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Neuroanatomical Alterations in Higher-Order Thalamic Nuclei of Fetuses With Down Syndrome

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Title: Neuroanatomical alterations in higher-order thalamic nuclei of fetuses with Down syndrome.

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ABSTRACT

Objectives. Down syndrome (DS) is a genetic condition characterized by cognitive disability starting from infancy. Children with DS exhibit deficits in several cognitive domains, including executive function, i.e., a set of cognitive processes that heavily depend on higher-order thalamic nuclei. The goal of this study was to establish whether executive function-related thalamic nuclei of fetuses with DS exhibit neuroanatomical alterations that may contribute to the defects in higher-order control processes seen in children with DS.

Patients and Methods. In brain sections from fetuses with DS and control fetuses (gestational week 17-22), we evaluated the cellularity in the mediodorsal nucleus (MD), the centromedian nucleus (CM), and the parafascicular nucleus (PF) of the thalamus and the density of proliferating cells in the third ventricle.

Results. We found that all three nuclei had a notably reduced cell density. This defect was associated with a reduced density of proliferating cells in the third ventricle, suggesting that the reduced cellularity in the MD, CM, and PF of fetuses with DS was due to neurogenesis impairment. The separate evaluation of projection neurons and interneurons in the MD, CM, and PF showed that in fetuses with DS the density of projection neurons was reduced, with no changes in interneuron density.

Conclusion. This study provides novel evidence for DS-linked cellularity alterations in the MD, CM, and PF and suggests that altered signal processing in these nuclei may be involved in the impairment in higher-order control processes observed in individuals with DS starting from infancy.

Key words: Down syndrome; fetal thalamus; higher-order nuclei; cellularity; projection neurons.

1. INTRODUCTION

Individuals with Down syndrome (DS) are characterized by impairment in cognitive functions starting from their earliest life stages. Studies aimed at discovering the causes of intellectual disability have shown that the trisomic condition is characterized by severe impairment in the process of neurogenesis at fetal stages of brain development (Contestabile et al., 2007, Guidi et al., 2008, Guidi et al., 2011, Stagni et al., 2018a). This defect accounts for the reduced cellularity and the reduced size of various brain regions in fetuses and children with DS (Stagni et al., 2018a). These early-occurring defects are thought to be major determinants of the cognitive disability that characterizes DS from early infancy.

Children with DS are characterized by deficits in several cognitive domains, including different facets of explicit memory (Vicari et al., 2005, Vicari et al., 2013), i.e., memory of places, objects, and events. These defects are consistent with various neuroanatomical alterations observed in fetuses with DS in key regions of the temporal lobe involved in long-term memory (Contestabile et al., 2007, Guidi et al., 2008, Guidi et al., 2018, Stagni et al., 2018b). In addition to memory defects, individuals with DS show poor performance in executive function that starts from infancy and persists in adulthood (Chapman and Hesketh, 2000, Lanfranchi et al., 2010, Grieco et al., 2015, Loveall et al., 2017). Executive functions are a set of cognitive processes that include attentional control, inhibitory control, working memory, and cognitive flexibility, and that are necessary for the cognitive control of behavior (Zelazo et al., 2003). They are necessary in adapting to new circumstances, rather than for well-learned behaviors or familiar routines. The thalamus is a crucial node of the cortico-striatal-thalamo-cortical circuits and its role in attention, arousal, attentional shift, attention to salient stimuli, and processing of salient information has been documented by studies in animal models and functional connectivity studies in humans (Metzger et al., 2013). While it is well established that the cortical structures of the medial temporal lobe are fundamental for explicit memory, certain thalamic nuclei are fundamental for higher-order control processes that subserve problem-solving, concept formation, task switching, inhibition, initiation of rapid and fluent responses, and planning. Most of the studies aimed at detecting early neuroanatomical

alterations in individuals with DS that may account for their cognitive impairment have focused on cortical structures (Stagni et al., 2018a). Thalamic nuclei involved in higher-order control processes, however, have been largely neglected.

The mediodorsal thalamic nucleus (MD) is reciprocally connected with the prefrontal cortex, amygdala, hippocampal region, and basal ganglia and is part of the cerebral circuits underlying the neuronal network for executive control. Consistently with its anatomical connection, a lesion of the MD leads to impairment of functions such as working memory, association memory, emotion, attention, and learning (see (Lee and Shin, 2016)). The MD is the most studied thalamic nucleus in relation to schizophrenia because its primary target, the prefrontal cortex, has been consistently implicated in schizophrenia (see (Byne et al., 2009)). Recent evidence shows that elderly people with DS have an MD with a reduced number of neurons (Karlsen et al., 2014). This reduction has been interpreted as the possible outcome of age-related neurodegeneration. Yet, considering the widespread neurogenesis alterations that characterize DS it is conceivable that this nucleus exhibits a reduced asset of neurons starting from fetal life stages. If so, this defect may contribute to explain the early impairment of cognitive functions in which this nucleus is involved in children with DS. The centromedian nucleus (CM) projects to the basal ganglia and, similarly to other intralaminar nuclei of the thalamus, sends diffuse excitatory projections to the cerebral cortex. The CM, in addition to being involved in attention and arousal, plays a role in the processing of verbal information, and integrates activity conveying both interoceptive and exteroceptive information to cortical systems involved in reward and cognition (see (Byne et al., 2009)). Decreased attention, hyperactivity, and impulsivity are frequently reported in children with DS (Ekstein et al., 2011), suggesting that alterations of the CM may take part in these behavioral defects. The parafascicular nucleus (PF) has been shown to influence behavioral flexibility (Brown et al., 2010) and to be involved in object recognition tasks (Castiblanco-Pineros et al., 2011). In view of its function, it is possible that alterations of the PF may be involved in the deficits of memory and executive function in DS.

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4 In view of the role of the MD, CM, and PF in various cognitive domains, it seems likely that
5 anatomical alterations of these thalamic nuclei contribute to the behavioral alterations that characterize
6 DS. Considering that thalamus-related behavioral impairment is already present in infants/children with
7 DS, it is conceivable that development of the thalamus is already damaged starting from prenatal stages.
8 Thus, in order to shed light on this issue, in the current study we examined the MD, CM, and PF of fetuses
9 with DS with the goal to establish whether these nuclei exhibit early neuroanatomical alterations.
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22 **2. MATERIALS AND METHODS**

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26 **2.1 Subjects**

27 For this study, we used fetuses of between 17 and 22 gestational weeks (GW). Brains were obtained after
28 prior informed consent from the parents and according to procedures approved by the Ethical Committee
29 of the St. Orsola-Malpighi Hospital, Bologna, Italy. Regulations of the Italian Ministry of Health and the
30 policy of Declaration of Helsinki were followed. All fetuses derived from legal abortion and were
31 collected with an average post-mortem delay of approximately 2 h. Seven euploid fetuses with no obvious
32 developmental or neuropathological abnormalities and seven fetuses with DS were used (Table 1). Some
33 of the fetuses (cases marked with an asterisk in Table 1) are the same used in previous studies (Guidi et
34 al., 2008, Stagni et al., 2018b). Trisomy was karyotypically proven from the results of genetic
35 amniocentesis procedures. All cases were classical trisomy 21 (free trisomy). Autopsies were performed at
36 the Institute of Pathology of the St. Orsola Malpighi Hospital (Bologna, Italy). The gestational age of each
37 fetus was estimated through menstrual history and crown-rump length.
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53 **2.2 Histological procedures**

54 Whole brains were fixed using subdural perfusion with Metacarnoy fixative (methyl alcohol: chloroform:
55 acetic acid 6:1:1) injected through the anterior and posterior fontanelles. After 24-48 h the brains were
56 removed, the region of the right hemisphere caudal to the mammillary bodies was dissected out and
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4 coronally sectioned in order to obtain three to four blocks with a thickness of 2-3 mm. The blocks were
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6 post-fixed in 4% buffered formaldehyde for 5-7 days and embedded in paraffin according to standard
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8 procedures. The first block was cut so as to obtain either 8 μm or 12 μm -thick sections, 440 μm or 360 μm
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10 apart, as well as five to six series consisting of three-four sections, 200 μm apart, with a thickness of 4 μm
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12 or 5 μm , as previously described (Guidi et al., 2008).

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15 *Nissl-staining.* Nissl-staining was carried out in the 8 μm and 12 μm -thick sections, as previously
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17 described (Guidi et al., 2008).

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20 *Ki-67 and calretinin immunohistochemistry.* Ki-67 and Calretinin (CR) immunohistochemistry was
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22 carried out in the 4 μm and 5 μm -thick sections, as previously described (Guidi et al., 2008, Guidi et al.,
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24 2018). Sections immunostained for CR and Ki-67 were counterstained with Mayer's Haematoxylin.
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26 27 **2.3 Measurements**

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29 *Image acquisition.* Nissl-stained and CR immunostained sections containing the MD, CM, and PF of the
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31 thalamus were scanned at 40x magnification with a digital whole-slide scanner (Nano-Zoomer S60,
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33 Hamamatsu Photonics, Japan). The software NDP.view2 software was used in order to extract low (4x)
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35 and high (40x) magnification images of the regions of interest, i.e., the MD, CM, and PF, from the
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37 originally acquired images. These images were used to evaluate the size of the regions of interest, cell
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39 density, and surface area of neuronal nuclei. A light microscope (Leitz) equipped with a motorized stage
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41 and focus control system and a color digital camera (Coolsnap-Pro; Media Cybernetics, Silver Spring,
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43 MD, USA) was used to take bright field images of Ki-67 immunostained sections. Measurements were
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45 carried out using the Image Pro Plus software (Media Cybernetics, Silver Spring, MD 20910, USA).
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49 *Delineation of the regions of interest.* In Nissl-stained sections, cytoarchitectonic determination of borders
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51 of the MD, CM, and PF was based on Allen's Atlas online atlas of the fetal brain, which is available for
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53 fetuses at the 15th and 21st postconceptional week (<http://www.brainspan.org/static/atlas>). The MD, CM,
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55 and PF are contained in a brain region that stretches from approximately 500 μm before the appearance of
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57 the hippocampus (Fig. 1A) to approximately the end of the rostral third of the hippocampus (Fig. 1B).
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4 *Number of sampled sections.* Complete series of sections across the whole regions of interest were not
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6 available due to tissue damage during histological processing. The number of intact sections containing
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8 the regions of interest was 5-8 per fetus for Nissl-stained section, 2-3 for sections immunostained for Ki-
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10 67, and 3 for sections immunostained for CR.

