

## **Calretinin interneuron density in the caudate nucleus is lower in autism spectrum disorder**

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## **Abstract**

Autism spectrum disorder is a debilitating condition with possible neurodevelopmental origins but unknown neuroanatomical correlates. Whereas investigators have paid much attention to the cerebral cortex few studies have detailed the basal ganglia in autism. The caudate nucleus may be involved in the repetitive movements and limbic changes of autism. We used immunohistochemistry for calretinin and neuropeptide Y in 24 age-gender matched patients with [autism spectrum disorder](#) and [controls](#) ranging in age from 13 to 69 years. Autism subjects had a 35% lower density of [calretinin+](#) interneurons in the [caudate](#) that was driven by loss of small calretinin+ neurons. This was not caused by altered size of the [caudate](#), as its cross-sectional surface areas were similar between diagnostic groups. Controls exhibited an age dependent increase in the density of medium and large [calretinin+](#) neurons, whereas [subjects with autism](#) did not. [Diagnostic groups](#) did not differ regarding [ionized calcium-binding adapter molecule 1+](#) immunoreactivity for [microglia](#), suggesting chronic inflammation did not cause the decreased [calretinin+](#) density. There was no statistically significant difference in the density of [neuropeptide Y+](#) neurons between [subjects with autism and controls](#). The decreased [calretinin+](#) density may disrupt the excitation/inhibition balance in the caudate leading to dysfunctional corticostriatal circuits. The description of such changes in [autism spectrum disorder](#) may clarify pathomechanisms and thereby help identify targets for drug intervention and novel therapeutic strategies.

## Introduction

Autism spectrum disorder has a global prevalence of 0.62% (Elsabbagh *et al.*, 2012) and causes a range of mild to severe motor, interpersonal, cognitive and behavioral manifestations (Geschwind and State, 2015). It probably has a neurodevelopmental etiology and is associated with several gene mutations and environmental insults such as perinatal inflammation (Depino, 2013; Ebrahimi-Fakhari and Sahin, 2015). There are no effective drugs or behavioral therapies for the disorder and the condition is lifelong with enormous personal and societal burdens (Knapp *et al.*, 2009).

A variety of neuroanatomical and histological abnormalities have been documented which have been difficult to reproduce in animal models. These include enlarged head and brain size (Sacco *et al.*, 2015; Libero *et al.*, 2016). In particular the cerebral cortex was shown to be increased in size (Hardan *et al.*, 2006; Smith *et al.*, 2016). Several studies have shown that Purkinje cells of the cerebellum are decreased in number in [subjects with autism](#) (Kern, 2003; Wegiel *et al.*, 2014b).

The vast majority of studies in [autism spectrum disorder](#) have focused on altered physiology and histopathology of the cerebral cortex (Fatemi *et al.*, 2002; Gogolla *et al.*, 2009; Oblak *et al.*, 2009; Morgan *et al.*, 2010; Oblak *et al.*, 2011; Hashemi *et al.*, 2016). However there is ample evidence that subcortical structures such as the basal ganglia may underlie some of the behavioral, motor and cognitive symptoms of [autism spectrum disorder](#) (Peca *et al.*, 2011; Rothwell *et al.*, 2014; Fuccillo, 2016; Glerean *et al.*, 2016). The caudate nucleus in particular is a nexus of converging circuits including a massive corticostriatal input and therefore it may serve as a node for the pathophysiology of [autism spectrum disorder](#) (Kohls *et al.*, 2012; Haber and Behrens, 2014; Fuccillo, 2016).

Interneurons, most of which are inhibitory  [\$\gamma\$ -Aminobutyric acid-ergic](#) (Cicchetti *et al.*, 2000; Gonchar *et al.*, 2007), have received much attention as being especially relevant for balancing excitation/inhibition in the brain and have been postulated as being disrupted in neuropsychiatric diseases such as schizophrenia (Uhlhaas, 2013; Rogasch *et al.*, 2014) and [autism spectrum disorder](#) (Rubenstein and Merzenich, 2003; Coghlan *et al.*, 2012). As distinct subpopulations of  [\$\gamma\$ -Aminobutyric acid-ergic](#) interneurons, [calretinin+](#) and [neuropeptide Y+](#) neurons constitute ~15% and ~0.5% of

the cortical  $\gamma$ -Aminobutyric acid-ergic interneurons, respectively (Gonchar and Burkhalter, 1997; Meskenaite, 1997). The proportion of interneurons is greater in the primate than rodent **caudate nucleus** (Graveland and DiFiglia, 1985) and most of the **caudate interneurons are calretinin+** (Wu and Parent, 2000). In this study we aimed to compare the density and distribution of the aforementioned interneurons in the **caudate nucleus** in **subjects with autism** in order to elucidate the putative striatal excitatory/inhibitory imbalance and contribution to the condition.

## **Materials and methods**

### **Subjects**

Subjects were included partly from a former study ( $n_{ASD}=6$   $n_{Ctl}=6$ ) (Kotagiri *et al.*, 2014) or newly selected from the Oxford Brain Bank (OBB) and the Netherlands Brain Bank (NBB), Netherlands Institute for Neuroscience, Amsterdam (open access: [www.brainbank.nl](http://www.brainbank.nl)). We worked with an age-gender matched cohort of 12 **subjects with autism spectrum disorder** and 12 **control** subjects. All material has been collected from donors from whom written informed consent had been obtained by the OBB or NBB for brain autopsy and use of material and clinical information for research purposes. The demographic characteristics of the cohort are shown in Table 1. The mean values of age, post mortem interval (PMI), time in paraformaldehyde, time in paraffin and processing time (time in paraformaldehyde plus time in paraffin) were very similar in both diagnostic groups (Table 1, Supplementary Table 1).

Sampling was done by the assistants of the OBB and NBB supervised by trained neuropathologists. Depending on availability of tissue blocks from **subjects with autism**, regions were selected containing the head of the **caudate nucleus** between 22.5 mm caudal and 13.3 mm rostral to the anterior commissure according to the Human Brain Atlas (Mai *et al.*) (Supplementary Fig. 1). Corresponding levels of the head of the **caudate nucleus** were chosen from control subjects. The average position of sampling was similar in both diagnostic groups (Supplementary Tables 1, 2).

