

# Regionalized Loss of Parvalbumin Interneurons in the Cerebral Cortex of Mice with Deficits in GFR $\alpha$ 1 Signaling

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Inhibitory interneurons are crucially important for cerebral cortex function and behavior. The mechanisms controlling inhibitory interneuron diversification and allocation to distinct cortical areas remain poorly understood. GDNF (glial cell line-derived neurotrophic factor) and its receptor GFR $\alpha$ 1 have been implicated in the development of GABAergic precursors but, because of the early lethality of null mutants, their roles in postnatal maturation and function of cortical interneurons are unknown. “*cis*-only” mutant mice lack GFR $\alpha$ 1 only in cells that do not express the RET signaling receptor subunit and survive to adulthood. At birth, both null mutants and *cis*-only mice showed a specific loss of GABAergic interneurons in rostro- and caudolateral cortical regions but not in more medial areas. Unexpectedly, the adult cortex of *cis*-only mice displayed a complete loss of parvalbumin (PV)-expressing GABAergic interneurons in discrete regions (PV holes) interspersed among areas of normal PV cell density. PV holes predominantly occurred in the visual and frontal cortices, and their size could be affected by neuronal activity. Consistent with deficits in cortical inhibitory activity, these mice showed enhanced cortical excitability, increased sensitivity to epileptic seizure, and increased social behavior. We propose that GFR $\alpha$ 1 signaling guides the development of a subset of PV-expressing GABAergic interneurons populating discrete regions of the cerebral cortex and may thus contribute to the diversification and allocation of specific cortical interneuron subtypes.

## Introduction

The cerebral cortex is comprised of both excitatory (glutamatergic) projection neurons and inhibitory (GABAergic) short-range interneurons that work together to maintain the balance between inhibition and excitation that is necessary for the correct functioning of the cortex. Cortical interneurons represent 20–30% of all neurons in the cortex and comprise a very heterogeneous group with varied morphologies, electrophysiological properties, and complement of neuropeptides, ion channels, and calcium-binding proteins (Markram et al., 2004). Abnormalities in inhibitory interneurons are apparent in several neurological conditions, including epilepsy and schizophrenia (Eyles et al., 2002; Cossart et al., 2005), and have also been proposed to be implicated in bipolar disorder and autism (Rubenstein and Merzenich, 2003; Levitt, 2005). Understanding the processes and molecules responsible for the diversification and cortical deployment of this important group of neurons is key to elucidating the

cause of such disorders and identifying therapeutic targets for their treatment.

GABAergic interneurons are born in the embryonic subpallium in the medial, lateral, and caudal ganglionic eminences between embryonic day 12.5 (E12.5) and E15.5 in the mouse, from where they undergo a protracted period of tangential migration toward the cerebral cortex, hippocampus, and olfactory bulb (Corbin et al., 2001; Marín and Rubenstein, 2003; Levitt et al., 2004; Wonders and Anderson, 2006). Two fundamental questions that remain unsolved regarding cortical interneuron development concern where and how interneuron diversity is generated, and the mechanisms that promote and guide their migration and allocation in the cortex. Although the large morphological, neurochemical, and functional diversity of cortical GABAergic interneurons does not manifest until the postnatal maturation of these cells, part of that diversity is currently believed to arise during their generation in the ventricular zone of the embryonic ventral telencephalon. Drawing parallels to mechanisms operating in the spinal cord, recent work has identified combinatorial codes of transcription factors delineating multiple subpallial progenitor domains (Flames et al., 2007; Fogarty et al., 2007; Liadis et al., 2007; Miyoshi et al., 2007). Genetic fate-mapping and micro-transplantation studies have converged in identifying the medial ganglionic eminence (MGE) as the site in which progenitors of parvalbumin (PV)- and somatostatin (SST)-expressing cortical interneurons are generated (Butt et al., 2005; Fogarty et al., 2007). Within the MGE, recent *in utero* transplantation studies have suggested that SST<sup>+</sup> interneurons originate from precursors located in the dorsolateral aspect

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of the MGE, whereas more medioventrally located domains may generate PV<sup>+</sup> interneurons (Flames et al., 2007). These advances, however, have only explained a small fraction of the diversity that is known to be present among mature cortical interneurons. In addition to transcription factors, a number of extrinsic cues can influence the development of GABAergic interneurons, including neurotrophins (Polleux et al., 2002), hepatocyte growth factor (Powell et al., 2001), semaphorins (Marín et al., 2001), and neuregulin-1 (Flames et al., 2004). However, it is not clear whether any of these factors influence the primary differentiation of MGE progenitors into GABAergic cells or their further diversification into different interneuron subtypes.

In previous work, we found that glial cell line-derived neurotrophic factor (GDNF) and its receptor GFR $\alpha$ 1 promote the differentiation and migration of immature GABAergic interneurons derived from the MGE (Pozas and Ibáñez, 2005). Members of the GDNF ligand family signal by binding to GPI-anchored receptors termed GFR $\alpha$ 1–4, in collaboration with signaling receptor subunits such as the RET tyrosine kinase or the p140<sup>NCAM</sup> isoform of the neural cell adhesion molecule NCAM (Airaksinen and Saarma, 2002; Paratcha et al., 2003). Mutant mice lacking GDNF or GFR $\alpha$ 1 display several deficits in subpopulations of sensory and enteric neurons, and die shortly after birth due to intestinal aganglionosis and kidney agenesis (Airaksinen and Saarma, 2002). At birth, these mutants show reduced numbers of GABAergic neurons in the cerebral cortex and hippocampus, demonstrating the importance of GDNF and GFR $\alpha$ 1 for GABAergic interneuron development. Intriguingly, neither RET or NCAM are required for GABAergic interneuron migration or differentiation (Pozas and Ibáñez, 2005), suggesting that other transmembrane effectors may mediate the effects of GDNF on these cells.

The early lethality of mice lacking GDNF or GFR $\alpha$ 1 prevented us from assessing the consequences of this loss for postnatal cortical development, and the organization and function of inhibitory networks in the mature cerebral cortex. In the present study, we set out to investigate these questions taking advantage of a line of mice in which the lethality caused by homozygous null mutation of *Gfral* has been rescued by expression of a *Gfral* cDNA from the *Ret* locus (Enomoto et al., 2004). These mice, known as “*cis*-only,” express GFR $\alpha$ 1 only in cells that express RET, such as enteric neurons and kidney, and therefore survive to adulthood. *cis*-only mice lack GFR $\alpha$ 1 in all cells that do not express RET, including a subset of GABAergic precursors in the MGE. No gross abnormalities in either the peripheral nervous system or the CNS were found in a previous analysis of these mice (Enomoto et al., 2004).