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12 *Cross-sectional area of the MD, CM, and PF.* In low magnification images (4x) of all available Nissl-
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14 stained sections, we measured the area of the MD, CM, and PF. Since serial sections across the regions of
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16 interest were not available, it was not possible to estimate the volume of the MD, CM, and PF. In order to
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18 obtain a rough estimate of their size, we averaged the areas traced in individual sections.
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22 *Cell counting.* In Nissl-stained sections, a series (6-9) of rectangular areas (283x226 μm ; area 63,958 μm^2)
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24 was sampled at high magnification (40x) in the MD, CM, and PF. In order to sample cells in the whole
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26 extent of each nucleus, these areas were semi-randomly placed in rows and columns that covered the
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28 whole medio-lateral and dorso-ventral extent of each nucleus (see, for instance, Fig. 2B). All cells present
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30 in the sampled areas were counted. The total number of cells in each sampled rectangular area ranged
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32 from approximately 70 to 300, according to genotype and thalamic nucleus. In each fetus, the total number
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34 of counted cells ranged from 3,500 to 6,000 according to genotype and thalamic nucleus. Cell density was
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36 expressed as cell number/ mm^2 .
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40 *Neuron and glial cell differentiation.* During counting, neurons were distinguished from glial cells based
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42 on the shape and size of the nucleus and the presence of one clearly visible nucleolus. Neurons had visible
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44 cytoplasm around the nucleus or a small rim of cytoplasm circling the entire nucleus. Most of the neurons
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46 in the examined thalamic nuclei had a rounded shape (Fig. 1C, panels c1, c2; Fig. 1D, panels d1, d2; Fig.
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48 1E, panels e1, e2) although there were also sparser ovoid-shaped nuclei (Fig. 1C, panel c2). Small cells
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50 with an ovoid-shaped nucleus, a smooth nuclear envelope, and light blue euchromatin were considered as
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52 astrocytes (Fig. 1C, panel c3; Fig. 1D, panel d3, Fig. 1E, panel e3). In astrocytes, the nucleolus, if visible,
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54 was attached to the nuclear envelope. Astrocytes could be satellites to a neuron (Fig. 1E, panel e3).
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56 Rounded nuclei with condensed chromatin and with a surface area smaller than 12 μm were considered as
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58 oligodendrocytes or microglia (Fig. 1C, panel c4; Fig. 1D, panel e4; Fig. 1E, panel e4). Astrocytes,
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oligodendrocytes, and microglia were counted as a single entity. The density of neurons and glia was expressed as cell number/mm² and as a percentage over total number of neurons plus glia.

Neuron subtypes. In three-four sampled areas from each thalamic nucleus, we measured the surface area of all neuronal nuclei by tracing the contour of the nucleus (in each fetus approximately 250-600 nuclei were measured in each region of interest). Since the cellular volume is related to the nuclear size (Webster et al., 2009), neurons with a nuclear area $\geq 30 \mu\text{m}^2$ were considered here as large neurons and neurons with a nuclear area $< 30 \mu\text{m}^2$ were considered as small neurons. Based on this criterion, we evaluated the percentage of large and small neurons. From the percentage, we calculated the total number of large and small neurons. By averaging the areas of the sampled neurons, we evaluated the mean surface area of the total neuronal population (small plus large neurons) and the surface area of large and small neurons.

Apoptotic cells. In Nissl-stained sections, apoptotic cells were recognized based on their morphology, characterized by a pyknotic darkly-stained nucleus and light or absent cytoplasm. The number of pyknotic cells was expressed as cells/mm² and as percentage over total cell number.

Percentage of CR-positive cells. In sections processed for CR immunohistochemistry, a series (4-6) of rectangular areas (283x226 μm ; area 63,958 μm^2) was sampled at high magnification (40x) in the MD. All CR-positive and CR-negative cells within these areas were counted. The number of CR-positive cells was expressed as a percentage of the total number of counted cells.

Density of Ki-67-positive cells. Proliferating cells were identified with immunostaining for Ki-67 (Rose et al., 1994, Seress et al., 2001). Ki-67-positive cells were evaluated in the ventricular zone (VZ) of the third ventricle and in the MD, CM, and PF, in images taken at a magnification of 500x. Since the brain had been cut into two halves before histological processing, the VZ of the thalamus was not intact and, in most cases, fragments of the VZ had remained attached to the midline. We evaluated the number of Ki-67-positive cells in these fragments (2-3 sections per case; 6-12 fragments per section) by tracing an area that encompassed the whole VZ of each fragment and counting all Ki-67-positive cells in this area. Since in Ki-67-immunostained sections the borders between the MD, CM, and PF were not clearly detectable, we considered the region containing these nuclei as a single entity. Using the tiling function of Image Pro-

plus we acquired large areas (about 300,000-450,000 μm^2) within this region (2 sections per case; 2-3 areas per section) and counted all Ki-67-positive cells in each sampled area. The number of cells counted in the VZ and in the region of the MD, CM, and PF was expressed as density per mm^2 .

2.4 Statistical analysis

Results are presented as mean $\pm$ standard deviation (SD). Statistical analysis was carried out using the unpaired two-tailed Student t-test. A probability level of $p \leq 0.05$ was considered to be statistically significant.

3. RESULTS

3.1 The mediodorsal nucleus of fetuses with DS

We found no differences between the mean cross-sectional area of the MD of fetuses with DS and that of euploid fetuses (Fig. 2C). An evaluation of cell density, however, showed that fetuses with DS had a notably reduced cell density (-40%) in comparison with euploid fetuses (Fig. 2E). During cell counting, we counted neurons and glial cells separately. Most of the cells in the MD (approximately 89%; see Fig. 2H) were neurons and only a limited number of cells had a glial phenotype. The separate evaluation of the density of neurons and glia showed that fetuses with DS had a notably reduced number of both neuronal (-39%; Fig. 2F) and glial (-42%; Fig. 2G) cells in comparison with euploid fetuses. A comparison of the percentage of neurons and glia showed no differences between fetuses with DS and euploid fetuses (Fig. 2H,I).

The MD of newborn and adult human subjects contains both large and small neurons (Dorph-Petersen et al., 2004, Abitz et al., 2007). The former are thought to be projection neurons and the latter are thought to represent local interneurons (Dorph-Petersen et al., 2004). Consistently with this evidence, we found neurons with small and large nuclei in the fetal MD (see Fig. 1). A comparison of the percentage of large neurons in fetuses with DS and euploid fetuses showed that fetuses with DS had a notably reduced

percentage of large neurons (Fig. 3A). In euploid fetuses, large neurons counted for approximately 60% of the overall neuronal population. This value is similar to that found in the MD of adult subjects (Karlsen et al., 2014). In fetuses with DS, however, large neurons made up only 34% of the overall neuronal population (Fig. 3A). Consequently, fetuses with DS had a larger percentage of small neurons in comparison with euploid fetuses (Fig. 3B). An evaluation of the absolute number of large and small neurons showed that in fetuses with DS the total number of large neurons per unit area was notably lower (-68%) than in control fetuses (Fig. 3C), but that there were no differences in the total number of small neurons (Fig. 3D). An evaluation of the surface area of all sampled nuclei (small plus large nuclei) showed that its value was significantly reduced (-15%) in fetuses with DS in comparison with euploid fetuses (Fig. 3E). No differences were found in the mean surface area of the large nuclei (Fig. 3F). The surface area of the small nuclei, however, was reduced in fetuses with DS in comparison with euploid fetuses (Fig. 3G).

An evaluation of the number of apoptotic cells showed no differences between euploid fetuses and fetuses with DS, both in terms of cell density (Table 2A) and percentage over total cell number (Table 2B).

Calretinin (CR), which is a marker of inhibitory GABAergic interneurons, has been shown to be expressed at early stages of embryonic (Gonzalez-Gomez and Meyer, 2014) and fetal (Bayatti et al., 2008) brain development. Observation of Fig. 4 shows a particularly high expression of CR in the thalamus in comparison with the other brain areas. Consistently with previous evidence (Kultas-Ilinsky et al., 2004), CR expression was not uniform throughout the thalamus, with some thalamic regions, including the CM, exhibiting very scarce immunoreactivity (see Fig. 4A). Since among the thalamic nuclei examined here the MD exhibited a very high immunoreactivity, we thought it of interest to evaluate the number of immunoreactive cells in this nucleus. We found that most of the cells (about 80%) in the MD were immunopositive for CR (Fig. 4B). A comparison of euploid fetuses and fetuses with DS showed no differences in the percentage of CR-positive cells (Fig. 4C).

3.2 The centromedian nucleus of fetuses with DS

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4 We found no differences between fetuses with DS and euploid fetuses in the mean cross-sectional
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6 area of the CM (Fig. 5C). In contrast, overall cell density was notably lower (-42%) in fetuses with DS in
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8 comparison with euploid fetuses (Fig. 5E). Similarly to the MD, most of the cells in the CM were neurons
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10 (see Fig. 5H) and only a few cells had a glial phenotype. The separate evaluation of the density of neurons
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12 and glia showed that fetuses with DS had a notably reduced number of both neuronal (-43%; Fig. 5F) and
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14 glial cells (-33%; Fig. 5G). A comparison of the percentage of neurons and glia showed no differences
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16 between fetuses with DS and euploid fetuses (Fig. 5H,I).
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19 We found that, similarly to the MD, the fetal CM was populated by neurons with small and large
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21 nuclei (see Fig. 1D). At variance with the MD (see above) and PF (see below), in euploid fetuses large
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23 neurons represented less than one half of the overall neuronal population (Fig. 6A). A comparison of the
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25 percentage of large neurons in fetuses with DS and euploid fetuses showed that fetuses with DS had fewer
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27 large neurons. While in euploid fetuses large neurons counted for approximately 46% of the overall
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29 neuronal population, in fetuses with DS large neurons made up only 31% (Fig. 6A). Consequently, fetuses
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31 with DS had a larger percentage of small neurons in comparison with euploid fetuses (Fig. 6B). An
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33 evaluation of the absolute number of large and small neurons showed that in fetuses with DS the total
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35 number of large neurons per unit area was notably lower (-62%) than in control fetuses (Fig. 6C) but that
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37 there were no differences in the total number of small neurons (Fig. 6D). An evaluation of the surface area
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39 of all sampled nuclei (small plus large nuclei) showed that its value was significantly reduced (-12%) in
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41 fetuses with DS in comparison with euploid fetuses (Fig. 6E). No statistically significant differences were
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43 found in the mean surface area of the large nuclei (Fig. 6F) or in that of small nuclei (Fig. 6G), although in
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45 fetuses with DS the surface area of the small nuclei was reduced in comparison with euploid fetuses (Fig.
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47 6G).
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53 An evaluation of the number of apoptotic cells showed no differences between euploid fetuses and
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55 fetuses with DS, both in terms of cell density (Table 2A) and percentage over total cell number (Table
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57 2B).
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3.3 The *parafascicular nucleus* of fetuses with DS

We found no differences in the mean cross-sectional area of the PF of fetuses with DS and euploid fetuses (Fig. 7C). In contrast, overall cell density was significantly lower (-30%) in fetuses with DS in comparison with euploid fetuses (Fig. 7E). Similarly to the MD and CM, most of the cells in the PF were neurons (see Fig. 7H) and only a few cells had a glial phenotype. The separate evaluation of the density of neurons and glia showed that fetuses with DS had a reduced number of both neuronal (-28%; Fig. 7F) and glial cells (-40%; Fig. 7G). A comparison of the percentage of neurons and glia showed no differences between fetuses with DS and euploid fetuses (Fig. 7H,I).

In the fetal PF, similarly to the MD and CM, we found neurons with small and large nuclei (see Fig. 1D). Similarly to the MD (see above), in the PF of euploid fetuses large neurons represented the larger fraction of the overall neuronal population (Fig. 8A). A comparison of the percentage of large neurons in fetuses with DS and euploid fetuses showed that fetuses with DS had significantly fewer large neurons. While in euploid fetuses large neurons represented approximately 52% of the overall neuronal population, in fetuses with DS large neurons made up 31% (Fig. 8A). Consequently, fetuses with DS had a larger percentage of small neurons in comparison with euploid fetuses (Fig. 8B). An evaluation of the absolute number of large and small neurons showed that in fetuses with DS the total number of large neurons per unit area was notably lower (-59%) than in control fetuses (Fig. 8C) but that there were no differences in the total number of small neurons (Fig. 8D). An evaluation of the surface area of all sampled nuclei (small plus large nuclei) showed that its value was significantly reduced (-19%) in fetuses with DS in comparison with euploid fetuses (Fig. 8E). No differences were found in the mean surface area of the large nuclei (Fig. 8F) or in that of small nuclei (Fig. 8G), although in fetuses with DS the surface area of both the large (Fig. 8F) and small (Fig. 8G) nuclei was reduced in comparison with euploid fetuses.