## **Immunohistochemistry**

Serial sections (6  $\mu\text{m}$  thick) were cut in the coronal plane from paraffin embedded blocks and mounted on slides. The slides were heated in a thermostate at 76° C for 20 min. Slides were dewaxed and treated with 3%  $\text{H}_2\text{O}_2$  solution (in PBS, pH=7.4) for 30 minutes. Antigen retrieval was applied by autoclaving the slides in citrate buffer (0.01M, pH=6.0) at 121° C for 10 min. The following primary antibodies were used: anti-calretinin (rabbit, 1:300, Chemicon, AB5054), anti-neuropeptide Y (rabbit, 1:250, Abcam, ab30914) and anti-ionized calcium-binding adapter molecule 1 (rabbit, 1:500, WAKO, 01919741) in TBS/TritonX (pH=7.4) for 1 hr. Sections were incubated with HRP-linked secondary antibody from the Envision Kit (DAKO, K-5007) for 60 min and labeling was visualized by DAB-Substrate-Chromogen solution from the same Kit according to manufacturer's protocol. During incubation of primary and secondary antibodies slides were put into a Sequenza System coverplates and rack (Thermo Scientific, 72110017, 73310017). Nuclear counterstain was applied by Haematoxylin for 20 sec. Slides were dehydrated and coverslipped by DePeX.

## **Image analysis, archiving and quantification**

Slides were digitized using a slidescanner (Aperio ScanScope AT Turbo, Leica Biosystems) at 20x magnification and stored on a server (msdlt-slide.dpag.ox.ac.uk). The regions of interest were outlined using the ImageScope programme (Aperio, v11.2.0.780) and the longest diameter of every calretinin+ and neuropeptide Y+ cell body in the caudate nucleus was manually measured. Two investigators contributed to the calretinin+ quantification and both were blinded to the diagnoses of the subjects, through random coding of the subject identifiers. In contrast to many other brain diseases where pathology is histologically evident, there were no major histopathological patterns recognizable in the autism cases by our immunohistochemical stainings, and it was not possible to tell based on any of our immunohistochemistries which subjects were controls versus autism spectrum disorder. Neuronal cell bodies with a diameter >6  $\mu\text{m}$  and a width >2  $\mu\text{m}$  were included in further statistical analysis (Supplementary Fig. 2). Calretinin+ neurons were also counted in the anterior cingulate cortex (ACC, Brodmann's area 24) throughout all layers. It is noted that this part of the ACC does not have an internal granular layer (layer 4) and has a relatively wide layer 6 (Chana *et al.*, 2003). Occasionally layer 5 pyramidal neurons exhibited a light calretinin staining (in 3 Ctl and 5 ASD) in line with previous observations (Barinka *et al.*, 2010). These neurons

were not included in the quantitative analysis. For the ionized calcium-binding adapter molecule 1 immunohistochemistry, the stained area fraction was obtained by using the Aperio Positive Pixel Count Algorithm (parameters are shown in Supplementary Table 3). The details of our statistical analyses can be found in the Supplementary Material. In brief, Mann Whitney U tests, repeated measures analysis of variance, univariate analysis of variance and Spearman's rank tests were used as appropriate.

## RESULTS

### Morphological assessment of calretinin+ neurons in the caudate nucleus of controls and subjects with autism.

We examined the caudate nucleus of 12 control subjects and 12 age and gender-matched subjects with autism. Three types of calretinin-immunopositive (calretinin+) neurons were distinguished in the caudate nucleus based on their characteristic morphology and diameter (Fig. 1), (Petryszyn *et al.*, 2014). We qualitatively examined the morphology of 1,080 neurons, 180 neurons from each subtype (small, medium, large) in both diagnostic groups (Fig. 1A, B). We next measured the diameters of all calretinin+ neurons present in the caudate nucleus, altogether 17,936 neurons in the control group and 12,677 neurons in the autism spectrum disorder group were counted (Supplementary Fig. 2). The small calretinin+ neurons in controls had round to oval cell bodies with most being round (Fig. 1I-M). Their diameter ranged from 6-15  $\mu\text{m}$ , with an average diameter of  $8.67 \pm 0.15 \mu\text{m}$  (15,510 neurons measured). The small calretinin+ neurons usually had one or two processes in the plane of the section. The medium-sized neurons in controls had multipolar or bipolar cell bodies (with multipolar dominance) and 2-3 processes (Fig. 1D-E). Their diameter ranged from 16-25  $\mu\text{m}$  with an average diameter of  $19.19 \pm 0.16 \mu\text{m}$  (1,503 neurons measured). The large neurons in controls had multipolar perikarya with 3-5 processes in the plane of the section (Fig. 1C). Their diameter ranged from 26-60  $\mu\text{m}$  with an average diameter of  $30.39 \pm 0.34 \mu\text{m}$  (923 neurons measured). Qualitative comparison of process numbers, branching and somal shape did not reveal conspicuous morphological differences between the control and autism spectrum disorder groups (Fig. 1F-H, N-R).

**The mean diameter and proportion of subgroups of calretinin+ neurons in the caudate nucleus are unchanged in subjects with autism.**

No significant differences were found between controls and subjects with autism in the average diameters of the calretinin+ neurons. Autism spectrum disorder calretinin+ neuronal diameters: small  $8.66 \pm 0.15 \mu\text{m}$  (10,717 neurons,  $p=0.99$  compared to Ctl), medium  $19.10 \pm 0.16 \mu\text{m}$  (1,238 neurons,  $p=0.84$  compared to Ctl), large  $30.41 \pm 0.31 \mu\text{m}$  (722 neurons,  $p=0.97$  compared to Ctl). The proportions of different calretinin+ neuron subtypes (small, medium, large) were also very similar in the groups (Ctl: 86.47%, 8.39%, 5.14%; ASD: 84.53%, 9.76%, 5.69%), in line with a previous report (Wegiel *et al.*, 2014b) where neuronal cell body volumes were not changed in the caudate nucleus in subjects with autism ranging from 11-60 years old. To confirm the similarities in calretinin+ size between controls and subjects with autism we measured the surface area of 40 randomly selected neurons from each case (960 neurons). Similar to the diameter, surface areas were not significantly different between the diagnostic groups: controls:  $97.41 \pm 5.89 \mu\text{m}^2$ , autism spectrum disorder:  $107.34 \pm 5.76 \mu\text{m}^2$ ,  $p=0.29$ . The proportion of the small, medium and large subtypes measured in this analysis were: controls: 87.29%, 8.95%, 3.75%; autism spectrum disorder: 87.5%, 6.79%, 5.7%. There was a very strong correlation between surface areas and diameters in controls ( $r=0.71$ ,  $p=7.03\text{E-}75$ ) and subjects with autism ( $r=0.78$ ,  $p=3.72\text{E-}93$ ) that confirmed the validity of diameter measurements and compatibility of the two approaches.

