Genetic Evidence for p75<sup>NTR</sup>-Dependent Tetraploidy in Cortical Projection Neurons from Adult Mice

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A subpopulation of chick retinal projection neurons becomes tetraploid during development, an event prevented by blocking antibodies against p75 neurotrophin receptor (p75<sup>NTR</sup>). We have used an optimized flow cytometric assay, based on the analysis of unfixed brain cell nuclei, to study whether p75<sup>NTR</sup>-dependent neuronal tetraploidization takes place in the cerebral cortex, giving rise to projection neurons as well. We show that 3% of these neurons express the immediate early genes Erg-1 and c-Fos, indicating that they are functionally active. Tetraploid cortical neurons (65–80%) express CTIP2, a transcription factor specific for subcortical projection neurons in the mouse neocortex. During the period in which these neurons are born, p75<sup>NTR</sup> is detected in differentiating neurons undergoing DNA replication. Accordingly, p75<sup>NTR</sup>-deficient mice contain a reduced proportion of both NeuN and CTIP2-positive neocortical tetraploid neurons, thus providing genetic evidence for the participation of p75<sup>NTR</sup> in the induction of neuronal tetraploidy in the mouse neocortex. In the striatum tetraploidy is mainly associated with long-range projection neurons as well since ~80% of tetraploid neurons in this structure express calbindin, a marker of neostriatal-matrix spiny neurons, known to establish long-range projections to the substantia nigra and globus pallidus. In contrast, only 20% of tetraploid cortical neurons express calbindin, which is mainly expressed in layers II–III, where CTIP2 is absent. We conclude that tetraploidy mainly affects long-range projection neurons, being facilitated by p75<sup>NTR</sup> in the neocortex.
synthesis in the cortical neuroepithelium during the period of neurogenesis of CTIP2-positive neurons, as previously described in the chick retina (Morillo et al., 2010). In contrast with this latter tissue, differentiating cortical neurons that reactivate the cell cycle were observed to express Rb. Finally, we show that in the striatum, neuronal tetraploidy is also associated with projection neurons, thus suggesting that neuronal tetraploidization in vertebrates mainly occur in this type of neurons.

**Materials and Methods**

**Animals.** Original breeding pairs of mice heterozygous for a mutation targeted to the third exon of the *Ngfr* gene encoding a form of p75NTR that lacks its capacity to interact with its ligands (B6.129S4-Ngfrtm1Ja/e j p75NTR+/−/ mice) (Lee et al., 1992) were obtained from The Jackson Laboratory. These functional knock-out mice were inbred to produce all three genotypes. Wild-type control (p75NTR+/+/+) mice and homozygous mice for the *Ngfr* mutation (p75NTR−/−) were used in the present study. Genotypes were determined by genomic PCR as previously described (Frade and Barde, 1999). Males and females were interchanged equally with no differences between genders. C57BL/6J mice (Harlan) were also used in this study. Pregnant females were identified by the presence of a vaginal plug. The day of plug observation was designed for embryonic day 0.5 (E0.5). Embryos were staged as described by Kaufman (1992). Fluorescent, ubiquitination-based cell cycle indicator (Fucci) mice [B6.B6D2-Tg(Fucci)504Bsi mice] (Sakaue-Sawano et al., 2008) were obtained from C57BL6/129 background. One month-old 10–12 g body weight) and killed with either i.p. or gavage injection of isoflurane before sacrificing with cervical dislocation. Brains were dissected in ice-cold 70% ethanol/PBS and fixed in 10% formaldehyde in PBS for 4 days before mechanical disaggregation by gently swirling of the vial. In some cases, pellets were resuspended in ice-cold 1% Triton X-100 and 10 µg/ml DNase I (Roche). Then sections were further washed twice with PBS and subjected to immunohistochemistry as described above.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.** Apoptosis was analyzed in cryosections (20 µm) and isolated nuclei using the *in situ* cell death detection kit (ROCHE) following the manufacturer’s instructions.

**Cell nuclei isolation.** Fresh-frozen mouse and chicken tissues (one mouse hemisec or one chick telencephalic hemisphere) were placed in 2.5 ml of ice-cold, DNase-free PBS containing 0.1% Triton X-100 (Sigma) and protease inhibitor mixture (Roche) (nuclear isolation buffer). Cell nuclei were then isolated by mechanical disaggregation using a dounce homogenizer. Undissociated tissue was removed by centrifugation at 200 × g for 1.5 min at 4°C. The supernatant was eightfold diluted with nuclear isolation buffer and centrifuged at 400 × g for 4 min at 4°C. Supernatant with cellular debris was discarded, and the pellet incubated at 4°C in 800–1000 µl of cold nuclear isolation buffer for at least 1 h before mechanical disaggregation by gently swirling of the vial. In some cases, pellets were resuspended in ice-cold 70% ethanol/PBS and fixed O/N at 4°C as described by Westra et al. (2010). The quality and purity of the isolated nuclei was analyzed microscopically after staining with 10 ng/ml DAPI.