## Materials and Methods

**Mice.** *cis*-only and *Gfral*<sup>TLZ/TLZ</sup> mice (Enomoto et al., 2004), Gad67-GFP mice (Tamamaki et al., 2003), *Gdnf*<sup>+/-</sup> mice (Pichel et al., 1996) and *Ret*<sup>flx/flx</sup>; *Nestin*-CRE (cAMP response element) mice (Kramer et al., 2006) have been previously described. *cis*-only and *Gfral*<sup>TLZ/TLZ</sup> mice have a mixed C57/129 genetic background. Wild-type (wt) and mutant mice used in the same experiment were always derived from the same litter. We note that the line of *Gfral* null mice used in the present study (denoted *Gfral*<sup>TLZ/TLZ</sup>) is different from the one used in our previous work by Pozas and Ibáñez (2005) (denoted *Gfral*<sup>-/-</sup>). However, both lines showed comparable reductions of cortical GABAergic neuron number at birth. Animal protocols were approved by Stockholms Norra djurförsöksetiska nämnd and in accordance with ethical guidelines of the Karolinska Institute.

**Immunohistochemistry.** Perfusion, fixation, and preparation of cryosections followed standard procedures. Immunohistochemistry was conducted in free floating sections according to standard protocols. Primary

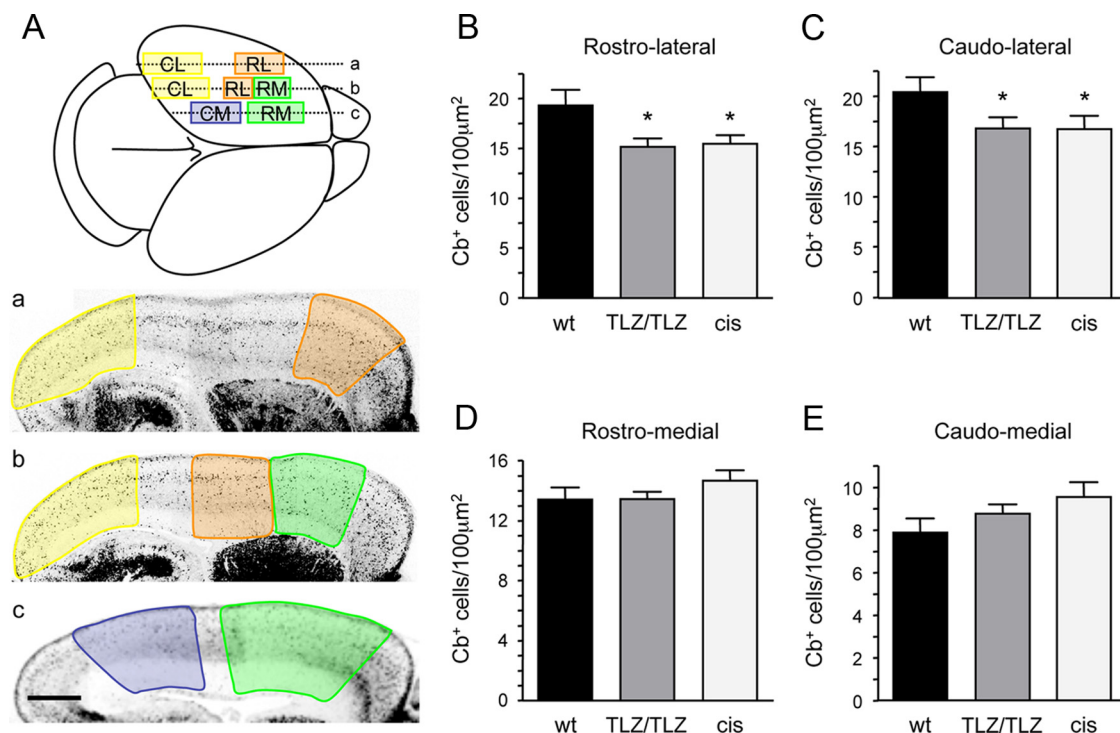
antibodies used were rabbit anti- $\beta$ -galactosidase (Cappel, 1:4000), rabbit anti-cleaved caspase-3 (Santa Cruz Biotechnology, 1:100), rabbit anti-calbindin (Swant, 1:1000), goat anti-calretinin (Swant, 1:1000), rabbit anti-GFAP (DAKO, 1:800), mouse anti-NeuN (Millipore Bioscience Research Reagents, 1:200), mouse anti-PV (Swant, 1:2000), rabbit anti-PV (Swant, 1:1000), rabbit anti-Lhx6 (V. Pachnis, National Institute for Medical Research, London, UK; 1:1000), rabbit anti-somatostatin-14 (Peninsula Laboratories, 1:1000), and rabbit anti-vGAT (Synaptic Systems, 1:2000) and lectins biotin anti-*Banderia simplicifolia* to visualize microglia (BS-1 lectin, Sigma, 1:50) and biotin anti-*Solanum tuberosum* to visualize blood vessels (Vector Laboratories, 20 mg/ml).

**Image analysis.** Serial sections (240 and 96 mm apart for adult and neonate brains, respectively) were processed for each antibody to ensure complete analysis of each cell type across the cortex. The mouse brain atlas by Paxinos and Franklin (2001) was used to identify functional regions of the cortex. Quantification of calbindin (Cb) cell profiles in neonatal cortex was performed in three sagittal sections from each hemisphere (Fig. 1A). Cells were counted in rostralateral, caudolateral, rostromedial, and caudomedial regions of 5 hemispheres per genotype from the pial surface to the underlying white matter. PV holes in the adult *cis*-only cortex were defined as spanning at least two consecutive sections 240 mm apart on the mediolateral axis and 150 mm in length on the rostro-caudal axis. PV holes were mapped in coronal and sagittal sections and then manually transferred onto the flattened cortical map from the Paxinos atlas (Paxinos and Franklin, 2001). Quantification of NeuN cells was performed in cortical layers 2/3 of PV holes (5 mice, 8 holes, 44 fields) and equivalent areas of wild-type cortex (6 hemispheres, 125 fields) from 20 $\times$  confocal images using automations made in OpenLab software (Improvision). Quantification of Gad67-GFP and Lhx6 cells was performed on sagittal sections using a grid of 0.04 mm<sup>2</sup> (Gad67-GFP) or 0.017 mm<sup>2</sup> (Lhx6) quadrants. Due to high variability between different animals, cell counts inside PV holes were normalized to counts obtained from an area of equivalent size surrounding each hole, and expressed as percentage cell loss. Identical areas in size and location were selected in wild-type brains (pseudoholes) and quantified for comparison. Quantification of vGAT intensity was performed in sections from PV holes and equivalent areas in wild-type controls that were processed at the same time and imaged by confocal microscopy under identical conditions. vGAT pixel intensity was measured in 6 quadrants from 2 separate images within each hole (or pseudohole) and individually adjusted for background using average pixel intensities from the underlying immunonegative corpus callosum in the same section. Unless otherwise indicated, Student's *t* test was used for statistical analysis.

**Optic nerve transection.** Within 48 h of birth, pups ( $n = 12$  *cis*-only,  $n = 4$  wild type) were anesthetized by hypothermia for 3–4 min. A vertical cut was made overlying each eye, the optic nerve severed using fine dissection scissors, and the eye removed. The overlying skin was then rejoined, and pups were warmed on a heat pad before being returned to the home cage. Pups were given two doses of buprenorphine (Temgesic, Schering-Plough, 0.2 mg/ml, s.c.) both before (–1 h) and after (+12 h) surgery to minimize pain and discomfort. This protocol resulted in 90% survival of pups. Pups remained with their mothers in the home cage until postnatal day 35 (P35), when they were genotyped and perfused. Mice raised in normal conditions ( $N = 8$  per genotype) were used as controls.