**The density of caudate nucleus calretinin+ neurons is lower in subjects with autism.**

The main finding of this study was a lower density of calretinin+ neurons in caudate nucleus in subjects with autism. There was a significant 36.1% decrease in the density of small calretinin+ neurons in subjects with autism compared to controls (ASD:  $878 \pm 77$  neurons/ $\text{cm}^2$ , Ctl:  $1,374 \pm 104$  neurons/ $\text{cm}^2$ ,  $p<0.001$ ) (Fig. 2). Regarding the medium and large subgroups, 25.9% and 27.0% lower densities were seen in the autism group, however these were not statistically significant ( $p=0.242$ ,  $p=0.590$ , respectively, Fig. 2). When the three subgroups were taken together, the autism group showed a statistically significant 34.8% lower density (Ctl:  $1,577 \pm 132$

neurons/cm<sup>2</sup>, ASD: 1,028±97 neurons/cm<sup>2</sup>,  $p=0.001$ , Fig. 2). There is a paucity of postmortem human brains from subjects with autism spectrum disorder (N of ~100 total worldwide). Nevertheless we were able to add one autism spectrum disorder case and several controls that could not be matched as those described in Table 1. With a final cohort of N=18 controls and N=13 autism spectrum disorder, the decrease in calretinin+ density again was highly significant ( $P=0.00002$ ). We extensively analyzed the potential effects of sex, PMI, fixation time, and other variables on calretinin+ density and found no significant interactions (Supplementary Results).

Previous work found significantly more calretinin+ interneurons rostrally than caudally (rodent) and in the caudate compared to the putamen (primate) (Wu and Parent, 2000). We found a significantly higher density of medium calretinin+ neurons in the anterior caudate nucleus (from -22.5 mm to -5.8 mm) compared to the posterior caudate nucleus (from -5 mm to 13.3mm) of controls (anterior: 183±28.4 neurons/cm<sup>2</sup>, posterior: 72±13 neurons/cm<sup>2</sup>,  $p=0.002$ , Mann Whitney U-Test). This anterior-posterior difference was not statistically significant in autism spectrum disorder (anterior: 112±23 neurons/cm<sup>2</sup>, posterior: 77±17 neurons/cm<sup>2</sup>,  $p=0.310$ ). Furthermore, there was a significant correlation between the density of medium calretinin+ neurons and the position of sampling in controls ( $r=-0.799$ ,  $p=0.002$ ) but not in autism spectrum disorder ( $r=-0.547$ ,  $p=0.066$ , Spearman's rank test). The small and large calretinin+ neurons were not significantly affected by position of sampling (Supplementary Results, univariate analysis of variance). Heatmap distributions of calretinin+ neurons revealed no conspicuous difference in hot spots of calretinin+ neurons in the caudate nucleus in both diagnostic groups (Supplementary Fig. 3). In support of this, cluster analysis in the coronal plane carried out with Matlab did not reveal any calretinin+ total, small, medium or large clustering in either diagnostic group tested with multiple algorithms (data not shown).

### **Calretinin+ density in the caudate nucleus increases with age in controls but not in subjects with autism spectrum disorder.**

We next examined the relationship between the density of calretinin+ neurons and age (Supplementary Fig. 4). There was a significant positive correlation between large and medium calretinin+ density and age in the caudate nucleus of controls



( $r=0.746$ ,  $p=0.005$ ;  $r=0.725$ ;  $p=0.008$ , large and medium, respectively). Interestingly, this correlation was not found in [autism spectrum disorder](#) ( $r=0.481$ ,  $p=0.114$ ,  $r=0.497$ ;  $p=0.100$ , large and medium, respectively). We found no correlation between small [calretinin+](#) neuron density and age in [controls](#) ( $r=0.352$ ,  $p=0.262$ ) or [subjects with autism](#) ( $r=0.327$ ,  $p=0.300$ ). Furthermore, we found a 308% increase in the density of large [calretinin+](#) [caudate nucleus](#) neurons in [controls](#) >35 years ( $N=5$ ) compared to younger individuals ( $N=7$ ) ( $p=0.018$ ), but this was not observed in [subjects with autism](#) ( $p=0.461$ ). Medium [calretinin+](#) neurons were also more abundant in [controls](#) >35 years (189% increase,  $p=0.048$ ), but not in [subjects with autism](#) ( $p=0.283$ ).

### **Caudate nucleus size was not significantly different between [subjects with autism](#) and controls.**

A total of 95 coronal cross-sectional surface areas (surface area) from 95 different slabs (50 from [controls](#) and 45 from [autism spectrum disorder](#)) were measured in our cohorts from 9 [controls](#) and 9 [subjects with autism](#) where multiple regions of the [caudate nucleus](#) were available. There was no significant difference in the average surface area between groups (Ctl:  $1.22 \pm 0.09$  cm<sup>2</sup>, ASD:  $1.19 \pm 0.09$  cm<sup>2</sup>  $p=0.863$ ). The average position of the sections was very similar between [controls](#) and [subjects with autism](#) (Ctl:  $-6.20 \pm 1.36$  mm, ASD:  $-5.10 \pm 1.60$  mm, from the anterior commissure,  $p=0.591$ ) and they corresponded well with the position of the original slides (Ctl:  $-8.06 \pm 1.35$  mm, ASD:  $-4.89 \pm 2.36$  mm).

There was no significant correlation between [caudate nucleus](#) surface area and age in either group (Supplementary Results, Ctl:  $r=0.492$ ,  $p=0.179$ ; ASD:  $r=0.209$ ,  $p=0.589$ ) nor significant difference in [caudate nucleus](#) surface area between younger ( $\leq 35$ ) or older ( $>35$ ) in [controls](#) ( $p=0.548$ ) or [subjects with autism](#) ( $p=0.905$ ). [Analysis of variance](#) revealed no statistically significant effects of any of the following variables on [caudate nucleus](#) surface area: diagnosis, gender, hemisphere, post-mortem interval and time in paraformaldehyde (Supplementary Table 6). These data indicate that there were no significant caudate size differences between [controls](#) and [subjects with autism spectrum disorder](#).