**Cell isolation.** Fresh mouse cortex or striatum was placed in Ca2+−/Mg2+−/free PBS containing 3 mg/ml bovine serum albumin (BSA), and then treated with 0.5 mg/ml trypsin ( Worthington) for 35 min at 37°C. Reactions were stopped by adding 0.5 mg/ml soybean trypsin inhibitor (Sigma). DNase I (10 µl) prepared at 1 µg/µl in PBS was then added; and the cells were subsequently dissociated by gentle trituration. Cells were centrifuged at 300 × g for 5 min at RT. Pellets were washed twice with PBS to remove cell debris, and then they were fixed O/N at 4°C in 70% ethanol/PBS.

**Flow cytometry.** Nuclear immunostaining was performed by adding primary and secondary antibodies to 400 µl of isolated unfixed nuclei containing 5% FCS and 1.25 mg/ml BSA. In control samples, the primary or secondary antibodies were excluded. Flow cytometry was performed as previously described (López-Sánchez et al., 2011). Bovine serum albumin (BSA) was added to 1% (w/v) in 70% ethanol/PBS. All samples were analyzed using a FACSAria cytometer (BD Biosciences) equipped with a two argon laser (488 nm) and helium-neon laser (633 nm). Data were collected by using a linear digital signal process. Emission filters used were BP 530/30 for Alexa 488, BP 616/23 for PI, and BP 660/20 for Alexa 647. Data were analyzed with FACSDiva (BD Biosciences) and Weasel 3.0.1 (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) software, and displayed using biexponential scaling. Electronic compensation for fluorochrome spectral overlap during multicolor immunofluorescence analysis was performed using Multicycle software. Data were collected in logarithmic (L) mode, which was clearly differentiated from nuclei due to its inability to incorporate PI, was gated and excluded from the analysis. DNA content his-
Fluorescent in situ hybridization. Isolated cell nuclei from cerebral cortex of 2-month-old mice were immunolabeled with anti-CTIP2 antibodies and counterstained with PI. Cell nuclei were then subjected to fluorescence-activated cell sorting (FACS) to isolate CTIP2-positive nuclei with 2C or 4C DNA content. Suspensions of nuclei were subsequently centrifuged at 300 g, and the pellets were then fixed with ethanol (Merck)/glacial acetic acid (Merck) (3:1) for 30 min. The fixed nuclei were dropped onto wet slides and dried overnight at room temperature. Fluorescent in situ hybridization (FISH) was performed using a Poseidon TK (11q€) mouse probe (Kreatech Diagnostics) following the indications of the manufacturer. This probe recognizes mouse chromosome 11. Nuclei were finally stained with 100 ng/ml DAPI for 2 min. After dehydration with 70% ethanol, 90% ethanol, and 100% ethanol, coverslips were air dried and mounted with ProLong Gold Antifade Reagent (Invitrogen).

Results

An optimized flow cytometric assay for the analysis of DNA content in fresh cell nuclei

An optimized procedure for measuring DNA content in brain cells was developed based on flow cytometry performed with fresh cell nuclei (Fig. 1). For this analysis, it was first required to discriminate homogeneous nuclear populations from cell debris using forward scatter/side scatter biplots (Fig. 1Aa, polygonal line). Then, the proportion of tetraploid nuclei within the gated nuclear population was quantified. Doublets of diploid nuclei were discarded using the standard pulse processing method (for review, see Nunez, 2001), thus assuring that the gated events with 2C or 4C DNA content. Suspensions of nuclei were subsequently centrifuged at 300 g, and the pellets were then fixed with ethanol (Merck)/glacial acetic acid (Merck) (3:1) for 30 min. The fixed nuclei were dropped onto wet slides and dried overnight at room temperature.

The mouse cerebral cortex contains tetraploid neurons

As indicated above, isolated unfixed nuclei can be immunolabeled with nuclear neuronal markers such as NeuN (Fig. 2A, B), and the DNA content present in singlet nuclei can be quantified by flow cytometry as a function of the intensity of PI labeling. In our experiments, nuclei were considered to be positive for NeuN only when the intensity of the signal was above that obtained with secondary antibody alone (Fig. 2B, dark gray). This procedure allowed us to reliably quantify the proportion of NeuN-positive nuclei in the cerebral cortex showing 4C DNA content (Fig. 2C). This analysis evidenced that most tetraploid nuclei were immunopositive for NeuN (Table 1; Fig. 2C€E), as previously observed in the retina (Morillo et al., 2010), thus demonstrating that tetraploidy in the murine cerebral cortex is mainly associated with neurons. Our results indicated that ~3% of cortical neurons contained a double amount of DNA at both postnatal day 0 (P0) and 2 months (Table 1), suggesting that the final proportion of neuronal tetraploidy is already reached at P0 and that it does not change substantially during adulthood.