**Electroencephalography.** Adult *cis*-only ( $n = 5$ ) and wild-type ( $n = 5$ ) mice of both sexes were deeply anesthetized with ketamine-xylazine (100 and 20 mg/kg, i.p.), placed in a stereotaxic frame, and a large craniotomy was made over the left cerebral hemisphere. Local field potential recordings were made with bipolar, tungsten electrodes guided to the V2 region of the cortex using stereotaxic coordinates (Paxinos and Franklin, 2001). Signals were amplified, digitized, and acquired using standard procedures.

**Seizure induction.** Adult *cis*-only ( $n = 16$ ) and wild-type ( $n = 28$ ) mice aged between 2 and 7 months, of both sexes were injected with pentylene-tetrazole (PTZ) (Sigma, 40 mg/kg in 0.9% saline, i.p.). Immediately after injection, each mouse was placed in a large Plexiglas cage and its behavior recorded with a Pentax digital video camera for 60 min. Seizure severity was recorded on a scale of 0–5 according to stages described by Lau et al. (2000). Statistical analysis was performed by Mann–Whitney



**Figure 1.** Region-specific loss of cortical GABAergic interneurons in newborn *Gfra1*<sup>TLZ/TLZ</sup> and *cis*-only mice. **A**, Scheme outlining the cortical regions used for quantification of Cb cells in wild-type and mutant newborn mice. Planes of sagittal sections are indicated by lines labeled **a–c** and shown below with cortical areas color-coded: rostralateral (RL, orange), caudolateral (CL, yellow), rostromedial (RM, green), and caudomedial (CM, blue). Scale bar, 500 μm. **B–E**, Calbindin cell counts in the indicated cortical areas of wt, *Gfra1* null mutants (TLZ/TLZ), and *cis*-only (*cis*) newborn mice. Results are expressed as average ± SD ( $n = 5$  hemispheres per genotype). \* $p < 0.05$ .

nonparametric analysis (severity), or Student's *t* test (latency). At the conclusion of behavioral observation, *cis*-only mice were perfused for retrospective histological analysis.

**Behavioral analysis.** SHIRPA and elevated plus maze followed previously established protocols (Rogers et al., 1997). For social interaction tests, adult *cis*-only ( $n = 16$ ) and wild-type ( $n = 14$ ) male mice were placed in a large arena for 11 min, the first minute with an opaque dividing wall in the center, for two consecutive days (habituation phase). On the third day, a second previously unknown mouse of the same age, weight, and genotype, that had been subjected to identical preconditioning, was introduced in the other half of the cage (experimental phase). The behavior of each mouse was assessed on days 1 and 3 by means of subsequent analysis of video footage using a Pentax digital video camera. Behavior was categorized into 11 broad categories—i.e., body care, digging, nonsocial exploration, explore from a distance, social investigation, threat, attack, avoidance/flee, defensive/submissive, sexual, and immobility—based on existing paradigms (Estelles et al., 2007). The latency to each event, number of events, and time spent within 7 cm (less than one body-length) was recorded.

## Results

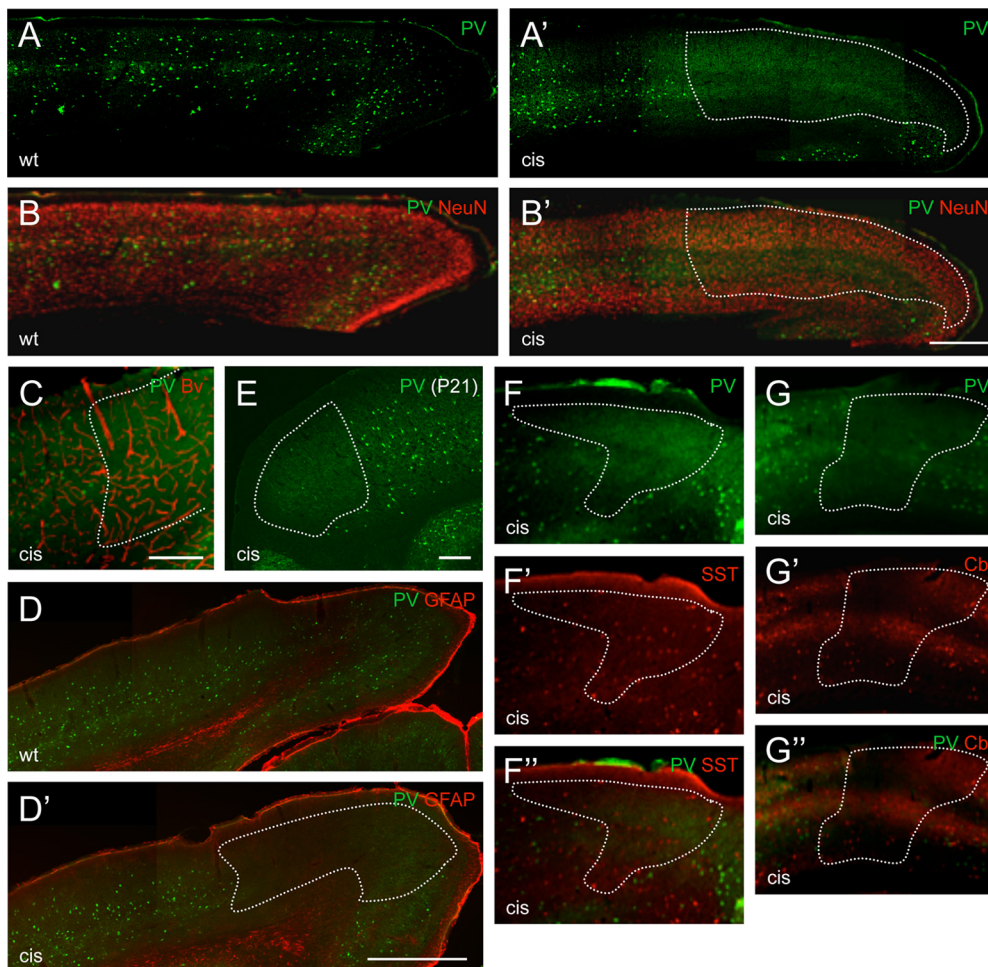
### Region-specific loss of cortical GABAergic interneurons in newborn *Gfra1*<sup>TLZ/TLZ</sup> and *cis*-only mice

In view of earlier findings indicating reduced numbers of cortical GABAergic neurons in newborn mice lacking GFR $\alpha$ 1 (Pozas and Ibáñez, 2005), we investigated whether this deficit was also present in newborn *cis*-only mice (*Gfra1*<sup>TLZ/TLZ</sup>; *Ret*<sup>Gfra1/+</sup>). Although our previous study was limited to the prospective somatosensory cortex, we have extended this analysis to other cortical areas in newborn *Gfra1*<sup>TLZ/TLZ</sup> (null) and *cis*-only mice (Fig. 1A). We found identical reductions in the number of cells expressing Cb<sup>+</sup>, a general marker of immature GABAergic precursors, in rostralateral and caudolateral areas (corresponding to prospective frontal/somatosensory and visual cortices, respec-

tively) in both *Gfra1*<sup>TLZ/TLZ</sup> and *cis*-only mice (Fig. 1B,C). This indicated that *cis*-only mice recapitulate the deficits in GABAergic neurons observed in the cortex of null mutants at birth. Unexpectedly, neither *Gfra1*<sup>TLZ/TLZ</sup> or *cis*-only mice showed any loss of Cb<sup>+</sup> cells in more rostromedial and caudomedial areas, corresponding to prospective motor and retrosplenial cortices (Fig. 1D,E). This indicated that not all regions of the cerebral cortex are equally affected by the loss of GFR $\alpha$ 1 expression.