### **The density of [calretinin+](#) neurons in the cerebral cortex**

The anterior cingulate cortex was present in a subset of the slides (5 Ctl and 5 ASD cases) therefore our analysis was extended to this region as well. The dominant calretinin+ neuronal type had elongated and bipolar morphology (Supplementary Fig. 5A, B). There were no obvious differences in calretinin+ neuron morphology or size between controls and subjects with autism spectrum disorder (Supplementary Fig. 5). We found markedly different densities of calretinin+ neurons in distinct layers of the cerebral cortex in both diagnostic groups (Supplementary Fig. 6). The frequency distribution of calretinin+ neurons in the human cortex was very similar to that observed in macaque (Gabbott, 2016). The density of calretinin+ neurons was not statistically different from controls even though the density was 33.3% lower overall in the autism group ( $p=0.151$ , Supplementary Fig. 5C). Comparing the layerwise distribution, there was a trend to a lower density of calretinin+ neurons in layer 2-6 but a greater density in layer 1 of subjects with autism compared to controls (Supplementary Figure 6A, C). Statistically significant lower density in autism spectrum disorder was only found in layer 5 (38.5%,  $p=0.032$ , Supplementary Figure 6C). We compared calretinin+ neuronal density in the caudate nucleus versus the cingulate cortex but found no statistically significant correlation in control ( $r=0.2$ ,  $p=0.747$ ) or autism spectrum disorder subjects ( $r=0.3$ ,  $p=0.624$ ).

#### **No evidence for altered corticostriatal circuitry in autism spectrum disorder.**

We carried out postmortem tractography on two subjects with autism, a 22 year old male (case 07/09) and a 60 year old female (case 29/12). Both showed a similar set of white matter tracts connecting the prefrontal cortex with the anterior/middle caudate nucleus (Supplementary Fig. 7, Supplementary Table 8). These results are consistent with previous studies demonstrating connectivity between the cortex and the caudate nucleus (Robinson *et al.*, 2012; Delmonte *et al.*, 2013).

#### **Morphology and density of **neuropeptide Y+** neurons did not significantly differ between **controls** and **subjects with autism**.**

Two types of **neuropeptide Y+** neurons were distinguished based on their characteristic morphology (Fig. 3). Many had round to oval perikarya usually with 1-2 primary processes (fusiform neurons). Fewer **neuropeptide Y+** neurons had obvious

pyramidal or multipolar perikarya usually with 2-3 primary processes (neurogliaform neurons). Both types of cell bodies ranged in diameter from 6-40  $\mu\text{m}$ . There were no conspicuous morphological differences in **caudate nucleus neuropeptide Y+** neurons between the **control** and **autism spectrum disorder** groups.

We quantified every **neuropeptide Y+** neuron present in the **caudate nucleus** in our sections. Altogether 4,977 neurons in the **control** group and 4,567 neurons in the **autism spectrum disorder** group were counted. No significant differences were found between the diagnostic groups in the average diameters of the **neuropeptide Y+** neurons (Ctl:  $14.33 \pm 0.32 \mu\text{m}$ , ASD:  $14.66 \pm 0.43 \mu\text{m}$ ,  $p=0.41$ ). We found a 12.7% lower density in **neuropeptide Y+** neurons in **autism spectrum disorder**, however it was not statistically significant (Ctl: 423.66 cells/ $\text{cm}^2$ , ASD: 369.66 cells/ $\text{cm}^2$ ,  $p=0.143$ ).

Similar to the lack of effect of diagnosis on the density of **neuropeptide Y+** neurons ( $F=3.055$ ,  $df=7, 16$ ,  $p=0.102$ ) there were no significant effects of other variables, such as gender, hemisphere, age or antero-posterior position of sampling (Supplementary Table 7). There were also no significant effects of potential confounders on the density of **neuropeptide Y+** neurons, such as post-mortem interval, fixation time in paraformaldehyde, time in paraffin and processing time (Supplementary Table 7).

### **Microglia were not activated in **autism spectrum disorder**.**

To determine if the decrease in calretinin+ density was associated with increased inflammation we carried out immunohistochemistry for the microglial marker **ionized calcium-binding adapter molecule 1** (Ito *et al.*, 1998). Qualitative assessment of **ionized calcium-binding adapter molecule 1+** microglial morphology in the **caudate nucleus** by three independent observers did not reveal obvious microglial morphology differences between **controls** and **autism spectrum disorder** (Fig. 4A,B), using previously established criteria (Goings *et al.*, 2008). Microglia in the **caudate nucleus** of both **controls** and **subjects with autism** had typical resting microglial shape with small cell bodies, fine ramified processes and moderate **ionized calcium-binding adapter molecule 1** immunohistochemistry (Fig. 4A,B).

Quantitative analysis revealed no statistical difference between controls and autism spectrum disorder in the ionized calcium-binding adapter molecule 1 immunopositive area fraction (Ctl: 3.16% $\pm$ 0.49%, ASD: 3.78% $\pm$ 0.63%,  $p=0.799$ , Mann-Whitney U test, Fig. 4C). The ionized calcium-binding adapter molecule 1 stained area fraction (percentage of ionized calcium-binding adapter molecule 1-immunopositive area per total area) did not have a significant effect on the density of calretinin+ neurons in either subjects with autism or controls ( $F=0.066$ ,  $df=7, 13$ ,  $p=0.803$ ) (Supplementary Table 4) or on the density of neuropeptide Y+ neurons ( $F=1.327$ ,  $df=7, 14$ ,  $p=0.274$ ) (Supplementary Table 7). Finally, there were no significant interactions between the ionized calcium-binding adapter molecule 1 stained area fraction and the density of small, medium or large calretinin+ neurons in either diagnostic group (univariate analysis of variance, Supplementary Table 5).