The stable proportion of neuronal tetraploidy as mice get older could also be explained in terms of continuous death of tetraploid neurons, which would be replaced by new diploid neurons undergoing DNA duplication. To test this alternative hypothesis, we first studied the presence of apoptotic cells in the cerebral cortex from mice of 1 month of age, an intermediate age between P0 and 2 months. This analysis demonstrated the absence of apoptosis in the cerebral cortex, as evidenced by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining in either sections (n = 2 mice) (Fig. 3A, bottom) or fresh cell nuclei subjected to flow cytometry (n = 2 mice) (Fig. 3B, bottom). In contrast, positive controls treated with DNase showed high levels of TUNEL staining (Fig. 3A,B, top). We next tested the capacity of cortical neurons to reactivate the cell cycle and duplicate their DNA. Since BrdU can be toxic if administered for long periods (Kimbrough et al.,...
survive in the adult brain. Neonatal rodents develop a large number of neurons in the cerebral cortex, mainly in the subventricular zone, and establish pathways to the developing thalamus and ventricles of the posthatch chick (Kaczmarek and Chaudhuri, 1997; Knapska and Kaczmarek, 2004), extensively used as markers for functional activity in neurons (Kaczmarek and Chaudhuri, 1997; Knapska and Kaczmarek, 2004), and show green nuclei in cells that undergo S/G2 phase (Sakaue-Sawano et al., 2008). These mice contain a similar proportion of tetraploid neurons in the cerebral cortex as those observed in wild-type mice (data not shown). As expected, cells located in the subventricular zone along the lateral ventricles of 1-month-old Fucci mice often contained green nuclei (Fig. 3C), likely representing adult neuronal precursors. In contrast, no evidence for green labeling in NeuN-positive nuclei was found in the cerebral cortex of these mice (18 sections from two different mice) (Fig. 3C). Unfortunately, flow cytometry could not be used for the detection of green labeling in fresh cell nuclei from Fucci mice since the mAG-fused protein is lost during the nuclear isolation process (data not shown). Overall, we conclude that the cortical tetraploid neurons observed in the adult brain are likely those born during development, which can survive in the adult brain.

The telencephalon of posthatch chick contains tetraploid neurons

To verify whether tetraploid neurons can also be detected in telencephalic derivatives of other species, a similar approach as that described above was performed with the telencephalon of the posthatch chick. Cell nuclei isolated from the telencephalon were immunostained with anti-NeuN antibodies (Fig. 2F,G), and subjected to flow cytometric analysis after PI labeling. In the case of the mouse cerebral cortex, most nuclei showing 4C DNA content were immunopositive for NeuN (Table 1; Fig. 2H–J), and ~3% of NeuN-positive nuclei isolated from the telencephalon of the posthatch chick contained a double amount of DNA (Table 1).

Tetraploid neurons in the murine cerebral cortex respond to environmental signals

The immediate early genes (IEG) Erg-1 and c-fos have been extensively used as markers for functional activity in neurons (Kaczmarek and Chaudhuri, 1997; Knapska and Kaczmarek, 2004),
including aneuploid projecting neurons (Kingsbury et al., 2005). These IEGs are rapidly and transiently expressed in response to a variety of stimuli, including ongoing synaptic activity in the adult brain (Loebrich and Nedivi, 2009). To determine whether tetraploid neurons can express these markers, nuclei isolated from the cerebral cortex of 2-month-old mice were double labeled for NeuN and either Erg-1 (Fig. 4A) or c-fos (data not shown), labeled with PI, and then subjected to flow cytometric analysis discarding doublets and analyzing ploidy as described above. Figure 4, B–E, illustrates the gating procedure for identifying the different nuclear populations based on the expression of NeuN and Erg-1. For this analysis controls with only secondary antibodies, or secondary antibodies with each primary antibody, were used for determination of the threshold for each signal (Fig. 4B–D). DNA content analysis for each of the different populations demonstrated that 88.50 ± 1.69% (mean ± SEM; n = 9) of the tetraploid neurons expressed Egr-1 above background (Fig. 4G). This percentage was slightly higher than the proportion of diploid neurons showing Erg-1 expression (80.25 ± 3.56%; mean ± SEM; n = 9; p < 0.005, Student’s t test) (Fig. 4G). Likewise, a similar analysis performed with antibodies against c-Fos demonstrated that 89.67 ± 3.56% (mean ± SEM; n = 9) of the tetraploid neurons expressed this IEG above background while c-Fos was detected only in 85.69 ± 5.81% (mean ± SEM; n = 9) of diploid neurons. Overall, these results indicate that tetraploid neurons are functionally active and can respond to environmental signals in the adult cerebral cortex.

