investigated hemispheres, the post-mortem interval, the brain weight and the fixation time.

Finally, it should be mentioned that the outcome of the present study was the same when the older control cases (i) C10, (ii) C9 and C10 or (iii) C8 to C10 were disregarded. Furthermore, the results obtained on the brains cut at 200 µm section thickness showed no systematic deviation from those cut at 500 µm (Figs 4 and 5, as well as Figs S3 and S4 in Supplementary data). Moreover, the results obtained on the brains from the patients with a history of seizures showed no systematic deviation from those without a history of seizures (Figs 4 and 5, as well as Figs S3 and S4 in Supplementary data).

Discussion

This is the first study focusing on volume, neuron density, total neuron number and mean perikaryal volume of neurons in the FG of patients with autism and matched controls. The main findings of the present study include a significant reduction in the mean neuron density in layer III (−13.1%), a reduced mean total neuron number in layers III, V and VI (−23.7%, −14.3% and −10.6%, respectively) and a decreased mean perikaryal volume of neurons in layers V and VI in the FG (−21.1% and −13.4%, respectively) in the brains of the patients with autism compared to the controls. These alterations did not reflect general neuropathological alterations found in all cortical regions in autism, as demonstrated by the fact that no differences in these variables were found in area 17 or in the whole CGM. In addition, the age of the patients with autism was not correlated with any of the observed neuronal alterations, suggesting that the alterations found in the FG might be of neurodevelopmental origin. The mean volumes of the FG and CGM found in the present study agree with previous reports in the literature (McDonald *et al.*, 2000; Kreczmanski *et al.*, 2007). Although this study consists of a relatively small sample, it is, besides the series investigated by Schumann and Amaral (2006; nine patients with autism versus 10 controls), larger than all other autism post-mortem brain series studied in the past 20 years (Bauman and Kemper, 1985; Raymond *et al.*, 1996; Bailey *et al.*, 1998; Blatt *et al.*, 2001; Fatemi *et al.*, 2001; Schumann and Amaral, 2006).

Compared to the controls, we did not find alterations in the mean volumes of the whole hemispheres and the CGM in the brains from the patients with autism. The observed lack of increase in brain volume in patients with autism at older ages is in accordance with some, but not all, related MRI studies (Piven *et al.*, 1995; Courchesne *et al.*, 2001; Hardan *et al.*, 2001; Aylward *et al.*, 2002; Palmen *et al.*, 2004*b*). Although it has been suggested that abnormal brain development is a typical feature of autism regardless of IQ (Aylward *et al.*, 2002), the differences in outcome between the present and previous studies may be explained by the influence of several factors associated with smaller brains such as mental retardation and epilepsy (Mosier *et al.*, 1965; Theodore *et al.*, 2003), which are the most common co-morbid features of autism (Guerin *et al.*, 1996; Canitano, 2007). Despite the fact that exact IQ data were not available in our study, all patients with autism investigated here were classified as high functioning patients in the clinical records.

Incomplete pruning during brain development, resulting in overabundant synapses and neurons, has been suggested to result in the larger brain size reported in some patients with autism (Frith, 2003; Belmonte *et al.*, 2004). As suggested elsewhere (Courchesne *et al.*, 2004), this could indicate improper function of overabundant synapses and neurons in patients with autism, that is eventually followed