## DISCUSSION

We have discovered one of the most striking examples of altered neuronal numbers in the forebrain in autism spectrum disorder; a highly significant decrease in calretinin+ neuronal density in the caudate nucleus. Rather than sampling, we counted all calretinin+ cells in our material, a total of 30,613 calretinin+ neurons. In line with the observations of (Petryszyn et al., 2014) we confirmed that there are three populations; small, medium and large calretinin+ interneurons in the human caudate nucleus. Furthermore, our data on the proportions of small, medium and large calretinin+ neurons in controls correspond well with previous stereological data (Bernacer et al., 2012). Several reports claim that autism spectrum disorder is associated with increased head and brain size and expansion of particular brain subregions (Courchesne et al., 2003). Wegiel and colleagues reported increased caudate volume in autism spectrum disorder (Wegiel et al., 2014a; Wegiel et al., 2014b). If our autism spectrum disorder cases had expanded caudate nucleus, it could have contributed to the decreased calretinin+ cell density. However, we carried out careful measurements and found that the cross-sectional surface areas of the caudate nucleus were very similar between controls and patients with autism. Others also found that the caudate volume is not significantly different in autism spectrum disorder compared to controls (McAlonan et al., 2002; Hardan et al., 2003; Herbert et al., 2003; Ecker et al., 2012; Langen et al., 2012; Hua et al., 2013; Lin et al., 2015;

Sussman *et al.*, 2015).

There is a remarkable dearth of information about the connectivity, electrophysiological properties and function of **calretinin+** neurons in the caudate nucleus. However, in the cerebral cortex, bipolar **calretinin+** neurons preferentially target layer 2/3 multipolar **calretinin+** neurons and layer 5 pyramidal neurons, whereas multipolar **calretinin+** neurons maintain theta-oscillations via inhibition of parvalbumin+ neurons that innervate layer 2/3 pyramidal neurons (Meskenaite, 1997; Gonchar and Burkhalter, 1999; Caputi *et al.*, 2009). Other important results suggest calretinin is a calcium-modulator limiting hyperexcitation of Purkinje cells (Schiffmann *et al.*, 1999) and maintaining long term potentiation (LTP) in the dentate gyrus (Schurmans *et al.*, 1997). **Calretinin+** neuronal function in the **caudate nucleus** may be poorly understood because they account for only 0.5% of neurons in rodents (Rymar *et al.*, 2004). Remarkably, they are estimated to comprise 10% of **caudate nucleus** neurons in non-human primate (Deng *et al.*, 2010) and humans (Wu and Parent, 2000). However, **calretinin+** neuron density in the rostral **caudate nucleus** is comparable across species (Wu and Parent, 2000). Given our results, future studies are warranted to elucidate neuronal inputs to **calretinin+** neurons in the **caudate nucleus** and whether they innervate local interneurons or medium spiny neurons. If they primarily regulate local interneurons they may disinhibit medium spiny neurons. If they directly innervate medium spiny neurons they may inhibit the principal cells of the striatum. It will also be important to determine if the large, medium and small **calretinin+** neurons have different connectivity and thus segregate function.

A major question that arose during our studies was if the decreased density of **calretinin+** neurons could be generalized to multiple brain areas or if it was specific to the **caudate nucleus**. Our results revealed a lower density of **calretinin+** neurons in the anterior cingulate cortex of autism spectrum disorder subjects that was not statistically significant compared to controls. A comparable decrease was found in the CNTNAP2-KO mouse model of autism spectrum disorder in the somatosensory cortex (Penagarikano *et al.*, 2011). However, in autism the human dorsolateral prefrontal cortex (Brodmann's area 9 and 46) and the ventrolateral prefrontal cortex (Brodmann's area 47) did not exhibit decreased densities of **calretinin+** neurons (Hashemi *et al.*, 2016). It will be important to determine if **calretinin+** interneurons in other cortical regions may exhibit altered densities in autism spectrum disorders.

Based on our results, thus far it appears that the caudate nucleus is the site of the greatest decreases in calretinin+ cell densities.

It will be important to probe the interrelated functions of dopaminergic neurotransmission and the large calretinin+/cholinergic neurons. Interestingly 90% of the large calretinin+ neurons are cholinergic (Massouh *et al.*, 2008) and cholinergic caudate nucleus interneurons profoundly influence dopaminergic neurotransmission in the caudate nucleus (Threlfell *et al.*, 2012). The density of calretinin+ neurons is altered after 6-OHDA and L-DOPA treatment in rat (Mura *et al.*, 2000; Ma *et al.*, 2014), suggesting they are responsive to dopaminergic inputs. Non-human primates have similar calretinin+ caudate nucleus neurons to humans (Petryszyn *et al.*, 2014) and primate models of autism spectrum disorder are being developed (Liu *et al.*, 2016) which may also help elucidate the role of calretinin+ caudate neurons. Rodent models may also help to reveal calretinin+ neuronal function, for example, caudate nucleus function is disturbed in mice with mutations in Shank3 a gene associated with autism spectrum disorder (Peca *et al.*, 2011).

An important aspect of these findings is that the relatively late changes in density of medium and large calretinin+ neurons in autism spectrum disorder may not contribute to pathophysiology in the first few years of life. It is also possible that these late changes reflect a secondary compensatory mechanism to other events in autism spectrum disorder. For example calretinin+ neuron density or expression could be modulated by seizures which are a common co-morbidity in autism. Several studies discovered increased calretinin+ neuron density in the context of seizures in humans (Blumcke *et al.*, 1999; Kuchukhidze *et al.*, 2015; Wimmer *et al.*, 2015). Interestingly, animal models of neonatal hypoxic brain injury induce the subventricular zone neurogenic niche to generate calretinin+ neurons that migrate to the striatum (Yang *et al.*, 2008). These studies suggest that indeed calretinin+ neuron numbers can be modulated, but these scenarios increased calretinin+ numbers and would not explain the decreased density of calretinin+ neurons we observed in this study.

The decreased calretinin+ neuron density correlates with expected effects of genetic mutations in autism spectrum disorder (Gene Scoring Module of the SFARI website). The CALB2 gene (encoding calretinin) is a downstream target of the following autism spectrum disorder syndromic genes: PAX6, ARX, TCF and TBR1 (high confidence

gene). Mutations in these genes decrease CALB2 (calretinin) expression in animal models and cells (Bedogni *et al.*, 2010; Quille *et al.*, 2011; Ha *et al.*, 2012; Wisniewska, 2013; Huang and Hsueh, 2015), however, calretinin messenger ribonucleic acid or protein levels have not been investigated in humans carrying the aforementioned mutations. We do not know the genetic profile of the cases investigated in this study and they were fixed in paraformaldehyde or embedded in paraffin for many years which hinders molecular investigation.