Most tetraploid neurons in the mouse cerebral cortex express CTIP2

To get insight into the identity of the tetraploid neurons present in the mouse cerebral cortex, and whether they constitute a population of long-range projection neurons, as it occurs in the retina (Morillo et al., 2010), flow cytometric analyses were performed after immunolabeling of isolated cortical cell nuclei with an antibody specific for CTIP2 (Fig. 5A, B). This antibody recognizes a transcription factor specific for subcortical projection neurons present in layers V and VI of the mouse neocortex (Arlotta et al., 2005). This analysis evidenced that ~65% of tetraploid nuclei showed intense CTIP2 immunolabeling (Table 2; Fig. 5C), indicating that neuronal tetraploidy in the cerebral cortex is largely associated with projection neurons. Moreover, this analysis demonstrated that ~5% of total CTIP2-positive nuclei were tetraploid at both P0 and 2 months (Table 2; Fig. 5C–E), thus demonstrating that only a subpopulation of projection neurons becomes tetraploid, as observed in the retina where most RGCs show a 2C DNA content in their nuclei (Morillo et al., 2010). These results also indicate that, as in the case of the NeuN-positive nuclei with 4C DNA content, the proportion of CTIP2-positive tetraploid nuclei did not change during adulthood.

Table 1. Percentage of tetraploid nuclei with NeuN-specific immunolabeling and NeuN-positive nuclei with tetraploid content in mouse cerebral cortex and chick telencephalon

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% NeuN + nuclei in the 4C population (mean ± SEM)</th>
<th>% NeuN + nuclei with 4C DNA content (mean ± SEM)</th>
<th>n</th>
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<tbody>
<tr>
<td>Mouse neocortex (P0)</td>
<td>98.66 ± 0.61</td>
<td>3.17 ± 0.15</td>
<td>8</td>
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<tr>
<td>Mouse neocortex (2 month old)</td>
<td>95.21 ± 0.71</td>
<td>3.12 ± 0.29</td>
<td>13</td>
</tr>
<tr>
<td>Chick telencephalon (posthatch)</td>
<td>98.30 ± 0.40</td>
<td>2.98 ± 0.22</td>
<td>5</td>
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The presence of CTIP2-positive cell nuclei with double the normal amount of DNA in the cerebral cortex was confirmed by SBC, a method able to reliably quantify DNA in tissue sections (Mosch et al., 2007; Morillo et al., 2010). This procedure provides spatial information about the areas in which CTIP2-positive nuclei showing 4C DNA content can be detected. To this aim, parasagittal cryosections including the cerebral cortex of mouse embryos were immunostained with an anti-CTIP2 antibody and counterstained with DAPI. We used embryos because of the lower background of DAPI staining when compared with that in adult tissues. E17.5 was chosen because at this stage all neurons from cortical layers V–VI and most neurons from other layers have already been generated (Caviness, 1982). Immunostaining with CTIP2 antibodies resulted in a dual pattern characterized by high levels of CTIP2 protein in neurons from layer V, while CTIP2 expression level in layer VI neurons was much lower (Fig. 5F), as previously described by McKenna et al. (2011) for postnatal cortical stages. The relative DNA content in the nuclei of CTIP2-positive neurons was estimated as a direct function of total DAPI intensity levels (see Materials and Methods). Using this approach we could observe CTIP2-positive nuclei with DNA content near 4C (Fig. 5F), located in both layer V (Fig. 5F, H) and VI (data not shown) of the cerebral cortex. Tetraploid cell nuclei were detected in the visual cortex (Fig. 5F–H) as well as in the other cortical areas that were studied such as the prefrontal and motor cortex (data not shown), indicating that tetraploid neurons are widely located throughout the whole cortex. Several areas within the different cortical regions analyzed were observed to contain an elevated proportion of overlapping nuclei, which forced us to exclude them from the analysis. Since we cannot be sure that these regions contain a similar proportion of tetraploid neurons as in the low nuclear density areas, we cannot provide a reliable quantification of neuronal tetraploidy in cortical CTIP2-positive nuclei using the SBC method.

Finally, FISH analysis performed on CTIP2-positive neuronal nuclei isolated by FACS further confirmed the presence of both diploid (Fig. 5I) and tetraploid nuclei (Fig. 5J) in these neurons.