An evaluation of the number of apoptotic cells showed no differences between euploid fetuses and fetuses with DS, both in terms of cell density (Table 2A) and percentage over total cell number (Table 2B).

3.4 Reduced density of proliferating cells in the VZ and MD, CM, and PF of fetuses with DS

Cells populating the thalamus derive from neural progenitor cells (NPCs) in the VZ of the third ventricle. NPCs leaving the VZ retain proliferative capacity while migrating to their final destination where they differentiate into neurons. Proliferating cells can be detected with Ki-67 immunohistochemistry, because this endogenous marker is expressed during all phases of the cell cycle. We previously found fewer proliferating NPCs in various neurogenic niches of fetuses with DS in comparison with euploid fetuses (Contestabile et al., 2007, Guidi et al., 2008, Guidi et al., 2011, Stagni et al., 2018b). In the current study, we examined the density of Ki-67-positive cells in the VZ of the third ventricle (Fig. 9A) and that of migrating Ki-67-positive cells (neuroblasts) in the region of the MD, CM, and PF (Fig. 9C). Since the borders between these nuclei were scarcely detectable in sections immunostained with Ki-67, they were considered as a single entity in the evaluation of the density of Ki-67-positive cells.

Results show that in fetuses with DS the VZ of the third ventricle had fewer Ki-67-positive cells/mm² in comparison with euploid fetuses (-45%; Fig. 9B), although the difference only approached statistical significance (p=0.0543, two-tailed t-test). Scattered Ki-67-positive cells could be detected in the region of the MD, CM, and PF. A comparison of euploid fetuses and fetuses with DS showed that in fetuses with DS there were significantly fewer (-60%; Fig. 9D) Ki-67-positive cells/mm² in comparison with euploid fetuses. Taken together, these results suggest an impairment in the size of the population of the actively dividing NPCs in the VZ of the third ventricle of fetuses with DS. Consistently with this reduction, fetuses with DS had a reduced density of migrating new cells in the region of the MD, CM, and PF.

4. DISCUSSION

4.1 Reduced number of neuronal and glial cells in the MD, CM, and PF of fetuses with DS

We found that the density of the cells populating each of the three investigated thalamic nuclei was notably reduced in fetuses with DS in comparison with euploid fetuses. In contrast, no differences between euploid fetuses and fetuses with DS were found in the cross-sectional area of these nuclei. The finding of cell density reduction in the presence of a similar nuclear size strongly suggests that in the MD, CM, and PF of fetuses with DS there is a reduction in the total number of cells forming these nuclei. In all examined nuclei, the separate evaluation of the density of neurons and glia showed that fetuses with DS had fewer neurons and fewer glial cells in comparison with their euploid counterparts. In absolute terms, in the fetal MD, CM, and PF neurons largely outnumbered glial cells. The production of neurons and glia occurs at discrete time points during fetal brain development. Gliogenesis follows neurogenesis and persists long after neurogenesis has ceased (Lee et al., 2000, Sauvageot and Stiles, 2002, Yamaguchi et al., 2016). The timing of neuron and glia generation accounts for the small glia/neuron ratio found here and in previous studies (Guidi et al., 2008, Guidi et al., 2018, Stagni et al., 2018b) in the fetal brain in comparison with the large glia/neuron ratio that characterizes the adult brain. A comparison of the percentage of neuronal and glial cells in the MD, CM, and PF of fetuses with DS and euploid fetuses showed that fetuses with DS had a similar ratio to that of euploid fetuses, indicating a similar impairment in the production of both cell types.

We found here a reduced number of proliferating cells in the VZ of the third ventricle. This fits with previous demonstration of a reduction in the size of the pool of neural progenitor cells in the VZ of the temporal lobe, hippocampus, subiculum, and parahippocampal gyrus, and in the dentate gyrus and cerebellum of fetuses with DS (Contestabile et al., 2007, Guidi et al., 2008, Guidi et al., 2011, Guidi et al., 2018, Stagni et al., 2018b). The thalamic neurons (and glia) derive from neural progenitor cells lining the third ventricle (Wang et al., 2011). The conspicuous cellularity reduction found here in the MD, CM, and PF of fetuses with DS strongly suggests that it is underpinned by impairment in the proliferation rate of neural progenitors in the thalamic ventricular zone. This conclusion is strengthened by the observation that fetuses with DS did not show an increase in cell death in comparison with euploid fetuses.

Two reports provide evidence for a reduction in the number of neurons and glial cells in anterior thalamic nuclei (Perry et al., 2018) and the MD (Karlsen et al., 2014) of aged people with DS. Since individuals with DS are bound to develop Alzheimer's disease, this reduction has been attributed to neurodegeneration. Our study, suggests that the reduced number of thalamic neurons (and glia) in adults with DS is attributable to impairment of neurogenesis during fetal development. It cannot be ruled out that neurodegeneration at adult life stages may add up, causing a further neuronal loss.

4.2 Reduced number of projection neurons in the MD, CM, and PF of fetuses with DS

In all thalamic nuclei examined here, fetuses with DS exhibited a reduction in the total number of neurons in comparison with euploid fetuses. The separate evaluation of large and small neurons showed that fetuses with DS had a notably reduced number of large neurons. In contrast, there were no differences in the number of small neurons. The former are thought to be projection neurons and the latter are thought to represent local interneurons (Dorph-Petersen et al., 2004). The factors that drive the formation of different subtypes of thalamic neurons remain to be established. Several studies have shown the importance of the Sonic hedgehog (Shh) pathway for cell fate specification during thalamic development (see (Leyva-Diaz and Lopez-Bendito, 2013)). In addition, it has been shown in the developing spinal cord that when the Shh signaling is blocked the generation of motor neurons is impaired and neural progenitors differentiate into interneurons (Ericson et al., 1996). Reduction of Shh levels has been detected in the brain of fetuses with DS (Trazzi et al., 2011), suggesting that this defect may be involved in the impairment of cell fate specification in the thalamus.

While interneurons subserve local signal processing and their activity regulates the excitability of the projection neurons, projection neurons send their axons away from the site of origin and take part in the activity of cerebral circuits. The large interneuron/principal neuron ratio in the MD, CM, and PF of fetuses with DS suggests a possible excitation/inhibition imbalance, which, in turn, may alter the responsivity of the principal neurons to incoming signals. The reduced number of projection neurons in the MD, CM, and PF of fetuses with DS implies a reduction in the output from these nuclei. Excitability

alterations of the projection neurons in conjunction with a reduction in the amount of output signals may concur to alter the processing of signals in the circuits that include the MD, CM, and PF and, consequently, to modify their function.

4.3 Neuronal hypotrophy in the MD, CM, and PF of fetuses with DS

In each thalamic nucleus that we investigated, we found an overall reduction in the surface area of neuronal nuclei from fetuses with DS in comparison with euploid fetuses. This defect appeared to be mainly due to small neurons (interneurons), because the nuclear size of large neurons was relatively well preserved. A recent study demonstrates that cortical interneurons generated from induced pluripotent stem cells (iPSCs) in DS have a reduced size in comparison with euploid iPSCs (Huo et al., 2018). Moreover, a reduction in the size of granule cell nuclei has been found in the hippocampal dentate gyrus of the Ts65Dn model of DS (Mojabi et al., 2016). Taken together, these data suggest that the trisomic condition may negatively affect the size of neuronal nuclei. The factors that govern nuclear size and the reasons behind the reduced nuclear size of neurons in DS remain to be established.

4.4 High percentage of CR-expressing neurons in the fetal MD

CR has been shown to be expressed early in thalamic development and to be unevenly distributed in the thalamus (Kultas-Ilinsky et al., 2004). In the rat thalamus, CR is expressed early during embryonic development in a subpopulation of post-mitotic neurons and most of the thalamic neurons expressing CR during embryonic life continue to express this protein throughout the postnatal development and in adulthood (Frassoni et al., 1998). A heterogeneous pattern of distribution of CR also appears to be retained in the adult human thalamus, with a strong expression in the MD and midline nuclei (Fortin et al., 1998). Confirming this evidence, we found that CR was unevenly expressed in different thalamic nuclei and had a very high expression in the MD, where about 80% of the cells were CR-positive. The high number of CR-expressing neurons in the MD is at variance with the low CR expression in the fetal temporal cortex, subiculum, and hippocampal formation, where CR-positive cells represent less than 5% of the total cell

population (Guidi et al., 2018, Stagni et al., 2018b). The role of the high expression of CR in certain thalamic nuclei needs to be further investigated. Unlike in the temporal cortex, subiculum, and hippocampus (Guidi et al., 2018, Stagni et al., 2018b), in the MD of fetuses with DS the percentage of CR-expressing cells was similar to that found in euploid fetuses, indicating that differences in the expression levels of CR may be region-specific.

4.5 Conclusions

The cognitive deficits of DS have been mainly attributed to an overall reduction in the size of the cerebral hemisphere, hypocellularity, and disorganization of various cortical regions. Remarkably, these defects are present in fetuses and children with DS, indicating that neurodevelopmental alterations are an early occurring event in DS (Stagni et al., 2018a). Since little attention has been devoted to thalamic structures involved in cognition in fetuses with DS, in the current study we thought it of interest to examine the cellularity of three thalamic nuclei involved in higher order function. We found that, similarly to other brain regions of fetuses with DS, the three thalamic nuclei examined here exhibited a notably reduced number of cells in comparison with euploid fetuses.