### Parvalbumin holes in the cerebral cortex of adult *cis*-only mice

During the first postnatal weeks, GABAergic neurons cease their migration and begin to mature functionally. Several different classification systems for mature interneurons have been proposed, based on their morphology, electrophysiological properties, or expression of ion channels, calcium-binding proteins, and neuropeptides (Markram et al., 2004). We used a combination of PV, Cb, and calretinin markers, labeling largely nonoverlapping subpopulations of mature interneurons, to assess the distribution of GABAergic neurons in the cortex of adult *cis*-only mice. It should be noted that, although widely expressed across different classes of GABAergic precursors during early stages of development, Cb labels a restricted subset of mature interneurons in the adult cortex (Alcantara et al., 1996). In the wild-type cortex, PV neurons normally comprise ~40% of all interneurons, and their large cell bodies are distributed throughout layers 2–6 across the entire cortex (Fig. 2A,B). Unexpectedly, the cortex of *cis*-only mice displayed areas completely devoid of PV neurons (herein referred to as PV holes) interspersed among regions of normal PV cell density (Fig. 2A',B'). PV holes were present in the majority (82%) of *cis*-only brains analyzed, and either encompassed only the upper layers of the cortex (e.g., Fig. 2E,F), or the full depth of



**Figure 2.** Parvalbumin holes in the cerebral cortex of adult *cis*-only mice. **A, B**, PV and NeuN immunohistochemistry of sagittal sections of wild-type (**A, B**) and *cis*-only (**A', B'**) adult cortex. PV holes are delineated by white dotted lines. Scale bar (in **B'**) **A, B, A', B'**, 500  $\mu$ m. **C**, PV and blood vessel (Bv) immunohistochemistry in PV hole and adjacent areas of a sagittal section through the adult *cis*-only cortex. **D**, PV and GFAP immunohistochemistry of sagittal sections of wild-type (**D**) and *cis*-only (**D'**) adult cortex. Scale bar, 500  $\mu$ m. **E**, PV immunohistochemistry of a PV hole and adjacent areas in a sagittal section through the cortex of P21 *cis*-only mice. **F**, PV and SST immunohistochemistry of a PV hole and adjacent areas in a sagittal section through the adult *cis*-only cortex. **G**, PV and Cb immunohistochemistry of a PV hole and adjacent areas in a sagittal section through the adult *cis*-only cortex. Scale bars (in **C, E, F, G, F', G''**) **C, E, F, G**, 200  $\mu$ m.

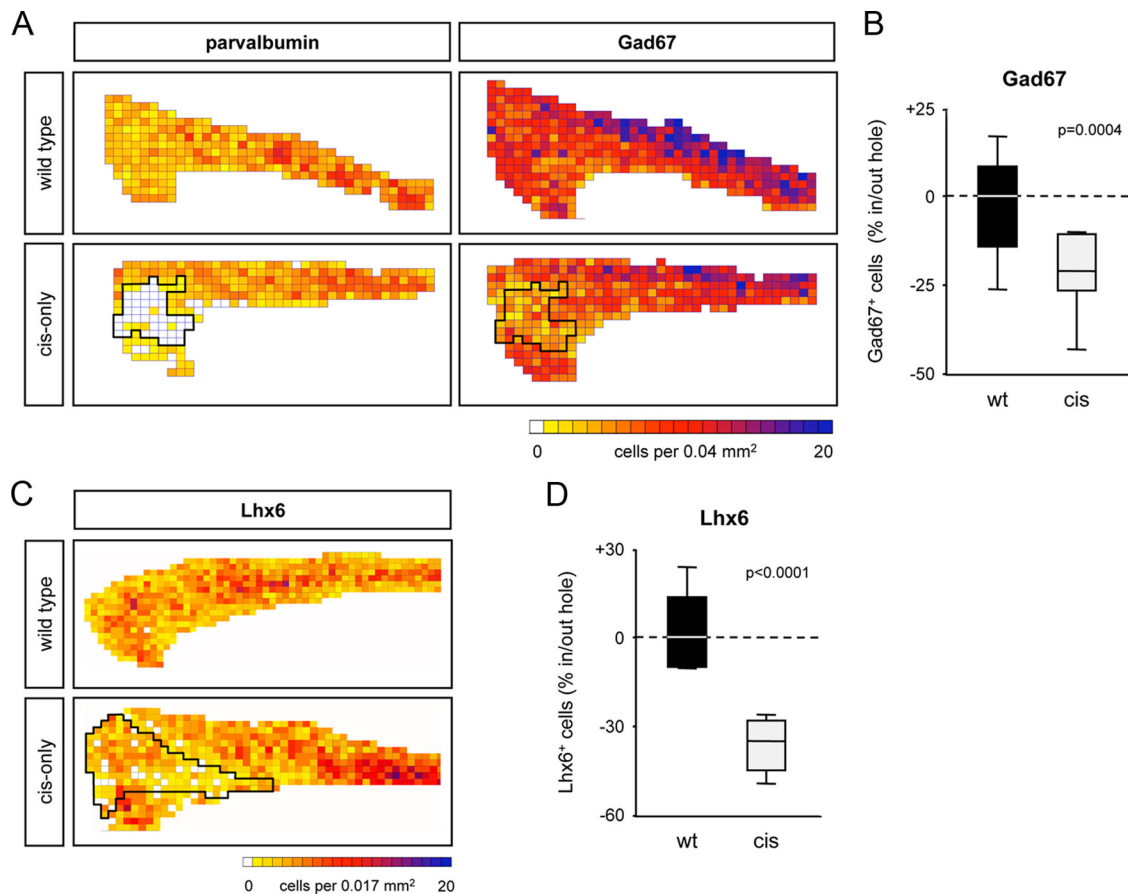
the cortical plate (e.g., Fig. 2*A', C, D, G*). No PV holes were observed in the cortex of wild-type or *Ret<sup>Gfra1</sup><sup>+/+</sup>* mice. Smaller, isolated PV holes, which never reached the full depth of the cortex, were observed in a fraction (<40%) of *Gfra1<sup>TLZ</sup><sup>+/+</sup>* heterozygous mutants, irrespectively of the presence of the *Ret<sup>Gfra1</sup>* allele. Also 40% of *Gdnf<sup>+/-</sup>* heterozygous mutants analyzed displayed cortical PV holes (data not shown). No PV holes were observed in brains of adult *Ret<sup>flx/flx</sup>; Nestin-CRE* conditional mutants lacking RET in the CNS (data not shown).