The telencephalon of posthatch chick contains CTIP2-positive tetraploid neurons
Flow cytometry was also performed with cell nuclei isolated from the telencephalic derivatives of posthatch chick to evaluate the presence of tetraploid neurons expressing CTIP2 in the telencephalon of this species (data not shown). This analysis indicated that ~80% of tetraploid nuclei showed intense immunolabeling for CTIP2 (Table 2). In addition, ~4% of CTIP2-positive nuclei were observed to show 4C DNA content (Table 2). Therefore, the chick telencephalon has a similar proportion of CTIP2-positive tetraploid neurons as the mouse cerebral cortex.
p75\textsuperscript{NTR} is expressed in the neuroepithelium of the mouse telencephalon during the generation of CTIP2 cortical neurons

We have previously shown that the use of blocking antibodies against p75\textsuperscript{NTR} during the period of RGC genesis is able to reduce the proportion of tetraploid neurons in the developing chick retina (Morillo et al., 2010). Altogether, these results suggest that p75\textsuperscript{NTR} may participate in the generation of tetraploid neurons in the cerebral cortex.

p75\textsuperscript{NTR}-specific staining could also be detected in a population of CTIP2-positive postmitotic neurons, as evidenced by double immunostaining performed in sections from cerebral cortex of E12.5, E14.5, and E16.5 mouse embryos using antibodies specific for both p75\textsuperscript{NTR} and CTIP2. At E12.5 only a small number of CTIP2-positive nuclei could be observed at the marginal zone, most of them being positive for p75\textsuperscript{NTR} (Fig. 7A). At E14.5 and E16.5, p75\textsuperscript{NTR} was observed in a subpopulation of CTIP2-positive cells (Fig. 7B, C). In contrast, the strong p75\textsuperscript{NTR} labeling during development, the cerebral cortex of 1-month-old mice showed only background staining (Fig. 7D).

Cell cycle-reactivating cortical neurons express Rb

In contrast with the chick retina, where RGCs becoming tetraploid lack retinoblastoma (Rb) protein (Morillo et al., 2010), differentiating cortical neurons that reactivate the cell cycle (i.e., βIII-tubulin-positive, BrdU-positive cells) were observed to express Rb (Fig. 6B). Therefore, the mechanism regulating neuronal tetraploidization in cortical neurons seems to differ from that observed in the chick retina. As expected from their capacity to incorporate BrdU, these neurons also showed immunoreactivity for Rb phosphorylated at Ser 795 (Fig. 6C), the preferred phosphorylation site of cdk4/cyclin D1 in the molecule (Pan et al., 1998). This observation is consistent with the known expression of cdk4 and cyclin D1 by cortical neurons (Sumrejkanchanakij et al., 2003).

p75\textsuperscript{NTR}-positive subplate neurons (SPNs) are diploid

SPNs constitute a transient population of the first neurons that are born in the mouse cerebral cortex (McQuillen et al., 2002). These neurons express p75\textsuperscript{NTR} (Allendoerfer et al., 1990), suggesting that they may be susceptible to become tetraploid during development, as it occurs with the RGCs (Morillo et al., 2010) and the CTIP2-positive cortical neurons (this study). SPNs are known to express Sox5 (Kwan et al., 2008; Kanold and Luhmann, 2010), a nuclear marker that can also be observed in layer V/VI neurons (Kwan et al., 2008). Therefore, we have taken advantage of this observation to identify the SPNs as those cortical cells expressing Sox5 but lacking CTIP2-specific immunostaining. By using this criterion we were able to analyze whether a subpopulation of SPNs shows 4C DNA content. To this aim, fresh cell nuclei were isolated from the cerebral cortex of P0 mice, a stage in which SPNs are still present (McQuillen et al., 2002). These cell nuclei were then immunolabeled with both anti-Sox5 and anti-
Figure 5. CTIP2-positive neurons with 4C DNA content in the mouse cerebral cortex. A, Cell nuclei isolated from the cerebral cortex of 2-month-old mice immunostained with an anti-CTIP2-specific antibody (green) and counterstained with DAPI (blue). Arrow, A CTIP2-positive nucleus. B, Cell nuclei immunostained with the anti-CTIP2 antibody (anti-CTIP2) or with the secondary antibody alone (Control) were PI stained and subjected to flow cytometric analysis. Right, Illustrates the threshold used for the discrimination of CTIP2-positive nuclei. C, DNA content (Propidium Iodide-A) plotted against CTIP2 signal intensity (FITC-A) demonstrates that most nuclei with 4C content are positive for CTIP2 immunolabeling. D, DNA content histogram from CTIP2-negative nuclei. E, DNA content histogram from CTIP2-positive nuclei. See Table 2 for quantitative data. F, Coronal cryosections (15 μm) obtained from cerebral cortex of E17.5 mouse embryos were immunostained with anti-CTIP2 antibody (green) and labeled with DAPI (blue) for DNA quantification. G, DNA content histogram in CTIP2-positive nuclei obtained by SBC. H, Top, A high magnification of box shown in F. Arrow, Nucleus with 4C DNA amount. Bottom, CTIP2-positive nuclei were used for DNA quantification only in those cases in which DAPI signal was clearly identified as a single nucleus (numbered nuclei). I, J, CTIP2-positive cell nuclei from the cerebral cortex of 2-month-old mice, isolated by FACS, subjected to FISH with a chromosome 11-specific probe (red), and counterstained with DAPI (blue). Arrows, Hybridization spots. Scale bars: A, H, I, J, 10 μm; F, 40 μm.
CTIP2 antibodies, and subjected to flow cytometric analysis. This study demonstrated that the nuclei of the SPNs (i.e., Sox5-positive/CTIP2-negative population) were diploid, whereas layer V/VI neurons (i.e., Sox5-positive/CTIP2-positive population) contained both diploid and tetraploid nuclei (Fig. 8). We concluded that neuronal tetraploidy cannot be generalized to all neuronal populations that express p75NTR. Moreover, this result further stresses the specificity of our flow cytometric procedure, able to discriminate the presence of tetraploidy between different neuronal populations.