The caudate nucleus regulates movement, emotion and reward; three modalities that are central in the pathophysiology of autism spectrum disorder. The caudate nucleus has been implicated as a nexus in the pathophysiology of schizophrenia (Simpson *et al.*, 2010), a neuropsychiatric disorder with genetic and behavioral features in common with autism spectrum disorder (Gao and Penzes, 2015; Kastner *et al.*, 2015; Li *et al.*, 2015). The caudate nucleus – especially its rostral part, the pre-commissural head – receives major axonal input from the ventromedial prefrontal cortex, orbitofrontal cortex, dorsolateral prefrontal cortex and anterior cingulate cortex (Haber and Behrens, 2014). These functional cortical inputs to the CN are being actively investigated in ASD and several are impaired (Fatemi *et al.*, 2002; Oblak *et al.*, 2009; Oblak *et al.*, 2011; Watanabe *et al.*, 2013; Hashemi *et al.*, 2016) or increased (Delmonte *et al.*, 2013). Interestingly, Delmonte and colleagues showed within their study that despite increased corticostriate functional connectivity there were no differences between controls and autism spectrum disorder in structural connectivity (Delmonte *et al.*, 2013). Thus far, our pilot work also suggests structural corticostriatal inputs may not be not altered, the present results show no obvious deviation from the typically developed architecture. However, considering the small number of subjects with available MRI data, it was not possible to carry out a quantitative diffusion tensor imaging analysis of white matter microstructure.

The nucleus accumbens is similar in connectivity and neuronal subtypes to the caudate nucleus and they have overlapping functions. Mouse models of repetitive movements show that the nucleus accumbens mediates the effects of the autism-associated gene neuroligin-3 (Rothwell *et al.*, 2014). The globus pallidus, a major target of the caudate nucleus, is reduced in volume compared to the cerebrum in autism spectrum disorder (Estes *et al.*, 2011; Sussman *et al.*, 2015). Thus it will be important to examine not only the cortical inputs to the caudate but also its



connectivity of the nucleus accumbens and globus pallidus.

Altered peripheral and central inflammation has been reported in [autism spectrum disorder](#) (Vargas *et al.*, 2005; Depino, 2013), and could have contributed to the reduced number of [calretinin+](#) neurons. Previously, activated microglia were found in a subset of [autism spectrum disorder](#) cases (Vargas *et al.*, 2005; Morgan *et al.*, 2010). However, we found no differences between [subjects with autism](#) and [controls](#) in the level of [ionized calcium-binding adapter molecule 1+](#) immunohistochemistry. The morphology of microglial cells in [controls](#) and [subjects with autism](#) was also very similar and typical of resting ramified microglia, suggesting lack of inflammation in the [caudate nucleus](#) at the time of death. Inflammation may have occurred at earlier time points and it is possible that other indices of inflammation are altered in [autism spectrum disorder](#). If inflammation mediated [calretinin+](#) neuron death were prevalent we would have expected to see some damaged or dysmorphic [calretinin+](#) cells but the cells generally appeared to be healthy in both groups, with well-defined edges and intact processes.

Our analysis was originally inspired by several findings. Firstly, we discovered that more ependymal cells [of the lateral ventricles](#) expressed GFAP in [autism spectrum disorder](#) compared to controls (Kotagiri *et al.*, 2014) suggesting neurogenic activation similar to stroke (Carlen *et al.*, 2009; Young *et al.*, 2013). Secondly, human postnatal neurogenesis may contribute [calretinin+](#) and [neuropeptide Y+](#) neurons to the [caudate nucleus](#), with a reported 2.7% median turnover rate per year in adulthood (Ernst *et al.*, 2014). We are unsure if the changes we observe are due to reduced neurogenesis but may partially be so. Medium and large [calretinin+](#) neuronal numbers significantly increased with age in [controls](#), but not in [subjects with autism](#). The simplest explanation for this is continuous addition of [calretinin+](#) neurons to the [caudate nucleus](#) via adult neurogenesis in [controls](#) but not in [subjects with autism](#). Another explanation may be [relatively greater calretinin](#) expression and immunoreactivity in [controls compared to subjects with autism](#), with neuronal density remaining unchanged. This would still be interesting as loss of calretinin inhibits LTP (Schurmans *et al.*, 1997) and changes in [its expression could disrupt the inhibitory/excitatory balance in the caudate nucleus](#). This is a possibility, but we believe it is not likely as the level of expression of calretinin in the caudate appeared to be equivalent in controls and subjects with autism. Unless the decrease in



expression occurred before the ages examined, we would have expected to see faint calretinin immunohistochemical detection in at least a subset of ASD neurons. In addition to calretinin and neuropeptide Y expressing interneurons, other populations such as parvalbumin and somatostatin expressing interneurons exist in the caudate nucleus and may have altered densities in subjects with autism. It will be important to determine in future studies if the effect in the caudate is also found in other interneuron populations.

We achieved our goal of determining potential differences in the caudate nucleus between subjects with autism and controls even though autism spectrum disorder is heterogeneous and the cases ranged across ages. Given the heterogeneity of ASD, it was all the more remarkable that we found significant differences and it suggests that the reduction in calretinin+ neuron density may occur in multiple subtypes of the disease. We found significant differences, and subsequent studies will determine if autism spectrum disorder subtypes differ in calretinin+ density. Interestingly, *Cntnap2*<sup>-/-</sup> mice have similarities with several core features of autism spectrum disorder and display decreased numbers of  $\gamma$ -Aminobutyric acidergic interneurons in the caudate nucleus and fewer calretinin+ interneurons in the cerebral cortex (Penagarikano *et al.*, 2011). Several laboratories have learned how to differentiate caudate interneurons from human induced pluripotent stem cells (Maroof *et al.*, 2013; Nicholas *et al.*, 2013). Thus, future studies using human induced pluripotent stem cells or animal models may help dissect the molecular mechanisms leading to reduced numbers of calretinin+ neurons and thus lead to pharmacological interventions.

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## **Supplementary material**

Supplementary material is available at *Brain* online.