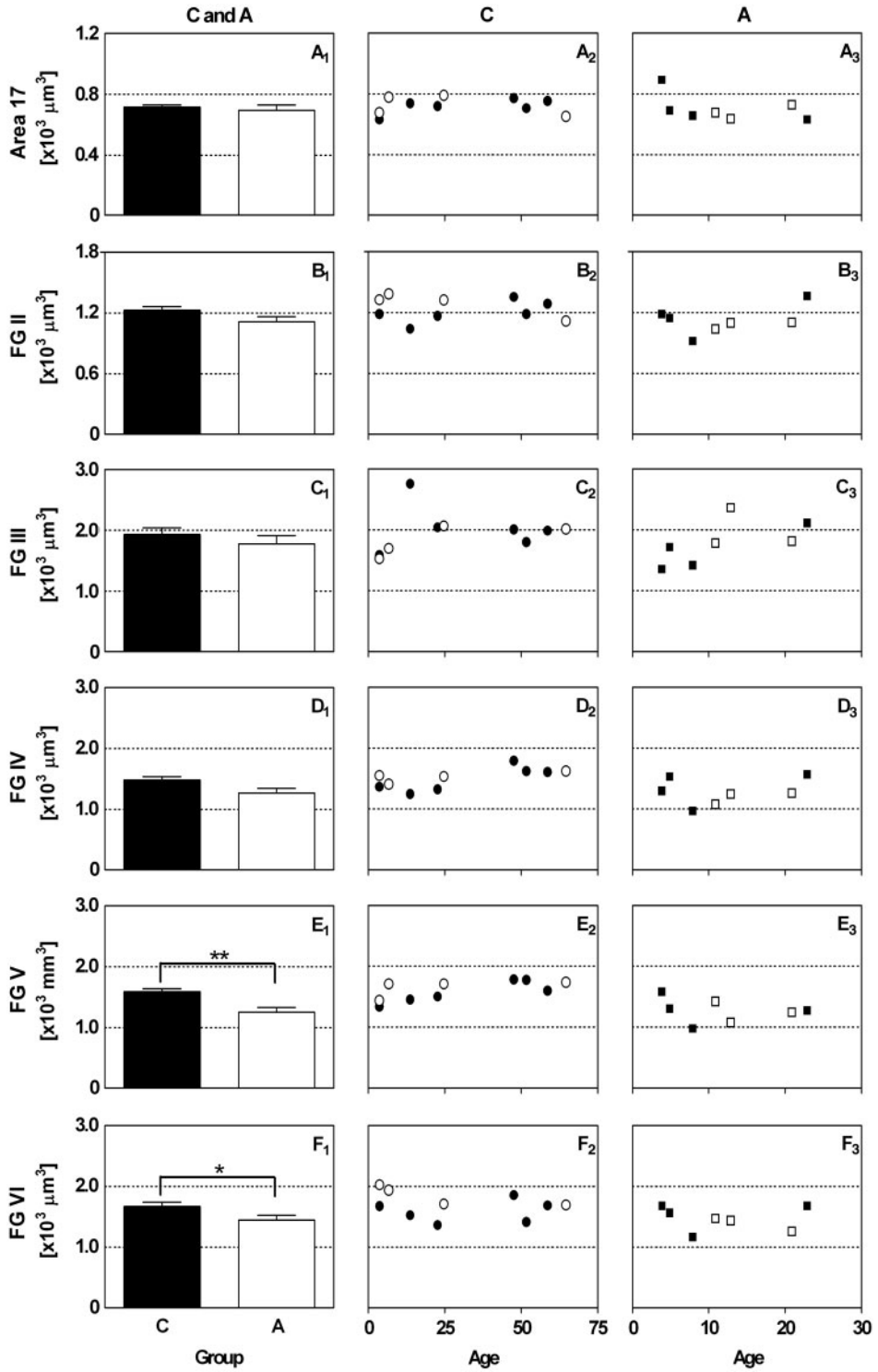

Fig. 5 Mean perikaryal volume of neurons in area 17 (**A_1, A_2, A_3**) and layers II, III, IV, V and VI of the fusiform gyrus (FG) (**B_1, B_2, B_3** to **F_1, F_2, F_3**, respectively), in the post-mortem brains from seven patients with autism (A; open bars in A_1 to F_1, dots in A_3 to F_3), and 10 controls (C; black bars in A_1 to F_1, squares in A_2 to F_2). In A_1 to F_1, data from patients with autism and controls are shown as mean and standard error of the mean. In A_2 to F_2, individual data from controls are shown as a function of the persons' age. Black dots represent data obtained on brains cut at 200 µm and open dots data obtained on brains cut at 500 µm. In A_3 to F_3, individual data from patients with autism are shown as a function of the persons' age. Black squares represent data obtained on brains from patients without history of seizures, and open squares data obtained on brains from patients with a history of seizures. $^{*}P < 0.05$ and $^{**}P < 0.01$ for the fixed factor diagnosis in general linear model multivariate analysis of variance (MANOVA).

in later childhood by death of neurons and subsequent normalization or even decrease in brain volume (and total neuron number in the CGM) in autism. We found no significant difference in the mean total neuron number in the CGM between the patients with autism and the controls. However, this does not provide evidence for or against the hypothesis that the total neuron number in the CGM could change with age in brains from patients with autism. This is due to the fact that our sample encompassed a rather wide age range (i.e. from 4 to 23 years). Differences in total neuron number in the CGM could still be there at a specific age or time period of development. Further research is necessary to address this question.