Neuronal tetraploidy is reduced in the cerebral cortex of p75NTR−/− mice
To directly study whether p75NTR participates in the creation of tetraploidy in murine cortical neuron, cell nuclei were isolated from the cerebral cortex of wild-type and p75NTR−/− littermates at both P0 and 2 months, and then they were immunostained with either anti-NeuN or anti-CTIP2 antibodies, followed by DNA labeling with PI. Flow cytometric analysis demonstrated that the proportion of NeuN-positive nuclei showing 4C DNA levels was significantly reduced in the p75NTR−/− mice compared with their wild-type littermates at P0, when the neurogenesis is just finished (Caviness, 1982), and in the adult mouse (Fig. 9A). Our study demonstrated that this is also the case for the subpopulation of CTIP2-positive neurons with 4C DNA content, since the proportion of these neurons is significantly reduced in the p75NTR−/− cerebral cortex compared with the cerebral cortex of the wild-type littermates at both P0 and 2 months (Fig. 9B).

Tetraploidy is enriched in long-term projection neurons from the striatum
To confirm that tetraploidy is mainly associated with long-range projection neurons in other brain structures we focused on the neostriatal-matrix spiny neurons. These neurons establish long-range projections to substantia nigra and globus pallidus (Kawaguchi, 1997) and they express both CTIP2 (Arlotta et al., 2008) and calbindin (Kawaguchi, 1997), a calcium-binding protein specifically expressed in subsets of adult neurons (Liu and Graybiel, 1992). This contrasts with the neocortex, where calbindin is predominant in neurons from layers II–III (Hof et al., 1999), a structure containing cortical interneurons and short-range, corticocortical projection neurons. The cytoplasmic localization of calbindin forced us to perform flow cytometric analyses similar to those described by Morillo et al. (2010) for retinal neurons. To this aim, cells were dissociated from both cerebral cortex and striatum of adult mice (3- to 3.5-month-old mice). These cells were then ethanol fixed and immunostained with an anti-calbindin-specific antibody before labeling their DNA with PI. As expected, ~80% of tetraploid striatal cells were observed to express calbindin (Fig. 10) whereas only ~20% of tetraploid cortical cells showed immunoreactivity for this calcium-binding protein. Therefore, neuronal tetraploidy seems to be associated with long-range projection neurons also in the striatum. These results, along with our previous observations in the retina (Morillo et al., 2010), suggest that neuronal tetraploidy predominates in subpopulations of large projection neurons throughout the whole vertebrate nervous system.

Discussion
We have optimized a flow cytometric method for DNA quantification in adult neurons, based on the analysis of unfixed isolated nuclei. Using this procedure we have demonstrated that the mouse cerebral cortex contains a small population of functionally active, tetraploid neurons. A substantial proportion of these neurons express CTIP2, a zinc finger transcription factor that specifies cortical neurons from layer V–VI projecting to subcortical

Table 2. Percentage of tetraploid nuclei with CTIP2-specific immunolabeling and CTIP2-positive nuclei with tetraploid content in mouse cerebral cortex and chick telencephalon

<table>
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<th>Tissue</th>
<th>% CTIP2+ nuclei in the 4C population (mean ± SEM)</th>
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<tr>
<td>Mouse neocortex (P0)</td>
<td>64.78 ± 3.34</td>
<td>5.18 ± 0.44</td>
<td>11</td>
</tr>
<tr>
<td>Mouse neocortex (2 month old)</td>
<td>63.19 ± 2.52</td>
<td>4.79 ± 0.61</td>
<td>13</td>
</tr>
<tr>
<td>Chick telencephalon (posthatch)</td>
<td>80.40 ± 2.60</td>
<td>4.17 ± 0.22</td>
<td>5</td>
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targets (Chen et al., 2008). These observations, confirmed by SBC and FISH, were extended to the chick telencephalon, suggesting an evolutionarily conserved mechanism for the generation of CTIP2-positive tetraploid neurons. Tetraploid neurons are likely to be generated during early stages of cortical development through a p75NTR-dependent mechanism, thus explaining the reduction of tetraploid neurons in the cerebral cortex of p75NTR−/− mice. Finally, in the striatum neuronal tetraploidy is largely associated with neurons that project to long distance targets, further supporting the hypothesis that tetraploidy in the nervous system mainly takes place in subpopulations of long-range projection neurons.