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## Figure Legends

**Fig. 1. The morphology of calretinin+ neurons in the caudate nucleus was similar in controls and autism spectrum disorder.**

Images were taken from 4 controls and 4 subjects with autism. (A) Representative field from controls and (B) from autism spectrum disorder (arrowhead-large neuron, arrow-small neuron). (C) Large neuron from a control. (D) Medium neuron with bipolar morphology from control. (E) Medium neuron with multipolar shape from control. (I-M) Small neurons from controls. (F) Large neuron from a subject with autism. (G-H) Medium neurons from subjects with autism. (N-R) Small calretinin+ neurons from subjects with autism. Scale bars = 30  $\mu$ m. (S) Frequency distribution of calretinin+ neurons in the caudate based on the longest diameter measured. Note the characteristic first peak of the calretinin+ population (small neurons). (T) Frequency distribution of calretinin+ neurons in the caudate showing the medium (15-25  $\mu$ m) and large (>25  $\mu$ m) populations.

**Fig. 2. The density of calretinin+ neurons is lower in subjects with autism than in controls.**

Graphs showing the number of calretinin+ neurons per square cm as a total population (A) and subdivided into the small (B), medium (C) and large (D) diameter populations. N=12 each controls and subjects with autism. \*\*p<0.01, \*\*\*p<0.001, Mann-Whitney U test.

**Fig. 3. The morphology of neuropeptide Y+ neurons in the caudate nucleus was similar in controls and subjects with autism.**

Images were taken from N=5 controls and N=5 subjects with autism. (A-E) Fusiform neuropeptide Y+ neurons in controls. (F-J) Fusiform neuropeptide Y+ neurons in subjects with autism. (K-O) Multipolar neuropeptide Y+ neurons in controls. (P-T) Multipolar neuropeptide Y+ neurons in autism spectrum disorder. Scale bars = C-E, H-J: 10  $\mu$ m, otherwise 30  $\mu$ m.

**Fig. 4. Ionized calcium-binding adapter molecule 1+ cells were similar between controls and subjects with autism.**

The distribution, intensity of immunostaining and morphology of ionized calcium-binding adapter molecule 1+ cells was not appreciably different between controls (A) and subjects with autism (B). Scale bars = 30  $\mu$ m. (C) The surface area occupied by ionized calcium-binding adapter molecule 1+ immunoreactivity in the caudate nucleus

was not significantly different between controls and subjects with autism spectrum disorder.

**Table 1.**

Main demographic characteristics of control and autism spectrum disorder subjects.

## **Supplementary materials and methods**

### **Autism spectrum disorder post-mortem tractography - magnetic resonance image analysis**

Scanning was performed at the Oxford FMRIB Centre on a Siemens Trio 3T scanner using a 12-channel head coil for signal reception. T1- weighted structural scans were acquired using a 3D balanced steady state free precession (BSSFP) sequence (TE/TR=3.7/7.4ms, bandwidth=302Hz/pixel, matrix size: 352x330x416, resolution 0.5x0.5x0.5mm). Images were acquired with and without radio frequency (RF) phase alternation to avoid banding artifacts. This protocol was repeated for 1-2 h to increase the signal to noise ratio. For more complete details see (Miller *et al.*, 2011).

Diffusion-weighted data were acquired using a modified spin-echo sequence with 3D segmented EPI (TE/TR=122/530ms, bandwidth=789 Hz/pixel, matrix size: 168x192x120, resolution 0.94x0.94x0.94mm). Diffusion weighting was isotropically distributed along 54 directions (b=4500s/mm<sup>2</sup>) with six b=0 images. This protocol takes approximately six hours and was repeated three times for 18 hours total diffusion imaging. For more complete details see (Miller *et al.*, 2011).

To investigate the structural connectivity between the caudate region of interest and cerebral cortex regions, we performed a probabilistic tractography analysis. FMRIB's Diffusion Toolbox (FDT), part of the FMRIB Software Library (FSL) (Smith *et al.*, 2004), was used to extract maps of fractional anisotropy (FA), mean diffusivity (MD), radial diffusivity (RD) and axial diffusivity (AXD). BEDPOSTX was used for Bayesian estimation of a two-fiber model using Markov chain-Monte Carlo (MCMC) sampling (Behrens *et al.*, 2007). Our region of interest (ROI) masks were hand-drawn on the structural scans, using the anterior commissure as a landmark to define the centre of the region of the caudate (as studied in histology). An "exclusion mask" was used to exclude the contralateral hemisphere. Tractography was performed from these ROI masks using the Probtrackx probabilistic tractography software (Behrens *et al.*, 2007).

### **Statistical analysis**

Numbers are presented as means  $\pm$  standard error of the mean. Statistical analyses were performed using SPSS software (version 22.0). One section per subject was used per immunostain. Two populations were analysed separately: i) *calretinin+*

neurons with three subgroups (small, medium and large size neurons) ii) **neuropeptide Y+** neurons as one class. For analysis of **calretinin+** neurons, in order to reveal possible significant effects of independent variables on density and whether these were dependent on the different subgroups (ie. small, medium and large **calretinin+** neurons), the General Linear Model of repeated measures analysis of variance was applied with the three subgroups (small, medium and large) as levels of the within-subject factor. Regarding the **neuropeptide Y+** neurons the General Linear Model of univariate analysis of variance was used. Significance level was set to 5% ( $\alpha=0.05$ ).

#### *Repeated measures analysis of variance of calretinin+ neurons*

The dependent variables were the densities of the small, medium and large-size neurons that were included in this analysis as levels of the within–subject factor. In order to be able to use **analysis of variance**, one outlier from the **control** group had to be removed (Ctl #15). Normality of dependent variables was tested by Shapiro-Wilk test. The independent categorical variables (diagnosis, gender and hemisphere) were tested as between-subjects factors and their effect and interactions were reported in Supplementary Table 4. The independent continuous variables, such as age and antero-posterior position of the sample were regarded as biological characteristics of the subjects and included as covariates in the analysis (Supplementary Table 4). In order to avoid erosion of data during analysis, the independent continuous variable of microglial presence (**ionized calcium-binding adapter molecule 1** stained area fraction) was tested separately due to the availability of data only from 22 subjects. Potential confounders, such as post-mortem interval, fixation time in paraformaldehyde, time in paraffin and processing time (from time of death until staining) were accounted for and tested as covariates in separate **repeated measures analysis of variance** analyses.