A growing body of evidence suggests that patients with autism have difficulties in face perception (Schultz, 2005). Recognition of persons, especially of their individual faces, is a key part of an individual's social experience and successful functioning within a social group. Virtually, all normal adults are experts in the recognition of faces (Tanaka and Gauthier, 1997), whereas patients with autism are consistently impaired in this task (Joseph and Tanaka, 2002). Most functional neuro-imaging studies have reported reduced activity in the FG during face processing tasks in autism (Schultz *et al.*, 2000; Hall *et al.*, 2003; Hubl *et al.*, 2003; Hadjikhani *et al.*, 2004; Pierce *et al.*, 2004; Piggot *et al.*, 2004). In addition, several studies demonstrated the involvement of a specific region located within the FG, the FFA (Schultz *et al.*, 2003; Schultz, 2005; Hadjikhani *et al.*, 2007). This region is more engaged by human faces than by any other object (Kanwisher *et al.*, 1997). In the present study, we did not observe differences in the mean volume of the FG between the patients with autism and the controls. The same was observed by Pierce *et al.* (2001) in a structural neuroimaging study on the FG in autism, whereas Waiter *et al.* (2004) reported an increased FG volume in autism.

With respect to the neurobiological basis of the reduced activation of the FG during face processing tasks in autism, the main finding of the present study was a significant reduction in mean total neuron numbers in both output layers III and V of the FG in the patients with autism compared to the controls. Notably, these alterations were not found in area 17 and the CGM. Cortical layer III is the principal source of corticocortical (association) connections, whereas layer V is the principal source of efferent fibres to sub-cortical regions (Jones, 1986).

Accordingly, our results suggest a disconnection of the FG or underdeveloped connections in face processing networks (shown in Fig. S6 in Supplementary data). Area 17 projects via the inferior occipital gyrus (IOG) to the FG. In addition, the IOG is also connected to the superior temporal gyrus (STG). Efferent fibres project from the FG to the amygdala (AMG) and to two regions in the frontal lobe, the inferior frontal gyrus (IFG) and the orbitofrontal cortex (OFC) (Fairhall and Ishai, 2007). Thus, there is evidence that the FG receives input from the visual cortex via the IOG and provides the major input into an extended system consisting of cortical regions (including IFG and OFC) and sub-cortical regions such as the AMG (Fairhall and Ishai, 2007).

Although individuals with autism do not show deficits in visual perception in complex object recognition tasks not involving faces, abnormalities in the visual system in autism could be a first sign of a failure to develop perceptual expertise for faces. Thus, there may be a cortical explanation for the deficits in face perception seen in patients with autism rather than an involvement of limbic structures (Schultz *et al.*, 2000). However, the present study found no differential effect in area 17 in patients with autism. This is supported by a recent finding showing no differences in activation of the visual cortex (areas V1 to V5) in eight patients diagnosed with autism spectrum disorder compared to four IQ-matched controls (Hadjikhani *et al.*, 2004). Rather the IOG and STG showed reduced activity in patients with autism (Pierce *et al.*, 2001), indicating that the altered function of the FG in patients with autism cannot be explained by abnormal input from area 17.

As mentioned above, Casanova *et al.* (2006) investigated a subset of the post-mortem brains investigated in the present study for possible alterations in the modular organization of cellular microdomains (minicolumns) in the pre-frontal cortex, primary motor cortex, primary sensory cortex and primary visual cortex. Casanova *et al.* (2006) found an increased neuron density and a slightly reduced mean neuron size in area 17 in the brains from the patients with autism compared with the controls. Although not directly comparable (because of methodological differences), the findings by Casanova *et al.* (2006) are in line with the results of the present study [as shown in Figs 5 and S4 (in Supplementary Data) of the present study].

Our finding of an age-related increase in the mean perikaryal volume of neurons in layer V of the FG was unexpected. In a sample of human post-mortem control brains with ages 4–4–7–14–23–25–48–52–59–65 (in years) as the one investigated here, one would not predict significant changes as a function of age, particularly if all cases were controlled for the absence of neurodegenerative diseases (as done in our sample). No design-based stereological studies have been published addressing the question of age-related alterations in perikaryal size of neurons in the human cerebral cortex. However, an earlier study by Schulz and Hunziker (1980) found no significant difference in the mean perikaryal size of cortical neurons between a group of people aged 19 to 44 and another group aged 65 to 74. Unfortunately, our sample did not include such age groups making a direct comparison between our data and the results by Schulz and Hunziker (1980) impossible. Additional research is necessary to evaluate the possible neurobiological repercussion of an age-related increase of the mean perikaryal volume of neurons in layer V of the FG in the human brain, but this was beyond the focus of the present study.