So far, the analysis of adult brain cells by flow cytometry has been hindered by intrinsic difficulties of this tissue derived from the interconnection of neural cells and the existence of myelin debris, which impairs the recovery of single cell suspensions and increases the background. In contrast, the use of unfixed cell nuclei for flow cytometry has several advantages compared with previous procedures (Morillo et al., 2010; Westra et al., 2010) as it reduces tissue dissociation difficulties, gives better analytical resolution, and reduces the presence of artifacts. Due to the purity of the nuclear preparations, our procedure facilitates a reliable and quantitative analysis whenever a specific nuclear marker is available. Moreover, our method allows representative and reproducible neuronal sampling, thus resulting in strong statistical analyses. Our method, which discriminates actual tetraploid nuclei from diploid nuclear doublets, has demonstrated the existence of tetraploid neurons in both murine and avian telencephalic derivatives. The specificity of our analysis is substantiated by the differential proportion of tetraploid neurons observed in cortical nuclei from wild-type mice versus p75NTR−/− mice.

The majority of cortical nuclei with 4C DNA content, both in mouse and in chicken, were observed to express NeuN, indicating that like in the retina (Morillo et al., 2010), most tetraploid cells are neurons. This observation agrees with previous studies showing tetraploid neurons in the human cerebral cortex (Mosch et al., 2007; Arendt et al., 2010). This also agrees with the study by Westra et al. (2009), who observed that, in humans, 17–48% of frontal cortical cells with 4C DNA content express neuronal markers. Surprisingly, these latter authors failed to observe neuronal markers in nuclei with duplicated pairs of chromosomes (Westra et al., 2009), an observation that contrasts with previous results from this same laboratory demonstrating that ~1.1% of cortical and hippocampal neurons are tetrasomic for chromosome 21 (Rehen et al., 2005). Tetrasomic nuclei have also been detected in human cortical neurons (Mosch et al., 2007), and our results indicate that CTIP2-positive nuclei from the mouse cortex can contain four hybridization

Figure 7. Expression of p75NTR in CTIP2-positive neurons. Coronal cryosections (12 μm) from the cerebral cortex (A–D) or basal forebrain (E) of mouse at the indicated ages were immunostained with anti-p75NTR (red) and anti-CTIP2 (green) antibodies. Left, Rectangles represent the area illustrated in middle and right parts. Arrows, CTIP2-positive neurons expressing p75NTR; arrowheads, CTIP2-positive neurons lacking p75NTR expression. V, ventricle. Scale bar: A–C left, 30 μm; A middle and right, 50 μm; B, C middle and right, 20 μm; D left, 200 μm; D middle and right, 60 μm; E, 60 μm.
spots as revealed by FISH. Our results also indicate that a small proportion of tetraploid nuclei lacks NeuN-specific immunostaining. These cell nuclei could correspond to glial cells, in accordance with the presence of tetraploid and polyploid glia in humans (Westra et al., 2009) and *Drosophila* (Unhavaithaya and Orr-Weaver, 2012), respectively.

It is likely that the cortical neurons becoming tetraploid during development remain viable during adulthood since the same proportion of neurons with 4C DNA content were observed at P0 and 2 months. This view is consistent with the absence of dying cells in the cerebral cortex of 1-month-old mice, as previously observed by Angata et al. (2007). Furthermore, no evidence was found for the existence of cortical neurons undergoing S/G2 phase in Fucci mice, in agreement with previous studies in the cerebral cortex of adult mouse (Magavi et al., 2000) and rat (Dayer et al., 2005), thus ruling out a hypothetical turnover of dying tetraploid neurons being substituted by cortical neurons that duplicate their DNA. The existence of viable tetraploid neurons in the cerebral cortex was further substantiated by the observation that most tetraploid neurons express Erg-1 and c-Fos, two IEGs expressed in response to neuronal activity (Kaczmarek and Chaudhuri, 1997; Knapska and Kaczmarek, 2004). Therefore, tetraploid neurons appear to be functional and, probably, integrated into the brain circuits as occurs with aneuploid neurons (Kingsbury et al., 2005). The capacity of tetraploid neurons to integrate in brain circuits and innervate specific target areas is further substantiated by the experiments performed by Tompkins et al. (1984) using diploid/tetraploid frog chimeras. These authors demonstrated that the RGCs from a tetraploid eye grafted into a diploid frog can innervate broader regions of the target tissue as compared with the RGCs from the original diploid eye. Our data also indicate that a small proportion of both tetraploid and diploid neurons lack Erg-1 and c-Fos expression, an observation consistent with the variable expression of this transcription factor in the cerebral cortex (Loebrich and Nedivi, 2009).