#### *Univariate analysis of variance of neuropeptide Y+ neurons*

The dependent variable was the density of **neuropeptide Y+** neurons and independent categorical variables (diagnosis, gender and hemisphere) were tested as fixed factors. The independent continuous variables, such as age and antero-posterior position of the sample were included as covariates and reported in Supplementary Table 7. As detailed above, the **ionized calcium-binding adapter molecule 1** stained area fraction was tested separately by **univariate analysis of**

variance. Potential confounders were included into [univariate analysis of variance](#), as detailed in previous paragraph.

#### *Post-hoc analyses*

To further refine the interpretation of the results obtained by [repeated measures analysis of variance](#) in the context of subgroups of [calretinin+](#) neurons, [univariate analysis of variance](#) with Bonferroni's correction for multiple comparisons and the non-parametric Mann-Whitney's and Spearman's rank test were conducted. Regarding the non-parametric tests no outlier was removed from the dataset. For the [neuropeptide Y+](#) neurons Mann-Whitney's and Spearman's rank tests were used.

### **Supplementary results**

#### *Further analysis of [calretinin+](#) density*

[Repeated measures analysis of variance](#) revealed that the lower [calretinin+](#) neuron density in the [caudate nucleus](#) of [subjects with autism](#) compared to [controls](#) was observed as a main effect of diagnosis: 29.6% reduction in [autism spectrum disorder](#) compared to [controls](#) ( $F=8.955$ ,  $df=1, 13$ ,  $p=0.010$ , statistical power=0.79) with no significant effect by sex or hemisphere. No other significant effects or interactions were found for [calretinin+](#) neuronal density regarding age or antero-posterior position of sample (Supplementary Table 4).

No significant effects of covariates were observed on [calretinin+](#) neuronal density regarding post-mortem interval ( $F=0.0001$ ,  $df=1, 9$ ,  $p=0.99$ ), fixation time in paraformaldehyde ( $F=0.456$ ,  $df=1, 11$ ,  $p=0.514$ ), time in paraffin ( $F=1.132$ ,  $df=1, 11$ ,  $p=0.31$ ) and processing time ( $F=1.045$ ,  $df=1, 12$ ,  $p=0.327$ ) (Supplementary Table 4).

In order to explore the context-dependent effects of variables on the density of [calretinin+](#) neurons regarding the small, medium and large subgroups a post-hoc [univariate analysis of variance](#) was applied (Supplementary Table 5). Diagnosis had a significant effect on the density of small [calretinin+](#) neurons ( $F=10.651$ ,  $df=1, 13$ ,  $p=0.006$ , statistical power=0.854) but not on density of medium or large neurons (Supplementary Table 5). No significant effect of any of the other variables was seen on the density of small or large [calretinin+](#) neurons (Supplementary Table 5). There

was a significant effect regarding the antero-posterior position of sampling on the density of medium **calretinin+** neurons ( $F=7.717$ ,  $df=1, 13$ ,  $p=0.016$ , statistical power=0.729) (Supplementary Table 5).

### Supplementary figure legends

**Fig. S1:** Coronal positions of sections analyzed. Note the significant overlap in the positions sampled between **subjects with autism** and **controls**. Red line indicates the position of anterior commissure. **Figure** adapted with permission from (Mai *et al.*, 2008).

**Fig. S2:** (A) Example of sampling of caudate nucleus shown with red outline. (B) Example of field with small medium and large neurons and (C) their measurements. Scale bar = A: 500  $\mu\text{m}$ , B-C: 100  $\mu\text{m}$ .

**Fig S3:** Heatmaps showing relative distribution in the coronal sections of **calretinin+** neurons in the **caudate nucleus**. Note there were no obvious differences between **controls** and **subjects with autism**.

**Fig. S4:** Effect of age on **calretinin+** neuronal density. (A) Total cells. (B) Small cells. (C) Medium cells. (D) Large cells.

**Fig. S5:** Calretinin immunohistochemistry in the cerebral cortex.

(A) Photomicrograph showing bipolar calretinin+ neurons in the anterior cingulate cortex from layer 3 in a control subject. (B) Bipolar calretinin+ neurons in layer 3 of the cingulate cortex from an autism spectrum disorder case. Scale bars: 20  $\mu\text{m}$ . (C) Quantification of the cortical density of calretinin+ neurons between control and autism spectrum disorder subjects.

**Fig. S6:** Calretinin+ density varied in cerebral cortex layers. (A) Histogram showing the density results of cortical calretinin+ neurons based on their distance from the pial surface (100  $\mu\text{m}$  deep bins). Right Y axis shows how these bins correspond to approximate cortical layers. (B) Nissl staining of the anterior cingulate cortex in a

control subject showing the laminar structure of this region. Note that layer 4 is missing from this region, Brodmann's area 24. wm = white matter. Scale bar: 100  $\mu$ m.  
 (C) Histogram showing the layerwise comparison of cortical calretinin+ neuronal density between controls and subjects with autism. There was a statistically significant difference in case of layer 5 ( $p=0.032$ , Mann-Whitney).

**Fig. S7:** Diffusion tensor imaging of two subjects with autism spectrum disorder. Horizontal, sagittal and coronal planes shown. The ROI masks for each tract are indicated by the black lines. Tracts generated in the right (light blue - blue) and left (red - yellow) caudate are displayed as maximum intensity projections on top of the structural scan. Left brain: case 07/09, Right brain: case 29/12.

**Table S1:** Additional demographic characteristics of subjects.

**Table S2:** Average values of age, position of sampling, PMI, time in paraformaldehyde, time in paraffin and processing time in the control and autism spectrum disorder groups.

**Table S3:** Parameters of Aperio Positive Pixel Count algorithm used in the ionized calcium-binding adapter molecule 1 analysis.

**Table S4:** Repeated measures analysis of variance for calretinin+ neurons.

**Table S5:** Univariate analysis of variance for calretinin+ neurons.

**Table S6:** Univariate analysis of variance showing the effects of variables on the average surface area of the caudate nucleus.

**Table S7:** Univariate analysis of variance showing the effects of variables on the density of neuropeptide Y+ neurons.

**Table S8:** Mean value for tensor-derived parameters extracted from the generated tracts.