Unbiased cell counting requires the use of stereological methods. The optical fractionator is a stereology system that pairs the optical disector probe (a three-dimensional counting space) with a two-dimensional grid that provides an unbiased systematic interval sampling of the region of interest (Gundersen et al., 1999). Unfortunately, the fetal material available for the current study prevented the use of the optical fractionator for evaluation of total cell number, due to the small number of sections and small section thickness. Therefore, cell counting was carried out in planar probes randomly placed on the region of interest. Fetal material is very precious because it may help understand a given pathology and devise possible means to prevent/minimize its consequences. There is paucity of information regarding the development of the thalamus in fetuses with DS. Therefore, in spite of the limitations of our measurements, the results obtained here may provide useful insight within the framework of brain developmental alterations in DS. We found that in fetuses with DS all three examined thalamic nuclei

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4 differ from those of euploid fetuses in terms of overall cellularity, neurons and astrocyte number, and
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6 types of neurons, indicating impairment in thalamic development at the very beginning of brain formation.
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8 Little attention has been devoted so far to subcortical structures involved in cognition in DS. The current
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10 study shows that the cortical defects in fetuses with DS are complemented by defects in the organization
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12 of thalamic nuclei that diffusely project to the cortex. These thalamic alterations may contribute to the
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14 defects in executive function (and, possibly, other functions) that characterize infants/children with DS.
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26 **CONFLICT OF INTEREST**

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28 The authors declare that they have no conflict of interest.
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33 **ETHICAL APPROVAL**

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35 All procedures performed in studies involving human participants were in accordance with the ethical
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37 standards of the institutional research committee (Ethical Committee of the St. Orsola-Malpighi Hospital,
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39 Bologna, Italy, reference number 27/2006/U/Tess) and with the 1964 Helsinki declaration and its later
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41 amendments or comparable ethical standards.
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48 **INFORMED CONSENT**

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51 Informed consent, for the use of fetal material in this study, has been obtained from all women who
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53 underwent legal abortion.
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REFERENCES

- Abitz M, Nielsen RD, Jones EG, Laursen H, Graem N, Pakkenberg B (2007) Excess of neurons in the human newborn mediodorsal thalamus compared with that of the adult. *Cereb Cortex* 17:2573-2578.
- Bayatti N, Moss JA, Sun L, Ambrose P, Ward JF, Lindsay S, Clowry GJ (2008) A molecular neuroanatomical study of the developing human neocortex from 8 to 17 postconceptional weeks revealing the early differentiation of the subplate and subventricular zone. *Cereb Cortex* 18:1536-1548.
- Brown HD, Baker PM, Ragozzino ME (2010) The parafascicular thalamic nucleus concomitantly influences behavioral flexibility and dorsomedial striatal acetylcholine output in rats. *J Neurosci* 30:14390-14398.
- Byne W, Hazlett EA, Buchsbaum MS, Kemether E (2009) The thalamus and schizophrenia: current status of research. *Acta Neuropathol* 117:347-368.
- Castiblanco-Pineros E, Quiroz-Padilla MF, Cardenas-Palacio CA, Cardenas FP (2011) Contribution of the parafascicular nucleus in the spontaneous object recognition task. *Neurobiol Learn Mem* 96:272-279.
- Chapman RS, Hesketh LJ (2000) Behavioral phenotype of individuals with Down syndrome. *Ment Retard Dev Disabil Res Rev* 6:84-95.
- Contestabile A, Fila T, Ceccarelli C, Bonasoni P, Bonapace L, Santini D, Bartesaghi R, Ciani E (2007) Cell cycle alteration and decreased cell proliferation in the hippocampal dentate gyrus and in the neocortical germinal matrix of fetuses with Down syndrome and in Ts65Dn mice. *Hippocampus* 17:665-678.
- Dorph-Petersen KA, Pierri JN, Sun Z, Sampson AR, Lewis DA (2004) Stereological analysis of the mediodorsal thalamic nucleus in schizophrenia: volume, neuron number, and cell types. *J Comp Neurol* 472:449-462.
- Ekstein S, Glick B, Weill M, Kay B, Berger I (2011) Down syndrome and attention-deficit/hyperactivity disorder (ADHD). *J Child Neurol* 26:1290-1295.
- Ericson J, Morton S, Kawakami A, Roelink H, Jessell TM (1996) Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. *Cell* 87:661-673.

- 1
- 2
- 3
- 4 Fortin M, Asselin MC, Gould PV, Parent A (1998) Calretinin-immunoreactive neurons in the human
- 5 thalamus. *Neuroscience* 84:537-548.
- 6
- 7 Frassoni C, Arcelli P, Selvaggio M, Spreafico R (1998) Calretinin immunoreactivity in the developing
- 8 thalamus of the rat: a marker of early generated thalamic cells. *Neuroscience* 83:1203-1214.
- 9
- 10 Gonzalez-Gomez M, Meyer G (2014) Dynamic expression of calretinin in embryonic and early fetal
- 11 human cortex. *Front Neuroanat* 8:41.
- 12
- 13 Grieco J, Pulsifer M, Seligsohn K, Skotko B, Schwartz A (2015) Down syndrome: Cognitive and
- 14 behavioral functioning across the lifespan. *Am J Med Genet C Semin Med Genet* 169:135-149.
- 15
- 16 Guidi S, Bonasoni P, Ceccarelli C, Santini D, Gualtieri F, Ciani E, Bartesaghi R (2008) Neurogenesis
- 17 impairment and increased cell death reduce total neuron number in the hippocampal region of
- 18 fetuses with Down syndrome. *Brain Pathol* 18:180-197.
- 19
- 20 Guidi S, Ciani E, Bonasoni P, Santini D, Bartesaghi R (2011) Widespread Proliferation Impairment and
- 21 Hypocellularity in the Cerebellum of Fetuses with Down Syndrome. *Brain Pathol* 21:361-373.
- 22
- 23 Guidi S, Giacomini A, Stagni F, Emili M, Uguagliati B, Bonasoni MP, Bartesaghi R (2018) Abnormal
- 24 development of the inferior temporal region in fetuses with Down syndrome. *Brain Pathol* 28:986-
- 25 998.
- 26
- 27 Gundersen HJ, Jensen EB, Kieu K, Nielsen J (1999) The efficiency of systematic sampling in stereology--
- 28 reconsidered. *J Microsc* 193:199-211.
- 29
- 30 Huo H-Q, Qu Z-Y, Yuan F, Ma L, Yao L, Xu M, Hu Y, Ji J, Bhattacharyya A, Zhang S-C, Liu Y (2018)
- 31 Modeling Down Syndrome with Patient iPSCs Reveals Cellular and Migration Deficits of
- 32 GABAergic Neurons. *Stem Cell Reports* 10:1251-1266.
- 33
- 34 Karlsen AS, Korbo S, Uylings HB, Pakkenberg B (2014) A stereological study of the mediodorsal
- 35 thalamic nucleus in Down syndrome. *Neuroscience* 279:253-259.
- 36
- 37 Kultas-Ilinsky K, Fallet C, Verney C (2004) Development of the human motor-related thalamic nuclei
- 38 during the first half of gestation, with special emphasis on GABAergic circuits. *J Comp Neurol*
- 39 476:267-289.
- 40
- 41 Lanfranchi S, Jerman O, Dal Pont E, Alberti A, Vianello R (2010) Executive function in adolescents with
- 42 Down Syndrome. *J Intellect Disabil Res* 54:308-319.
- 43
- 44 Lee JC, Mayer-Proschel M, Rao MS (2000) Gliogenesis in the central nervous system. *Glia* 30:105-121.
- 45
- 46 Lee S, Shin H-S (2016) The role of mediodorsal thalamic nucleus in fear extinction. *J Anal Sci Technol*
- 47 7:13.
- 48
- 49 Leyva-Diaz E, Lopez-Bendito G (2013) In and out from the cortex: development of major forebrain
- 50 connections. *Neuroscience* 254:26-44.
- 51
- 52
- 53
- 54
- 55
- 56
- 57
- 58
- 59
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- 61
- 62
- 63
- 64
- 65

- 1
- 2
- 3
- 4 Loveall SJ, Conners FA, Tungate AS, Hahn LJ, Osso TD (2017) A cross-sectional analysis of executive
- 5 function in Down syndrome from 2 to 35 years. *J Intellect Disabil Res* 61:877-887.
- 6
- 7 Metzger CD, van der Werf YD, Walter M (2013) Functional mapping of thalamic nuclei and their
- 8 integration into cortico-striatal-thalamo-cortical loops via ultra-high resolution imaging-from
- 9 animal anatomy to in vivo imaging in humans. *Front Neurosci* 7:24.
- 10
- 11 Mojabi FS, Fahimi A, Moghadam S, Moghadam S, Windy McNerneny M, Ponnusamy R, Kleschevnikov
- 12 A, Mobley WC, Salehi A (2016) GABAergic hyperinnervation of dentate granule cells in the
- 13 Ts65Dn mouse model of down syndrome: Exploring the role of App. *Hippocampus* 26:1641-
- 14 1654.
- 15
- 16 Perry JC, Pakkenberg B, Vann SD (2018) Striking reduction in neurons and glial cells in anterior thalamic
- 17 nuclei of older patients with Down syndrome. *Neurobiol Aging* 75:54-61.
- 18
- 19 Rose DS, Maddox PH, Brown DC (1994) Which proliferation markers for routine immunohistology? A
- 20 comparison of five antibodies. *J Clin Pathol* 47:1010-1014.
- 21
- 22 Sauvageot CM, Stiles CD (2002) Molecular mechanisms controlling cortical gliogenesis. *Curr Opin*
- 23 *Neurobiol* 12:244-249.
- 24
- 25 Seress L, Abraham H, Tornoczky T, Kosztolanyi G (2001) Cell formation in the human hippocampal
- 26 formation from mid-gestation to the late postnatal period. *Neuroscience* 105:831-843.
- 27
- 28 Stagni F, Giacomini A, Emili M, Guidi S, Bartesaghi R (2018a) Neurogenesis impairment: An early
- 29 developmental defect in Down syndrome. *Free Radic Biol Med* 114:15-32.
- 30
- 31 Stagni F, Giacomini A, Emili M, Uguagliati B, Bonasoni MP, Bartesaghi R, Guidi S (2018b) Subicular
- 32 hypotrophy in fetuses with Down syndrome and in the Ts65Dn model of Down syndrome. *Brain*
- 33 *Pathol*.
- 34
- 35 Trazzi S, Mitrugno VM, Valli E, Fuchs C, Rizzi S, Guidi S, Perini G, Bartesaghi R, Ciani E (2011) APP-
- 36 dependent up-regulation of Ptch1 underlies proliferation impairment of neural precursors in Down
- 37 syndrome. *Hum Mol Genet* 20:1560-1573.
- 38
- 39 Vicari S, Bellucci S, Carlesimo GA (2005) Visual and spatial long-term memory: differential pattern of
- 40 impairments in Williams and Down syndromes. *Dev Med Child Neurol* 47:305-311.
- 41
- 42 Vicari S, Pontillo M, Armando M (2013) Neurodevelopmental and psychiatric issues in Down's
- 43 syndrome: assessment and intervention. *Psychiatr Genet* 23:95-107.
- 44
- 45 Wang L, Bluske KK, Dickel LK, Nakagawa Y (2011) Basal progenitor cells in the embryonic mouse
- 46 thalamus - their molecular characterization and the role of neurogenins and Pax6. *Neural Dev*
- 47 6:35.
- 48
- 49 Webster M, Witkin KL, Cohen-Fix O (2009) Sizing up the nucleus: nuclear shape, size and nuclear-
- 50 envelope assembly. *J Cell Sci* 122:1477-1486.
- 51
- 52
- 53
- 54
- 55
- 56
- 57
- 58
- 59
- 60
- 61
- 62
- 63
- 64
- 65

1
2
3
4 Yamaguchi M, Seki T, Imayoshi I, Tamamaki N, Hayashi Y, Tatebayashi Y, Hitoshi S (2016) Neural
5 stem cells and neuro/gliogenesis in the central nervous system: understanding the structural and
6 functional plasticity of the developing, mature, and diseased brain. J Physiol Sci 66:197-206.
7
8
9 Zelazo PD, Muller U, Frye D, Marcovitch S, Argitis G, Boseovski J, Chiang JK, Hongwanishkul D,
10 Schuster BV, Sutherland A (2003) The development of executive function in early childhood.
11 Monogr Soc Res Child Dev 68:vii-137.
12
13
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15
16
17
18
19
20
21
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23
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FIGURE LEGENDS

Figure 1. Experimental setting.