No alteration in the overall neuronal structure and layering of the cortex was observed in areas lacking PV neurons as assessed with the pan-neuronal marker NeuN (Fig. 2*B, B'*). The morphology of blood vessels was also normal (Fig. 2*C*, data not shown), indicating that loss of PV immunoreactivity was not a perfusion or fixation artifact. In addition, no glial reaction was observed in PV holes using markers of astrocytes (Fig. 2*D, D'*), oligodendrocytes and microglia (data not shown). Neither was any increase in cell death detected using antibodies against the activated form of caspase-3 at all stages examined (E12.5–E17.5, P0, P7, P14, P21, and adult) in the *cis*-only cortex (data not shown).

PV expression normally develops during the second and third postnatal weeks, and PV holes could be detected as early as P21 (Fig. 2*E*), the earliest time by which the distribution of PV

throughout the cortex begins to stabilize. It was also clear that, among the GABAergic subpopulations examined, PV interneurons were those mostly affected. No abnormalities could be detected in Calretinin-expressing interneurons, which are concentrated in the deeper layers of the cortex (data not shown). SST-expressing neurons, the other major interneuron subpopulation derived from the MGE, were also normally distributed throughout the *cis*-only cortex, including within PV holes (13 holes analyzed) (Fig. 2*F, F', F''*). Overall, the distribution Cb<sup>+</sup> interneurons was also normal in the *cis*-only cortex, including in the majority of PV holes (Fig. 2*G, G', G''*), although a fraction of these (14 of 54 analyzed) also lacked Cb-expressing cells in a smaller volume ( $62.31 \pm 5.29\%$ ) in the center of the hole (supplemental Fig. S1, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material).

Loss of PV interneurons in the *cis*-only cortex was accompanied by a proportional reduction in GABAergic markers that was restricted to the areas lacking PV expression. A GFP gene inserted in the *Gad67* locus marks the majority of GABAergic neurons in the adult cortex (Tamamaki et al., 2003), and we found a reduction of  $20.4 \pm 4.6\%$  in the number of *Gad67*-GFP<sup>+</sup> cells in PV holes of *cis*-only mice carrying this allele (Fig. 3*A, B*; supplemental Fig. S3*A*, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). Similarly, *Lhx6* specifically marks MGE-derived GABAergic neu-



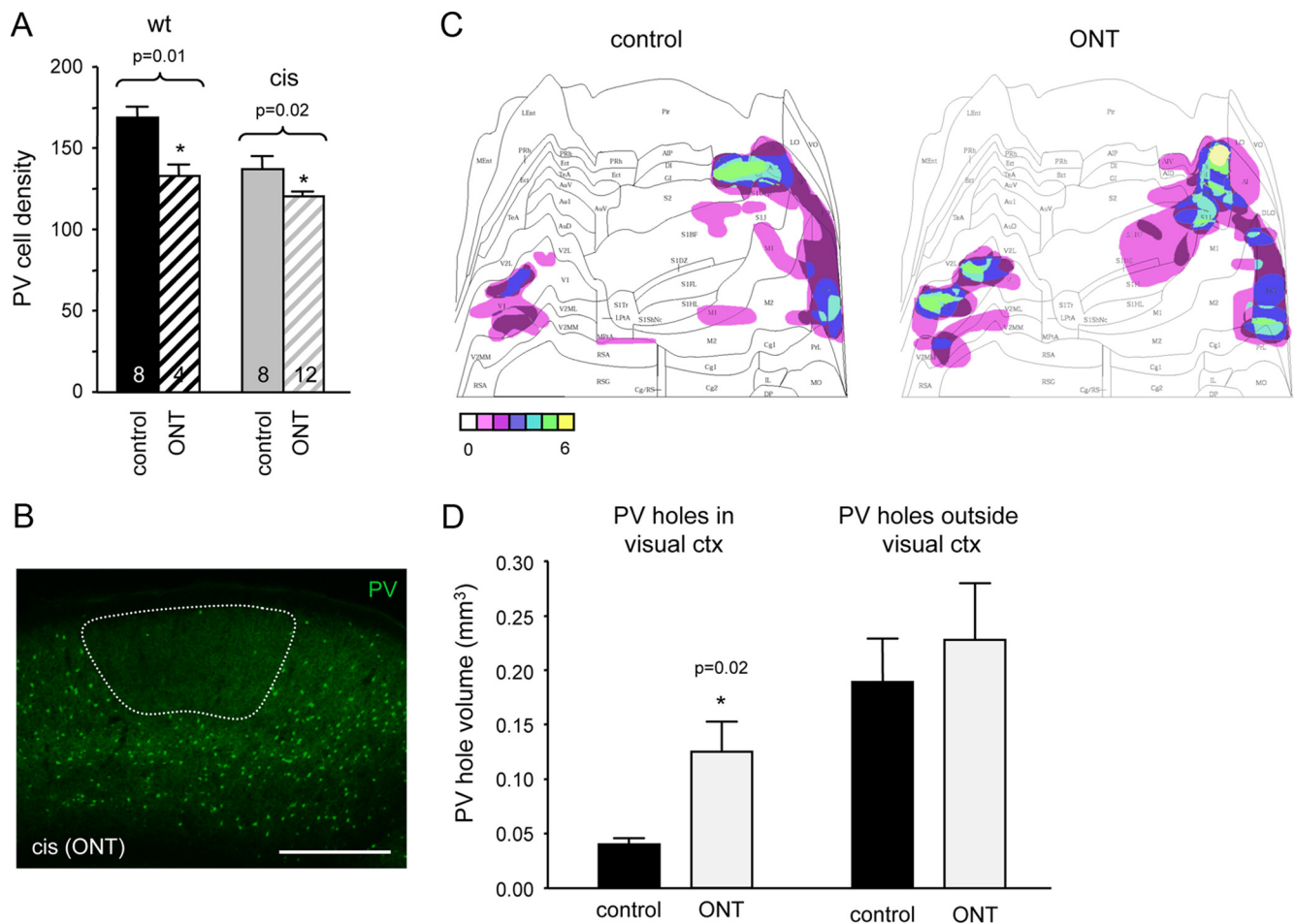
**Figure 3.** Neuronal loss in PV holes of *cis*-only mice. **A**, Quantitative analysis of PV and Gad67 cell density in sagittal sections of adult wild-type and *cis*-only cortex. Cell density in each quadrant is color-coded as per the scale shown, from 1 to 20 cells per 0.04 mm<sup>2</sup>. A PV hole is outlined in the *cis*-only section. **B**, Quantification of Gad67 cell number in PV holes of the adult *cis*-only cortex and equivalent areas of wild-type cortex.  $n = 6$  holes, *cis*-only;  $n = 30$ , wt. **C**, Quantitative analysis of Lhx6 cell density in sagittal sections of wild-type and *cis*-only adult cortex. Cell density in each quadrant is color-coded, from 1 to 20 cells per 0.017 mm<sup>2</sup>. A PV hole is outlined in the *cis*-only section. **D**, Quantification of Lhx6 cell number in PV holes of the adult *cis*-only cortex and equivalent areas of wild-type cortex.  $n = 7$  holes, *cis*-only;  $n = 6$ , wt.