Tetraploidy is usually associated with increased cell size (Edgar and Orr-Weaver, 2001; Ullah et al., 2009). Indeed, tetraploid neurons are known to contain larger cell somas, dendritic arbors, and innervation areas than the diploid counterparts (Tompkins et al., 1984; Szaro and Tompkins, 1987; Morillo et al., 2010).
increase in size likely facilitates the innervation of distant targets. Our results support the hypothesis that neuronal tetraploidy affects mainly to large projection neurons, usually born at initial stages of development. This is the case of the RGCs, the first neurons to be born in the retina (Sidman, 1961; Prada et al., 1991), which contains a subpopulation becoming tetraploid during development (Morillo et al., 2010). As in the case of the RGCs, neuronal tetraploidization in the cerebral cortex also affects to a subpopulation of early born neurons: the pyramidal cells from cortical layers V–VI (Caviness, 1982), but not to the SPNs.

We have demonstrated that p75NTR participates in neuronal tetraploidization in the cerebral cortex since the proportion of tetraploid nuclei expressing NeuN or CTIP2 becomes reduced in the cortex of 75NTR−/− mice. This observation is consistent with the expression of p75NTR by neuroepithelial cells at early stages of cortical development, when pyramidal cells from layer V–VI are born (Caviness, 1982). Some of these p75NTR-positive, positive neuroepithelial cells are newborn since they express the early neuronal marker βIII-tubulin at high levels. Importantly, a subpopulation of p75NTR-positive, newborn neurons were able to incorporate BrdU after a short 30 min pulse, suggesting that they were undergoing neuronal tetraploidization, as previously shown for tetraploid RGCs (Morillo et al., 2010). p75NTR-specific immunoreactivity was detected in a subpopulation of CTIP2-positive neurons at E14.5–E16.5, when these neurons project to their targets (De Carlos and O’Leary, 1992), extending previous observations by McQuillen et al. (2002), who reported that SPNs express this receptor. This raises the possibility that p75NTR could play a role in axonal projection in these neurons, as previously described to occur in differentiated RGCs (Yamashita et al., 1999). Only background levels of p75NTR were detected in the cerebral cortex of 1-month-old mice, as previously shown (Wang et al., 2011), suggesting that p75NTR is dispensable for long-lasting maintenance of subcortical projections.

Interestingly, a population of tetraploid neurons remain in the cerebral cortex of p75NTR−/− mice, like in the retina of chick embryos treated with blocking antibodies against p75NTR (Morillo et al., 2010). This suggests the existence of alternative mechanisms involved in the generation of tetraploid neurons, which may act synergistically with p75NTR to trigger neuronal tetraploidization in the normal and pathological nervous system (Frade and López-Sánchez, 2010).

Unlike in the chick retina (Morillo et al., 2010), newborn cortical neurons reactivating the cell cycle express Rb. In these neurons Rb was phosphorylated at Ser795, a known substrate for cdk4/cyclin D1 (Pan et al., 1998). This suggests that cell cycle reactivation in differentiating cortical neurons relies on the inhibition of Rb function. This hypothesis is consistent with the observations that differentiating cortical neurons incorporate BrdU in conditional Rb−/− mice (Ferguson et al., 2002), and that Rb−/− cortical cells become tetraploid in wild-type/Rb−/− chimeric mice (Lipinski et al., 2001); both effects are compatible with neuronal survival. Rb phosphorylation at Ser795 in differentiating cortical neurons is consistent with the expression of cyclin D1 and cdk4 in these cells (Sunrekjanchanakij et al., 2003). Nevertheless, phosphorylation of Rb in these neurons might also be triggered by p38 MAPK (Wang et al., 1999), JNK (Chauhan et al., 1999), or ERK (Guo et al., 2005), which are known to be activated by p75NTR (Casaccia-Bonnefil et al., 1996; Susen et al., 1999; Morillo et al., 2012).

In sum, we provide evidence that somatic tetraploidization represent a common mechanism creating neuronal variability in the normal nervous system of vertebrates. This mechanism, which in part depends on p75NTR signaling, is mainly associated with long-range projection neurons, such as RGCs, pyramidal cells from cortical layers V–VI, as well as neostriatal-matrix spiny neurons. Further studies will be required to fully understand the actual mechanism leading to cell cycle reactivation and tetraploidization in cortical neurons.

References