A,B: Nissl-stained coronal sections from the forebrain of a 20-week-old euploid fetus (case 27). Sections were taken at the rostral (A) and caudal (B) border of the regions of interest in the thalamus. The star, square, and triangle indicate the approximate location of the MD, CM, and PF, respectively. Calibration bar: 5 mm. C, D, E: Examples of cells in the MD (C), CM (D), and PF (E) of a 20-week-old euploid fetus (case 178). c1, d1 and e1 show a large neuron (white arrow); c2, d2, and e2 show a small neuron (orange arrow); c3, d3 and e3 show an astrocyte (red arrowhead); c4, d4 and e4 show a putative oligodendrocyte (green arrowhead). The orange asterisk in c2 indicates a small neuron with ovoidal shape. The white asterisk in e3 indicates a large neuron in contact with an astrocyte. Calibration: 10 μ m. Abbreviations: ACX, allocortex; CCX, cingulate cortex; CGE, caudal ganglionic eminence; CM, centromedian nucleus; d, dorsal; EC, entorhinal cortex; HF, hippocampal formation; HYP, hypothalamus; IC, internal capsule; LGE, lateral ganglionic eminence; MCX, motor cortex; l, lateral; m, medial; MD, mediodorsal nucleus; PCX, periallocortex; PF, PU, putamen; parafascicular nucleus; SCX, somatosensory cortex; TCX, temporal cortex; TH, thalamus; v, ventral.

Figure 2. Cell density in the MD of euploid fetuses and fetuses with DS.

A: Nissl-stained coronal section across the thalamus of a euploid fetus (case 27). Calibration: 2 mm. B: Higher magnification of the regions enclosed by a rectangle in (A). The dots indicate the outline of the MD. The small rectangles indicate the cell-sampling scheme. The rectangle had an area of 63,958 μ m² and was randomly positioned along rows and columns so as to sample cells in the whole nuclear area. Calibration: 2 mm. C: Cross-sectional area of the MD in euploid fetuses and fetuses with DS. D: Examples of cells in the MD of a euploid fetus (case 27) and a fetus with DS (case 133). E: Cell density in the MD of euploid fetuses and fetuses with DS. F, G: Density of neuronal (F) and glial (G) cells in the MD

of euploid fetuses and fetuses with DS. H, I: Percentage of neuronal (H) and glial (I) cells in the MD of euploid fetuses and fetuses with DS. Values are mean $\pm$ SD. ** $p < 0.01$ (two-tailed t-test). Abbreviations: ACX, allocortex; CCX, cingulate cortex; CGE, caudal ganglionic eminence; DS, fetuses with Down syndrome; Eu, euploid fetuses; HYP, hypothalamus; IC, internal capsule; LGE, lateral ganglionic eminence; MCX, motor cortex; MD, mediodorsal nucleus; PCX, periallocortex; PU, putamen; SCX, somatosensory cortex; TCX, temporal cortex; TH, thalamus.

Figure 3. Density and size of large and small neurons in the MD of euploid fetuses and fetuses with DS.

A-D: Percentage (A) and density (C) of large neurons and percentage (B) and density (D) of small neurons in the MD of euploid fetuses and fetuses with DS. E-G: Surface area of the nuclei of all sampled neurons (E), of large neurons (F) and of small neurons (G). Values are mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (two-tailed t-test). Abbreviations: DS, fetuses with Down syndrome; Eu, euploid fetuses.

Figure 4. Percentage of calretinin-positive cells in the MD of euploid fetuses and fetuses with DS.

A: Coronal section of the fetal brain (case 104) immunostained for calretinin and counterstained with Mayer's haematoxylin. The regions in brown are regions immunoreactive for calretinin. Calibration: 2.5 mm. B: Examples of calretinin-positive-cells (yellow arrows) and calretinin-negative cells (green arrow) in the MD (case 101). Calibration: 20 μ m. C: Percentage of calretinin-positive cells over total cell number in the MD of fetuses with DS and euploid fetuses. Values are mean $\pm$ SD. Abbreviations: CGE, caudal ganglionic eminence; CM, centromedian nucleus; DS, fetuses with Down syndrome; EU, euploid fetuses; HF, hippocampal formation; LGE, lateral ganglionic eminence MD, mediodorsal nucleus; PCX, periallocortex; PF, parafascicular nucleus; TCX, temporal cortex.

Figure 5. Cell density in the CM of euploid fetuses and fetuses with DS.

A: Nissl-stained coronal section across the thalamus of a euploid fetus (case 27). Same section as in Fig. 2. Calibration: 2 mm. B: Higher magnification of the regions enclosed by a rectangle in (A). The dots indicate the outline of the CM. The small rectangles indicate the cell-sampling scheme. The rectangle had an area of $63,958 \mu\text{m}^2$ and was randomly positioned along rows and columns so that cells in the whole nuclear area were sampled. Calibration 2 mm. C: Cross-sectional area of the CM in euploid fetuses and fetuses with DS. D: Examples of cells in the CM of a euploid fetus (case 27) and a fetus with DS (case 133). E: Cell density in the CM of euploid fetuses and fetuses with DS. F, G: Density of neuronal (F) and glial (G) cells in the CM of euploid fetuses and fetuses with DS. H, I: Percentage of neuronal (H) and glial (I) cells in the CM of euploid fetuses and fetuses with DS. Values are mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$ (two-tailed t-test). Abbreviations: ACX, allocortex; CCX, cingulate cortex; CGE, caudal ganglionic eminence; CM, centromedian nucleus; DS, fetuses with Down syndrome; Eu, euploid fetuses; HYP, hypothalamus; IC, internal capsule; LGE, lateral ganglionic eminence; MCX, motor cortex; PCX, periallocortex; PU, putamen; SCX, somatosensory cortex; TCX, temporal cortex; TH, thalamus.

Figure 6. Density and size of large and small neurons in the CM of euploid fetuses and fetuses with DS.

A-D: Percentage (A) and density (C) of large neurons and percentage (B) and density (D) of small neurons in the CM of euploid fetuses and fetuses with DS. E-G: Surface area of the nuclei of all sampled neurons (E), of large neurons (F) and of small neurons (G). Values are mean $\pm$ SD. ** $p < 0.01$; *** $p < 0.001$ (two-tailed t-test). Abbreviations: DS, fetuses with Down syndrome; Eu, euploid fetuses.

Figure 7. Cell density in the PF of euploid fetuses and fetuses with DS.

A: Nissl-stained coronal section across the thalamus of a euploid fetus (case 27). Same section as in Figs. 2 and 4. Calibration: 2 mm. B: Higher magnification of the regions enclosed by a rectangle in (A). The

dots indicate the outline of the PF. The small rectangles indicate the cell-sampling scheme. The rectangle had an area of 63,958 μm^2 and was randomly positioned along rows and columns so as to sample cells in the whole nuclear area. Calibration 2 mm. C: Cross-sectional area of the PF in euploid fetuses and fetuses with DS. D: Examples of cells in the PF of a euploid fetus (case 27) and a fetus with DS (case 133). E: Cell density in the PF of euploid fetuses and fetuses with DS. F,G: Density of neuronal (F) and glial (G) cells in the PF of euploid fetuses and fetuses with DS. H,I: Percentage of neuronal (H) and glial (I) cells in the PF of euploid fetuses and fetuses with DS. Values are mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$ (two-tailed t-test). Abbreviations: ACX, allocortex; CCX, cingulate cortex; CGE, caudal ganglionic eminence; DS, fetuses with Down syndrome; Eu, euploid fetuses; HYP, hypothalamus; IC, internal capsule; LGE, lateral ganglionic eminence; MCX, motor cortex; PCX, periallocortex; PF, parafascicular nucleus; PU, putamen; SCX, somatosensory cortex; TCX, temporal cortex; TH, thalamus.

Figure 8. Density and size of large and small neurons in the PF of euploid fetuses and fetuses with DS.

A-D: Percentage (A) and density (C) of large neurons and percentage (B) and density (D) of small neurons in the PF of euploid fetuses and fetuses with DS. E-G: Surface area of the nuclei of all sampled neurons (E), of large neurons (F) and of small neurons (G). Values are mean $\pm$ SD. * $p < 0.05$; *** $p < 0.001$ (two-tailed t-test). Abbreviations: DS, fetuses with Down syndrome; Eu, euploid fetuses.

Figure 9. Density of proliferating cells in the VZ of the third ventricle and the region of the MD, CM, and PF of euploid fetuses and fetuses with DS.

A: Examples of sections immunostained for Ki-67 and counterstained with Mayer's haematoxylin from the VZ of the third ventricle in a euploid fetus (case 104) and a fetus with DS (case 203). Calibration: 50 μm . B: Density of Ki-67-positive cells, expressed as number of cells/ mm^2 in the VZ of the third ventricle of fetuses with DS (cases 46, 77, 166, and 203) and euploid fetuses (cases 104, 118, 164, and 178). C:

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4 Examples of sections immunostained for Ki-67 and counterstained with Mayer's haematoxylin from the
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6 thalamic region of the MD, CM, and PF in a euploid fetus (case 104) and a fetus with DS (case 203).
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8 Calibration: 20 μm . D: Density of Ki-67-positive cells, expressed as number of cells/ mm^2 in the region of
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10 the MD, CM, and PF of fetuses with DS (cases 46, 77, 156, 166, and 203) and euploid fetuses (cases 101,
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12 104, 118, and 178). The red arrows in (A) and (C) indicate Ki-67-positive cells. Values are mean $\pm$ SD.
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15 (*) $p = 0.0534$; *** $p < 0.001$ (two tailed t-test). Abbreviations: DS, fetuses with Down syndrome; EU,
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17 euploid fetuses.
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Table 1. Cases of the present study.

Case		Age (weeks)	Sex	CRL (cm)	BW (gm)
Euploid fetuses					
C118*	+§	17	M	13	180
C164*	^§	19	F	14	285
C27*	^	20	F	17	230
C101*	+§	20	M	15	340
C104*	+§	20	M	15	330
C178*	^§	21	M	n.a.	n.a.
C198	^	22	n.a.	n.a.	n.a.
Fetuses with Down syndrome					
C203*	^+§	18	F	14.5	210
C166*	^§	19	F	14	240
C133*	^	20	M	16	300
C156*	+§	20	M	17	385
C134*	^	21	F	n.a.	n.a.
C46*	^§	21	M	22	355
C77*	+§	21	F	17.5	300

List of the cases used in the present study. Cases labeled with an asterisk are the same as in a previous study (Guidi et al., 2008). Cases labeled with ^ were used for cell count in Nissl-stained sections; cases labeled with + were used for CR immunohistochemistry; cases labeled with § were used for Ki-67 immunohistochemistry. Age refers to gestational age in weeks. CRL, crown-rump length; BW, body weight; F, female; M, male; n.a., not available.

Table 2. Number of pyknotic cells in the MD, CM, and PF of euploid fetuses and fetuses with DS

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	MD	CM	PF
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
Eu	10.83 $\pm$ 1.66	10.16 $\pm$ 0.61	9.98 $\pm$ 2.28
DS	10.26 $\pm$ 1.13	8.11 $\pm$ 1.30	8.95 $\pm$ 1.17
<i>p</i>	n.s.	n.s.	n.s.

B

	MD	CM	PF
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
Eu	0.31 $\pm$ 0.03	0.37 $\pm$ 0.10	0.34 $\pm$ 0.11
DS	0.54 $\pm$ 0.24	0.52 $\pm$ 0.17	0.45 $\pm$ 0.15
<i>p</i>	n.s.	n.s.	n.s.

Number of pyknotic cells in the mediodorsal nucleus, centromedian nucleus, and parafascicular nucleus expressed as number/mm² (A) and percentage over total cell number (B). n.s., not significant (two-tailed t-test). Abbreviations: CM, centromedian nucleus; DS, fetuses with Down syndrome; Eu, euploid fetuses. MD, mediodorsal nucleus; PF, parafascicular nucleus.