rons in the adult cortex (Lavdas et al., 1999), and we observed a decline of  $35.7 \pm 9.08\%$  in Lhx6<sup>+</sup> cell density in PV holes of *cis*-only mice by immunofluorescence (Fig. 3C,D; supplemental Fig. S3B, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). These values are in good agreement with the proportion of PV neurons among cortical interneurons and suggest a loss of PV cells, rather than loss of PV expression, in PV holes of the *cis*-only cortex. We also note that, outside PV holes, PV cell density in the *cis*-only cortex was not increased compared with wild-type cortex (Fig. 3A), arguing against the possibility that cortical PV interneurons were simply redistributed in the mutant. The analysis of GABAergic markers did not rule out the possibility that cells that failed to become GABAergic might still be present in place of PV neurons in the affected areas. To address this possibility, we counted the total number of neurons using the pan-neuronal NeuN marker in PV holes of *cis*-only mice and equivalent cortical areas of wild-type brains. We found a significant reduction of  $8.4 \pm 2.46\%$  ( $n = 44$  fields, *cis*-only;  $n = 125$  fields, wt;  $p = 0.01$ ) in the total number of neurons in PV holes of the *cis*-only cortex, a value that is in good agreement with the proportion of PV neurons present in the cerebral cortex (Tamamaki et al., 2003). We conclude from this analysis that PV holes represent cortical areas of the *cis*-only brain that lack PV interneurons.

### Preferential distribution of PV holes in the visual and frontal cortices

PV holes were variable in size (average volume  $0.30 \pm 0.44$  mm<sup>3</sup>). Their number and location also varied between different mutant brains, although during our initial analysis we noted a tendency for them to predominantly appear in the caudal- and frontal-most regions of the cortex. To obtain a more accurate record of their distribution, we systematically mapped the location of PV holes across serial sagittal sections in 36 hemispheres of adult *cis*-only brains according to their rostrocaudal and mediolateral position. These coordinates were transferred onto a flattened map of the mouse cortex (Paxinos and Franklin, 2001) (Fig. 4A), and layered upon each other to generate a heat map of PV holes in the adult *cis*-only cortex with a graded color scale showing the degree of overlap among holes in different hemispheres (Fig. 4B). This analysis revealed that the two cortical areas that were mostly affected by PV holes were the caudomedial visual cortex (particularly V2M and V1) and the frontal cortex, particularly the insula and frontal association cortices adjacent to the somatosensory cortex (Fig. 4B). Other areas, such as the motor, retrosplenial and auditory cortices, were either not at all or much less frequently affected (Fig. 4B). We note that this was in good correspondence with the regions showing reduced numbers of GABAergic neurons in the newborn *cis*-only cortex (Fig. 1), suggesting that the





**Figure 5.** Neuronal activity affects the size but not the distribution of PV holes. **A**, PV cell density in the cortex of control and ONT wild-type and *cis*-only mice at P35. Results are expressed as average  $\pm$  SD (number of hemispheres used is indicated). **B**, Example of a PV hole in the visual cortex of a P35 *cis*-only mouse after ONT. Scale bar, 500  $\mu$ m. **C**, Distribution of PV holes in P35 *cis*-only cortex of control (8 hemispheres superimposed) and ONT (13 hemispheres superimposed) mice. **D**, PV hole volume in visual cortex of control ( $n = 5$  holes) and ONT ( $n = 13$  holes) *cis*-only mice. PV hole volume outside the visual cortex of control ( $n = 14$  holes) and ONT ( $n = 28$  holes) *cis*-only mice.

collected from *cis*-only mice under ketamine-xylazine anesthesia. In wild-type mice, recordings from a single bipolar electrode inserted into the visual cortex showed the characteristic slow oscillatory activity (0.8–0.9 Hz) that has previously been described in cats and humans during normal slow wave sleep (Steriade et al., 1993) (Fig. 6*B*). In *cis*-only mice, this oscillatory activity was regularly interrupted by sharp, short-lasting (30–100 ms), negative potentials ( $n = 10$  electrodes, 5 mice) (Fig. 6*B*). These events only occurred during the depolarizing phase of the slow oscillation and are indicative of a state of enhanced cortical excitability (Lau et al., 2000). Retrospective PV immunohistochemistry was performed to confirm that PV holes were present at, or near, electrode insertion sites (data not shown). Together, these results indicate functional deficits in inhibitory cortical activity in *cis*-only mutants. In agreement with these observations, *cis*-only mice displayed a lower threshold for generation of seizures by a sublethal dose of the GABA antagonist pentylentetrazole (PTZ). Compared with wild-type controls, *cis*-only mice displayed a marked increase in seizure severity to the same dose of PTZ, with 11 of 16 mice (69%) reaching a stage 5 seizure that was always lethal in these mice (Fig. 6*C*). (The only wild-type mouse that reached stage 5 survived the seizure.) In addition, the latency to onset of seizure was significantly lower in *cis*-only mutants compared with wild-type mice (48 s *cis*-only vs 82 s wild type;  $p =$

0.05;  $n = 27$ , wt;  $n = 16$ , *cis*-only), which is also an indication of increased cortical excitability.