The reduced mean total neuron numbers in layers III and V of the FG and the reduced mean perikaryal volume of neurons in layers V and VI of the FG in the patients with autism could originate from pathological events primarily in the FG itself, or from loss of targets to which the FG projects. In this regard, it is important to note that the AMG receives input from the FG and is involved in face processing (as shown in Fig. S6 in Supplementary data) (Schultz *et al.*, 2000; Fairhall and Ishai, 2007). The AMG plays a role in the interpretation of faces (threatening or fearful) (Morris *et al.*, 1999), monitors eye gaze (Kawashima *et al.*, 1999) and has been implicated in autism because of its role in social behaviour and cognition (Adolphs, 2002). Structural imaging studies have reported increased (Howard *et al.*, 2000; Sparks *et al.*, 2002; Schumann *et al.*, 2004), decreased (Aylward *et al.*, 1999; Pierce *et al.*, 2001; Nacewicz *et al.*, 2006) or unchanged (Haznedar *et al.*, 2000; Palmen *et al.*, 2006) mean volumes of the AMG in autism [note that this discrepancy may be due to differences in the ages of the patients among the available studies; it was suggested by Schumann *et al.* (2004) that larger volumes are typically observed in young subjects, whereas no difference or smaller volumes are observed in older subjects. However, this question was beyond the focus of the present study]. In an earlier neuropathological study, neurons in the AMG were found to be abnormally small and densely packed in autism (Kemper and Bauman, 1993), whereas a recent design-based stereological study found no changes in mean neuron size but a significantly reduced mean total neuron number in the AMG overall and in its lateral nucleus in autism (Schumann and Amaral, 2006). The latter result suggests target loss of the FG in autism, which could contribute to reductions in mean total neuron numbers and mean neuronal size in the FG in autism as reported in the present study. The FG receives reciprocal input from the corticomedial nucleus of the AMG; however, these connections play a minor role during face perception (Fairhall and Ishai, 2007). Although no reduction in the mean total neuron number was found in this part of the AMG in patients with autism (Schumann and Amaral, 2006) and our data do not show alterations in the main input layers II and IV of the FG in autism, the results might point to an intact input from the AMG to the FG. In addition, because no alterations were found in area 17, input to the FG from the visual cortex seems to remain intact.

Finally, it should be noted that a reduced mean total neuron number in the lateral nucleus of the AMG is not specific for autism, as the same finding was recently reported for schizophrenia (Kreczmanski *et al.*, 2007). In this regard, it will be important to evaluate whether the FG also shows reduced mean total neuron numbers in schizophrenia (as in autism). The mean volume of the FG is however comparable in patients with schizophrenia and controls (McDonald *et al.*, 2000). There is indeed evidence that face processing deficits are also present in schizophrenia (Pinkham *et al.*, 2005), yet patients with schizophrenia do not show reduced haemodynamic responses in the FG during face perception tasks studied with fMRI (Yoon *et al.*, 2006).

In summary, although based on a relatively small sample of post-mortem brains, the present study provides novel insight into the neuropathology of autism. Specifically, reduced mean total neuron numbers and smaller neurons in the main output layers of the FG in patients with autism might be involved in impaired face processing in autism. Although the precise interpretation of the reported FG hypoactivity in fMRI studies in autism has not yet been clearly established, Pierce *et al.* (2001) suggested that face processing could also occur outside the FG and FFA. In this regard, both the IFG (semantic aspects) (Leveroni *et al.*, 2000) and the OFC (facial attractiveness and sexual relevance) (O'Doherty *et al.*, 2003; Kranz and Ishai, 2006) belong to the cortical networks mediating face processing (Fairhall and Ishai, 2007) and are related to autism. Interestingly, imaging studies found a reduced activation of the IFG (Just *et al.*, 2004; Harris *et al.*, 2006; Koshino *et al.*, 2007) and a decreased volume of the OFC in autism (Hardan *et al.*, 2006; Girgis *et al.*, 2007). It will therefore be of interest to investigate total neuron numbers and neuron densities in the IFG and OFC in post-mortem brains of patients with autism as well. Further studies are needed to test the hypothesis that there is a causal relationship between abnormal activation of the FG and related cortical areas in face processing in autism and the neuropathological findings reported in the present study.

Supplementary material

Supplementary material is available at *Brain* online.

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