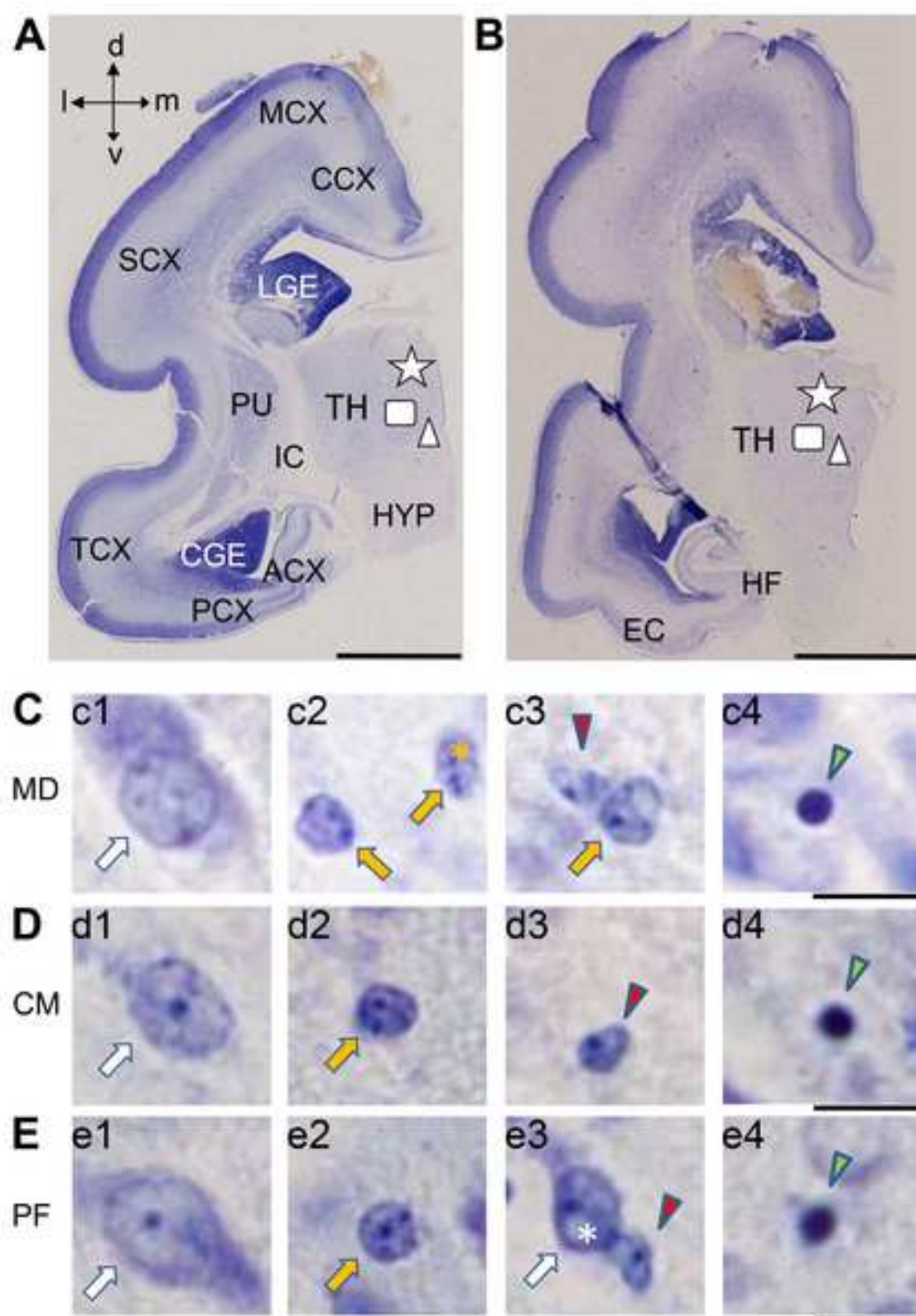


Fig. 1

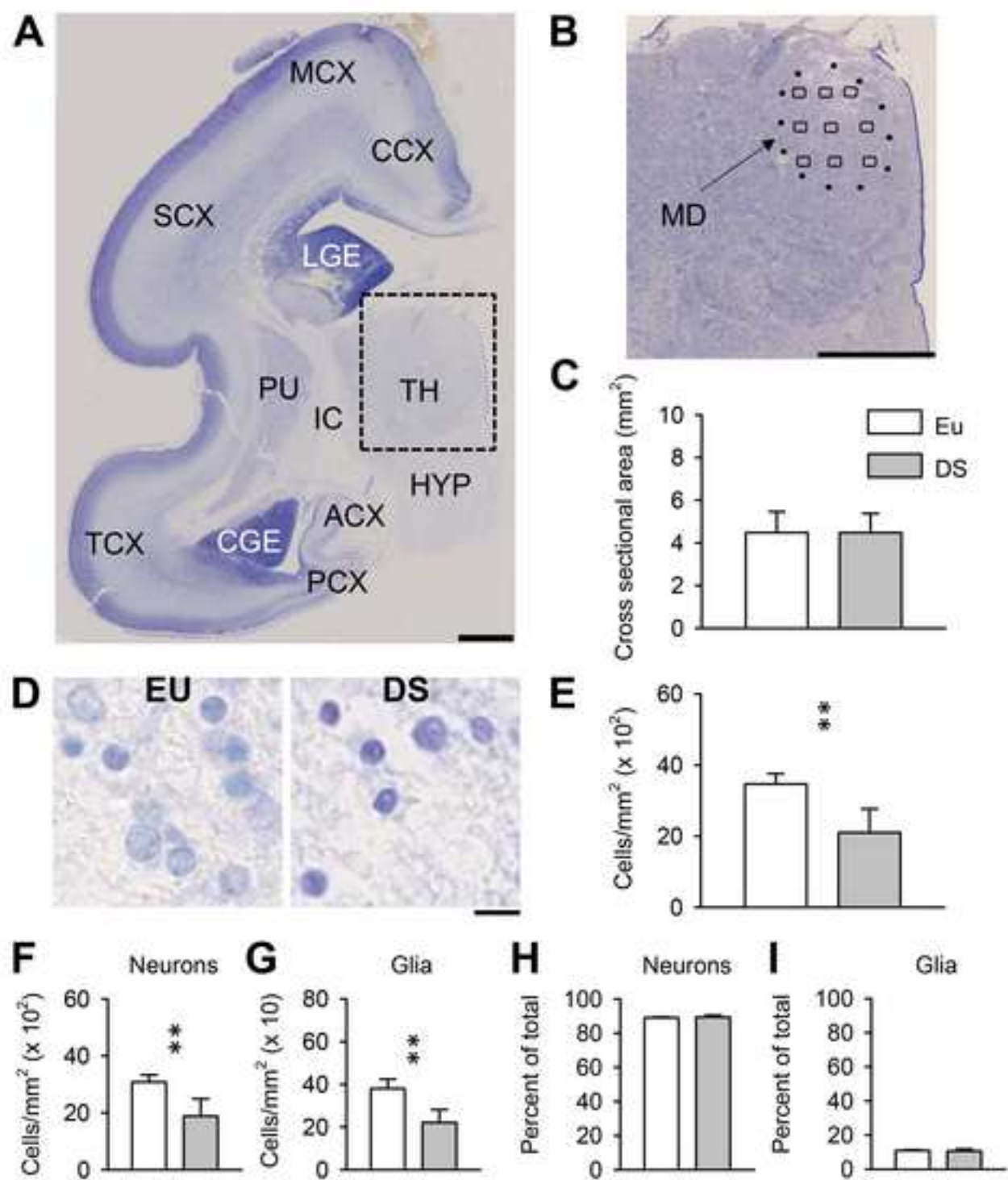


Fig. 2

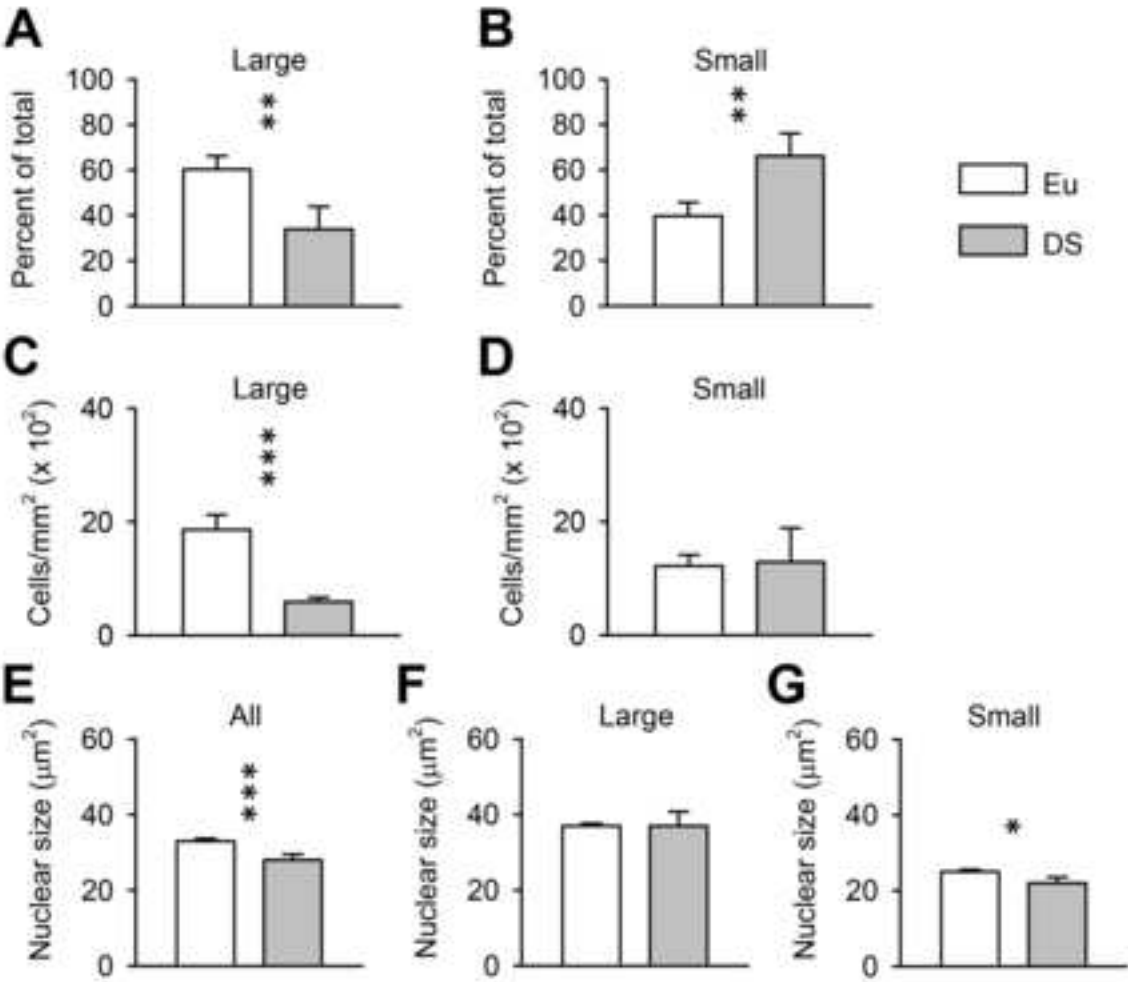


Fig. 3

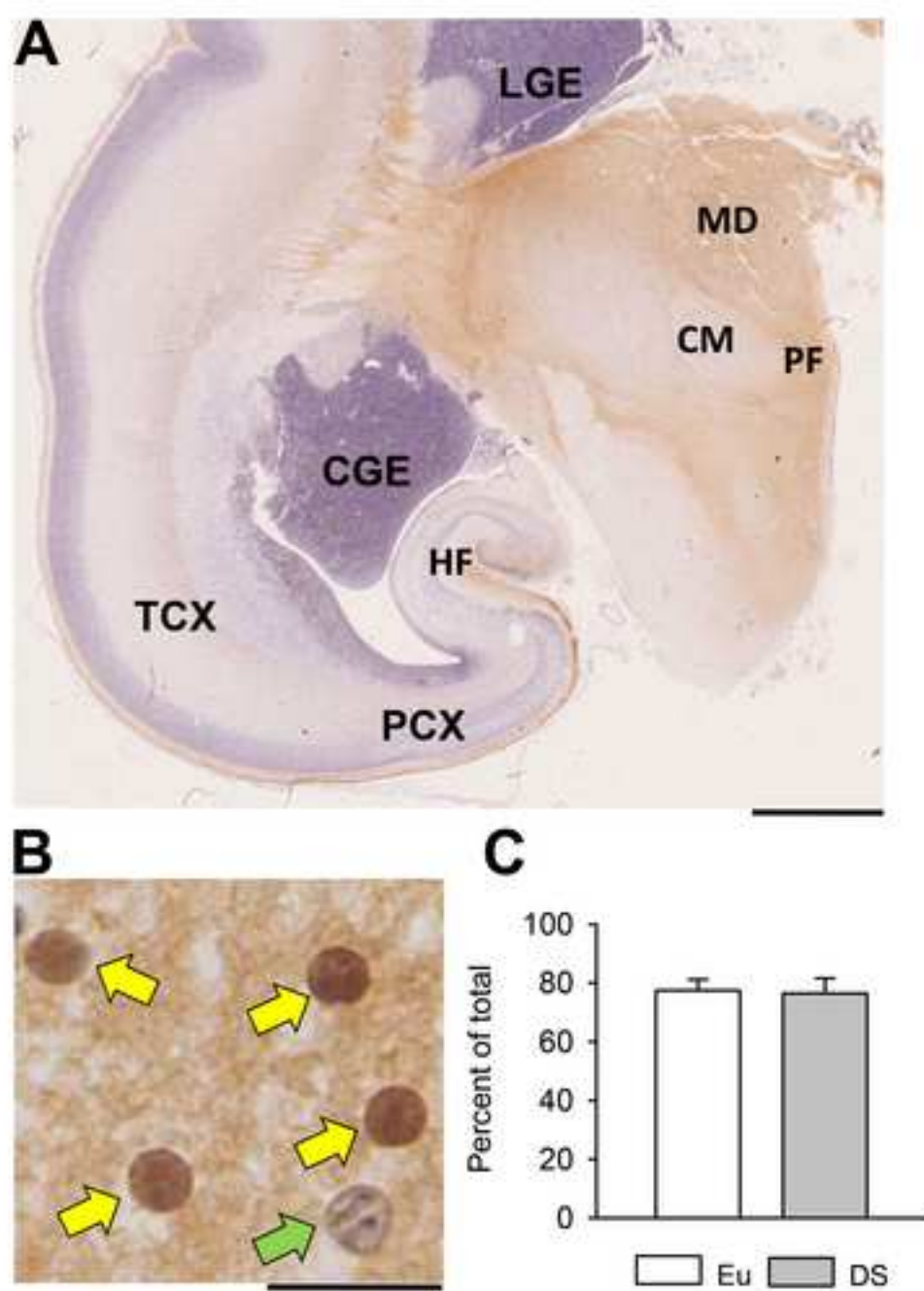


Fig 4

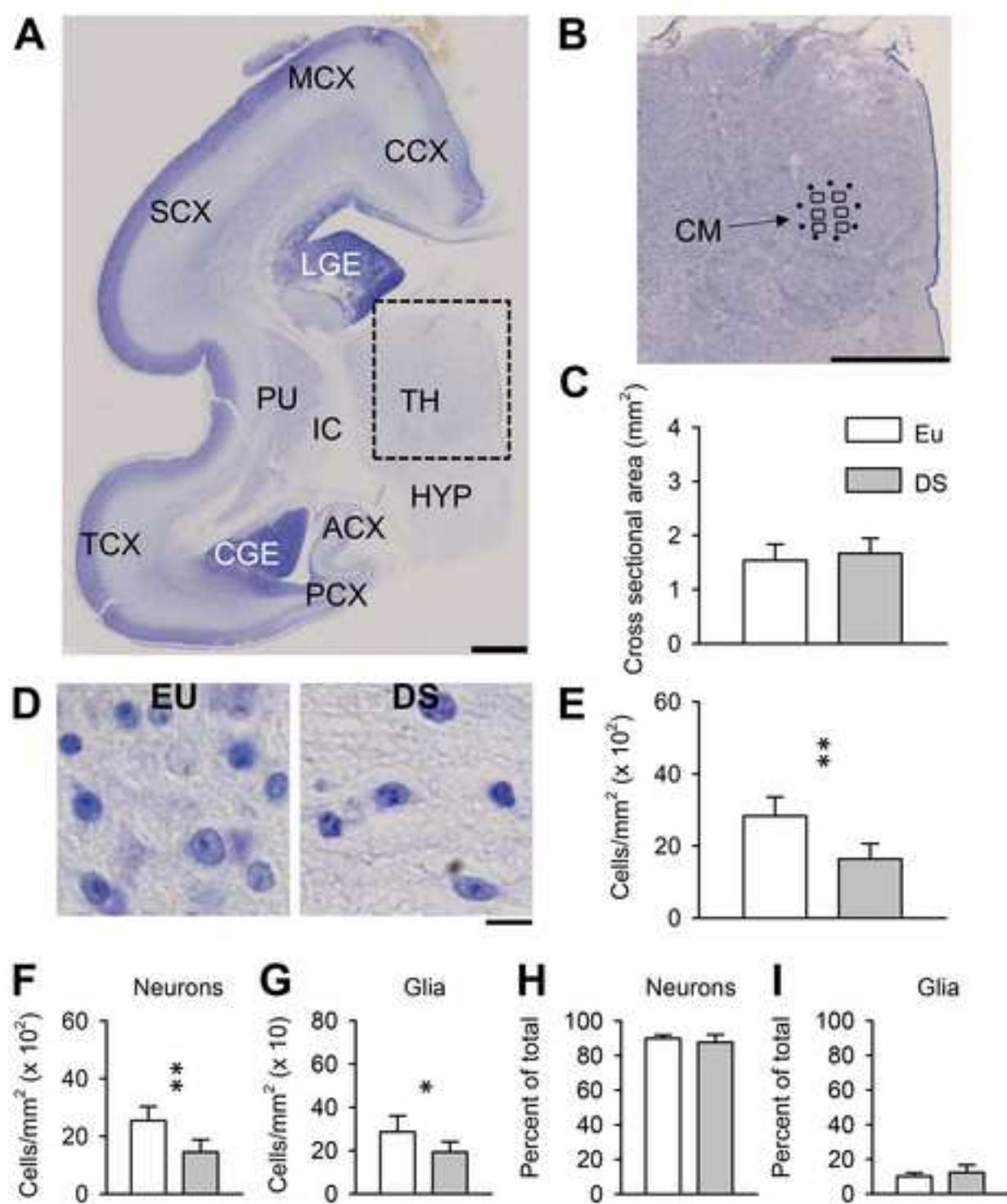


Fig. 5

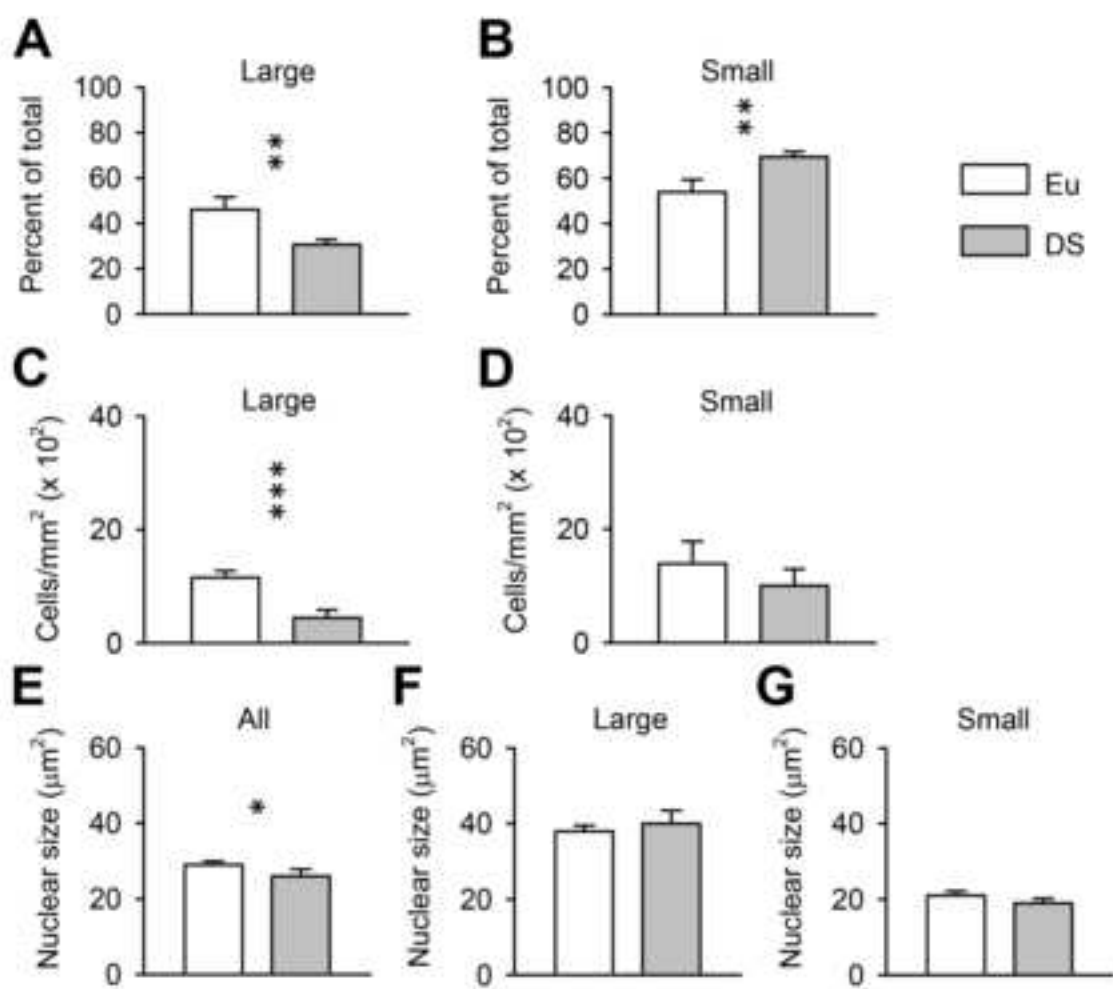


Fig. 6

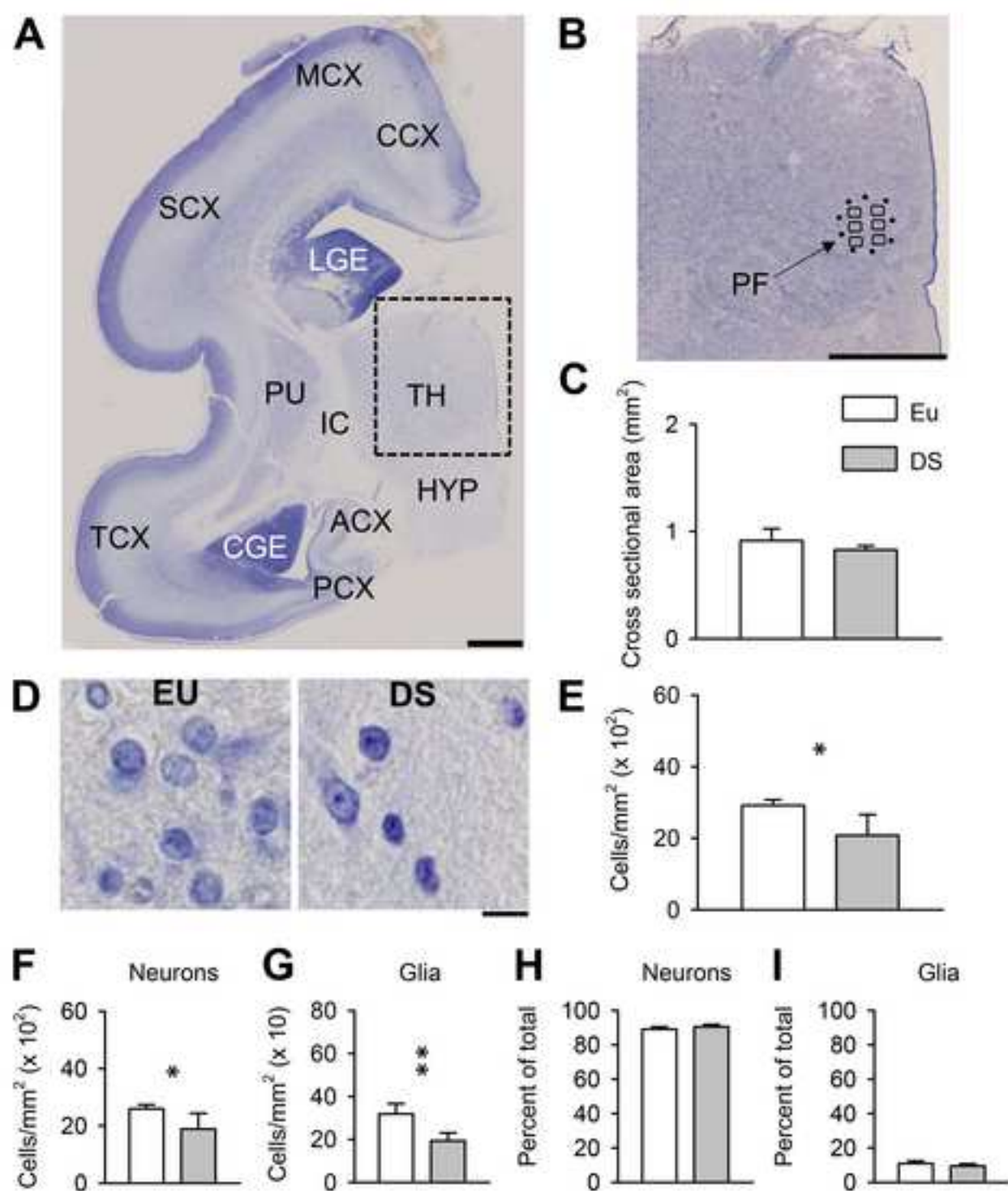


Fig. 7