#### *cis*-only mice show increased social behavior

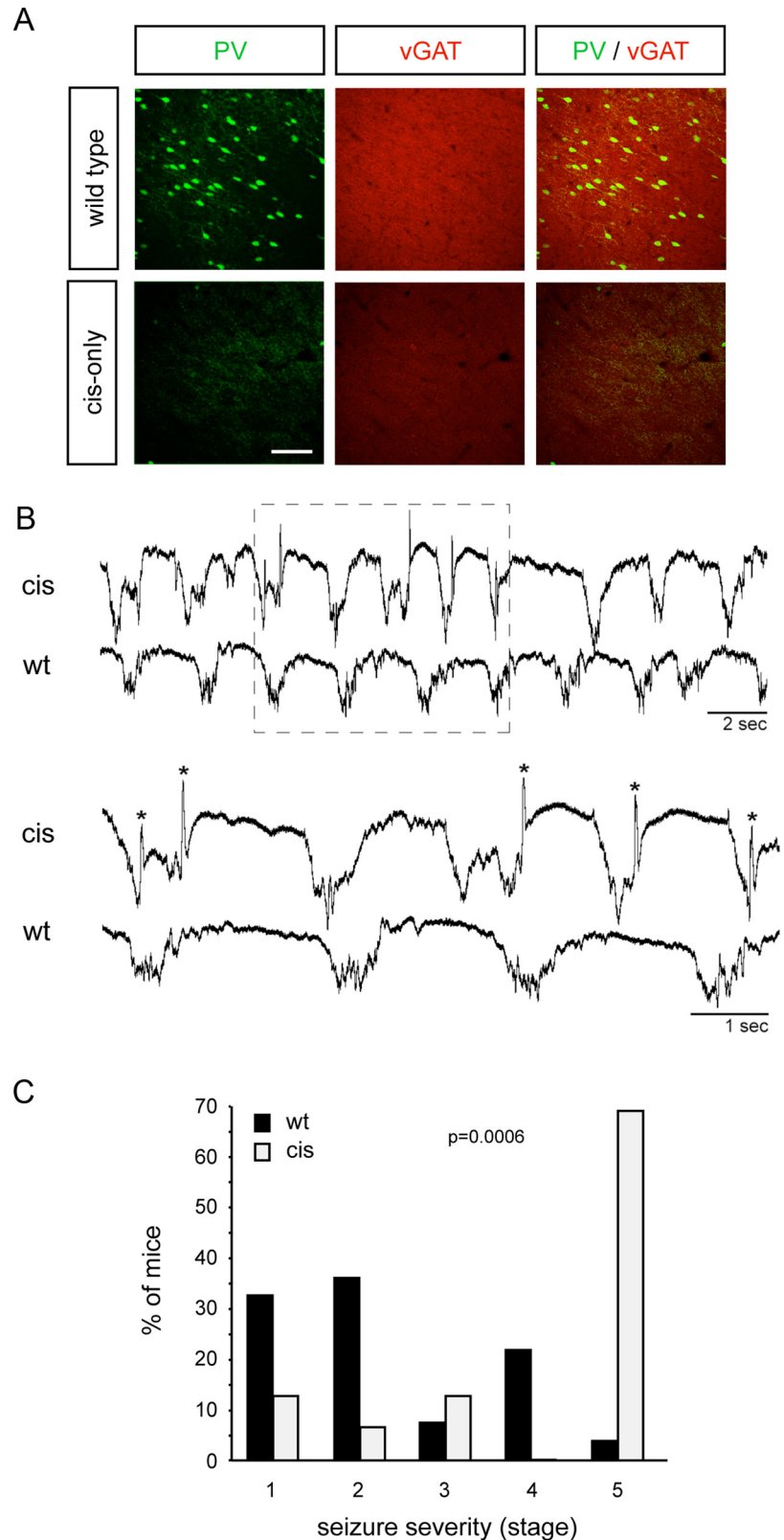
Human neurological conditions associated with abnormalities in cortical interneurons, such as schizophrenia, bipolar disorder and autistic spectrum disorders, present characteristic behavioral tendencies affecting anxiety state and social behavior. We subjected *cis*-only mice ( $n = 37$ ) and their wild-type littermates ( $n = 23$ ) to a primary SHIRPA screen to obtain a behavioral observation profile of the mutants (Rogers et al., 1997). The most prominent difference between wild type and mutants was found in the touch escape paradigm, in which *cis*-only males showed a heightened response ( $p = 0.05$ ). Such responses can sometimes be associated with increased anxiety. However, no significant differences could be found in the elevated plus maze, a standard behavioral test to detect changes in anxiety, although there was a tendency for *cis*-only mice to spend more time in the closed arms of the maze (data not shown). Because our animals originate from a mixed C57/129 genetic background, it is possible that this difference becomes statistically significant in mice that are genetically homogenous.

To investigate the possibility of altered social behavior, we performed a social interaction test in which two animals of the

same age, sex, and genotype that have not been in previous contact are placed together in a large open field. Before the test, each mouse had been habituated to the open field during 10 min for two consecutive days. Both wild-type and *cis*-only mice showed identical habituation profiles to the new environment in the sessions before the test, as assessed by the frequency of body care, digging, and rearing events (data not shown). On the third day, two mice were put together and the time spent in social interaction (within less than one-body length from each other) was measured during a period of 10 min. We found that *cis*-only mice spent significantly more time in close contact with each other compared with wild-type littermates, as assessed by the time spent within one body-length of each other during a 10 min period (total interaction time 207 s *cis*-only vs 155 s wild type;  $p = 0.04$ ;  $n = 7$  pairs, wt;  $n = 8$  pairs, *cis*-only). Moreover, and in contrast to wild-type mice, they showed no habituation to the presence of an unfamiliar mouse during the testing period (Fig. 7B). We conclude from this analysis that *cis*-only mice display an abnormal increase in social behavior.

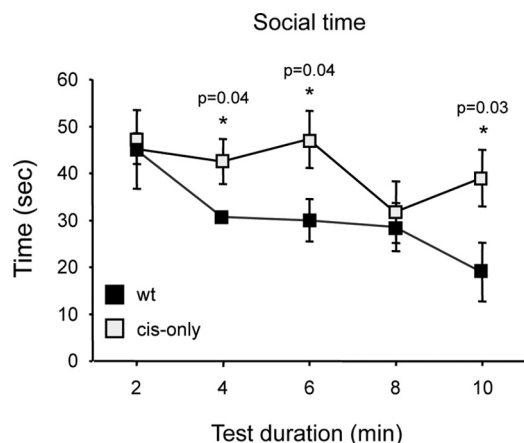
## Discussion

In this study, we investigated the importance of the GDNF receptor  $GFR\alpha 1$  for the diversification and allocation of cortical GABAergic interneurons. To this end, we used a line of mutant mice with a selective deficit in  $GFR\alpha 1$  expression restricted to cells that lack the RET signaling receptor subunit. Unlike null mutants, *cis*-only mice survive to adulthood, likely because of restoration of  $GFR\alpha 1$  expression in kidney and enteric neurons (Enomoto et al., 2004). This allowed us to investigate the fate and function of GABAergic interneurons in the postnatal and adult cortex of these mice. In newborn mutant animals, both null and *cis*-only, we found a specific loss of GABAergic neurons in rostralateral and caudal areas of the cortex but not in other cortical regions. Two striking observations were made in adult *cis*-only mutants. First, we found a loss of cortical GABAergic interneurons that was mostly restricted to PV-expressing cells, sparing calretinin-, somatostatin-, and most calbindin-expressing interneurons. Second, the extent of this loss was quite unusual, in that it was restricted to discrete patches, which we have called PV holes, interspersed among an otherwise normal-looking cortex. PV holes were most frequent in the same areas affected in newborn ani-



**Figure 6.** Decreased inhibitory activity in the cerebral cortex of *cis*-only mice. **A**, Loss of vGAT immunoreactivity in PV holes of *cis*-only mice. Scale bar, 100  $\mu$ m. **B**, EEG traces from adult *cis*-only and wild-type cortex. Region boxed in the top is shown enlarged below. Asterisks on the *cis*-only trace denote sharp negative potentials. **C**, Induction of seizures in wild-type and *cis*-only mice by PTZ injection. Shown are the percentages of mice displaying different stages of seizure severity. Seizure stages are based on Lau et al. (2000).  $p = 0.0006$ , Mann–Whitney nonparametric analysis.