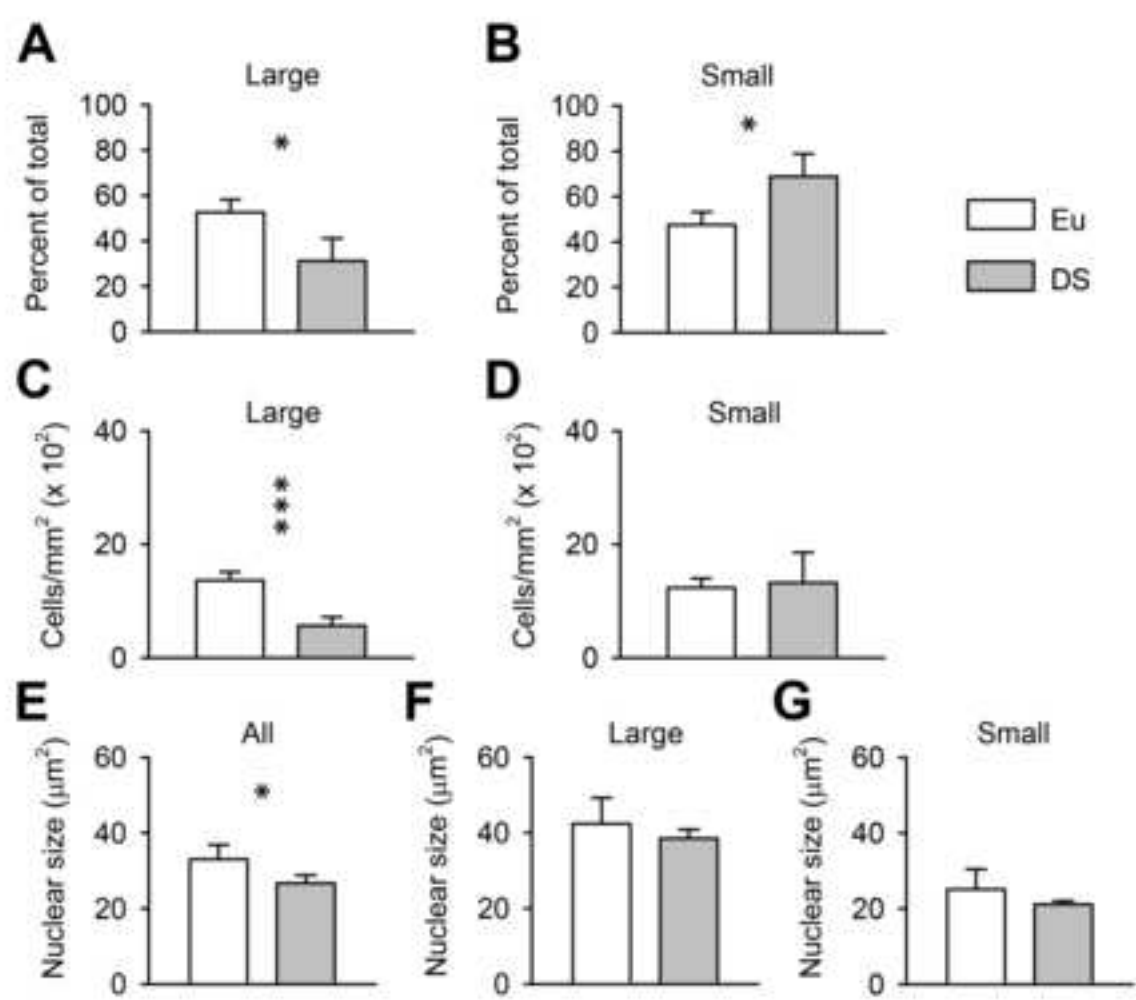


Fig. 8

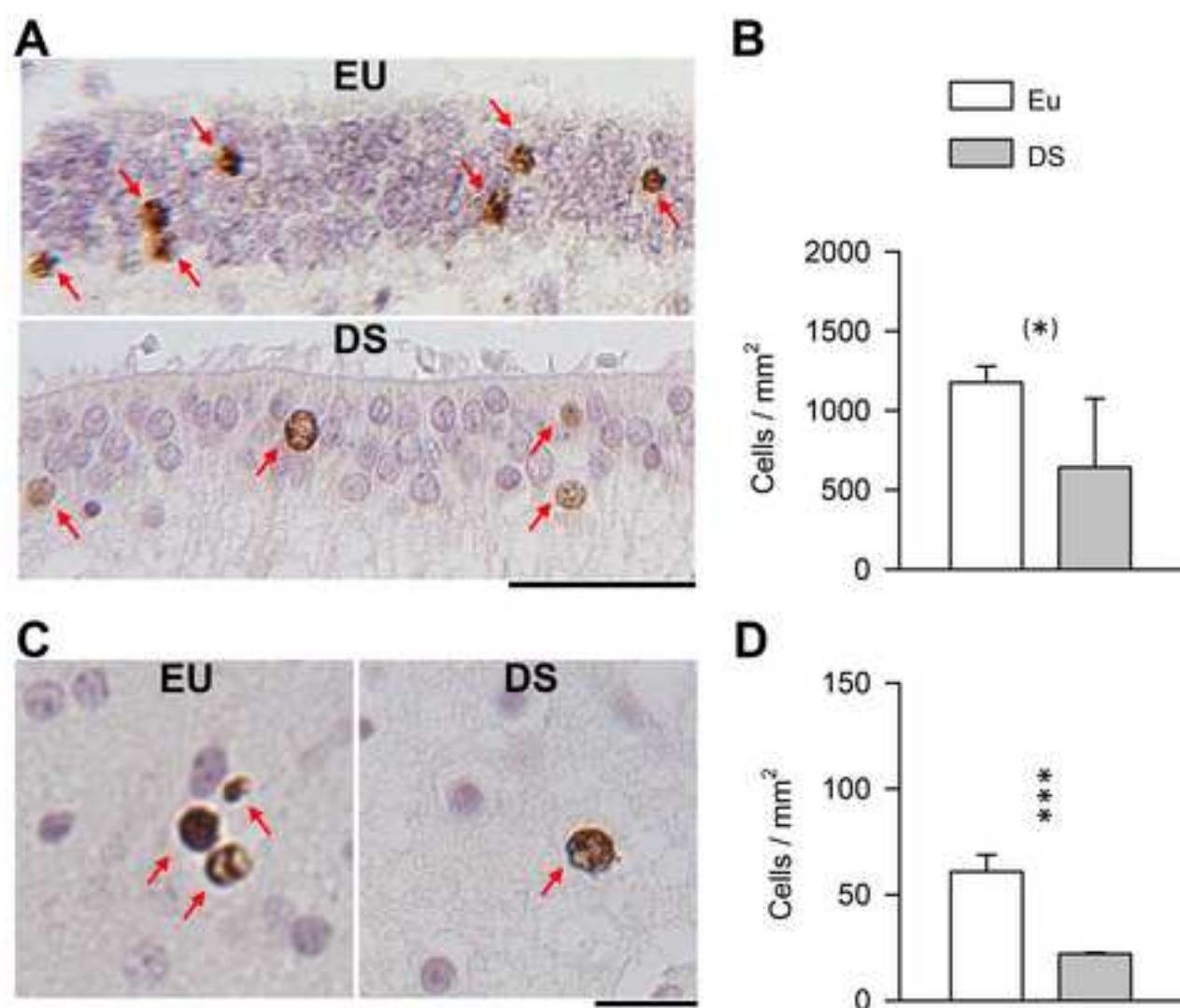


Fig. 9

Credit Author Statement

Fiorenza Stagni: carried out histological procedures, immunohistochemistry, evaluated cell density of Ki67-positive cells in VZ, and wrote the manuscript.

Andrea Giacomini: carried out statistical analysis of experiments.

Marco Emili: evaluated cell density and calretinine-positive cells in MD.

Beatrice Uguagliati: evaluated cell density in CM and PF.

Maria Paola Bonasoni: carried out the autoptic exam of fetuses.

Renata Bartesaghi: conceived and designed the experiments and wrote the manuscript.

Sandra Guidi: supervised the experiments and wrote the manuscript.