**Figure 7.** *cis*-only mice display increased social behavior. Social interaction time plotted at 2 min intervals during the test period of 10 min.

mals, namely the frontal and visual cortices. These findings have important implications for our understanding of GDNF signaling, the diversification of GABAergic interneurons, and mechanisms of neocortical regionalization.

#### RET-independent GDNF signaling in GABAergic interneuron development

Our previous studies had demonstrated that mice lacking GDNF or *GFRα1* show a reduction in the number of cortical GABAergic interneurons at birth (Pozas and Ibáñez, 2005). Since this phenotype was not reproduced in animals lacking RET, we suggested the participation of RET-independent GDNF signaling in GABAergic interneuron development. Detailed analysis of the CNS of *cis*-only mice has demonstrated a perfect correspondence between RET and *GFRα1* expression and a complete absence of *GFRα1* in cells lacking RET, without any ectopic *GFRα1* expression (H. Enomoto, unpublished observations). Since these mice lack *GFRα1* only in cells that do not normally express RET, processes that are controlled or regulated in a cell-autonomous manner by GDNF signaling through RET should not be affected by the *cis*-only mutation. In the initial analysis of these mice, no abnormalities could be observed in kidney histogenesis, enteric nervous system development or neuromuscular connectivity (Enomoto et al., 2004). Moreover, the CNS of these animals appeared grossly normal. Our present results, demonstrating profound abnormalities in cortical GABAergic neuron distribution, cortical excitability and behavior in *cis*-only mice, provide strong evidence for the physiological importance of RET-independent signaling *in vivo*. The specificity of these defects was further confirmed by their absence in conditional mutants lacking RET in the CNS, or *Ret<sup>Gfra1+</sup>* mice, expressing an additional copy of *GFRα1* from the *Ret* locus. Although the p140 isoform of NCAM can also function as a signaling receptor subunit for GDNF together with *GFRα1* (Paratcha et al., 2003), no deficits in cortical GABAergic interneurons have been observed in the newborn cortex of mice lacking this receptor (Pozas and Ibáñez, 2005). Therefore, *GFRα1* may contribute to GABAergic neuron development by partnering with other transmembrane molecules or through alternative mechanisms, such as ligand-induced cell adhesion (Ledda et al., 2007).

#### PV holes and neocortical regionalization

One of the most striking observations made in the present study is the pattern of GABAergic interneuron loss in the adult cortex of

*cis*-only mice. Although several other mutants have also shown deficits in cortical GABAergic interneurons, *cis*-only mice are, to the best of our knowledge, the first to display PV holes or any such pattern of interneuron loss in the cortex. Previous studies have analyzed the effects of mutations in genes affecting large numbers, if not the majority, of GABAergic neurons or their progenitors, and found reductions in the number of interneurons across large areas of the cortex (Levitt et al., 2004; Wonders and Anderson, 2006; Liodis et al., 2007). In contrast, the deficits observed in *cis*-only mice were mostly restricted to PV interneurons and, among these, primarily those normally residing in the visual and frontal cortices.

The reduction of GABAergic and pan-neuronal markers observed in PV holes is consistent with cell loss, rather than abnormal differentiation, and suggests—in the absence of detectable cell death—that it has a developmental origin. This is in agreement with the overall correspondence between the predominant location of PV holes and the distribution of deficits in null and *cis*-only mutants at birth. Together, our observations suggest that these defects originate during prenatal cortical development. During the last week of embryonic development, GABAergic neurons that had entered the cortex by tangential migration from the ganglionic eminences disperse through the marginal, intermediate and subventricular zones before invading the cortical plate (Tanaka et al., 2006). This process goes on over several days and involves tangential migration over long distances, up to 3 mm in the marginal zone (Tanaka et al., 2006), well in the range of the average size of PV holes. Although GABAergic neurons have been seen to disperse in multiple directions in the developing cortex (Tanaka et al., 2006), it is currently unknown whether different interneuron subtypes have distinct dispersion routes or migratory behaviors. The predominant locations of the deficits observed in newborn and adult *cis*-only mutants, at the caudal and rostral poles of the cortex, suggests a role for *GFRα1* signaling in the allocation of PV interneurons to those cortical areas. Since the caudal and frontal cortices are the regions furthest away from the ganglionic eminences, a general decrease in neuronal migration could in theory result in specific deficits in those areas. However, PV holes have not been observed in other mutants affected in general aspects of cortical interneuron migration or development, such as *uPAR* (Powell et al., 2001), *ErbB4* (Flames et al., 2004), or *Lhx6* (Liodis et al., 2007) knock-out mice. We thus favor the idea that *GFRα1* plays a more specific role in the allocation of PV interneurons to distinct cortical regions. *GFRα1* may function in a cell-autonomous manner in a subpopulation of GABAergic precursors of the MGE, directing their differentiation and migration to specific cortical areas. Alternatively, *GFRα1* may contribute to cortical interneuron allocation in a non-cell-autonomous manner, perhaps by regulating the interaction of interneurons with other cells or axonal pathways along their migratory routes. Because GABAergic neurons no longer express *GFRα1* once in the cortex, deciding between these alternatives will require additional studies, including genetic fate-mapping of *GFRα1*-expressing precursors and conditional ablation of *GFRα1* in MGE cells. Regardless of the mechanisms involved, our results present the first indication of compartmentalization of a distinct GABAergic interneuron subtype in the cerebral cortex, and reveal a level of cortical regionalization that has previously remained underappreciated.

After birth, cortical interneurons undergo a protracted period of maturation during which they acquire their adult biochemical and functional properties. In the visual cortex, this stage coincides with the critical period of ocular dominance and other

forms of plasticity, during which neural activity has the strongest influence on cortical maturation. Our observation that visual deprivation during the first 5 postnatal weeks did not affect the distribution of PV holes in the *cis*-only cortex is also in agreement with an earlier developmental origin for this defect, as discussed above. On the other hand, the increased size of PV holes in the visual cortex after ONT suggests that neuronal activity can influence the extent of PV interneuron loss in the affected areas. During cortical maturation, and as interneurons incorporate into functional circuits, differences in neuronal activity between cortical areas may affect interneuron distribution by recruiting cells toward the area undergoing faster activity-driven maturation. Such a process could possibly contribute to the amplification and sharpening of interneuron deficits initially established earlier in development. In agreement with our findings, an activity-driven process of this kind would not be expected to take place in identical manner in different brains, although strong correlations should be observed between hemispheres of the same brain.

### PV interneurons and cortical function

The *cis*-only cortex displayed a major decrease in inhibitory innervation in the affected areas, abnormal EEG profiles, and markedly enhanced sensitivity to PTZ-induced seizures. All these alterations are consistent with increased cortical excitability in the *cis*-only brain. Changes in excitatory/inhibitory balance are believed to contribute to the pathogenesis of several cognitive disorders, including epilepsy, schizophrenia, autism, and bipolar disorder (Eyles et al., 2002; Rubenstein and Merzenich, 2003; Cossart et al., 2005). In the case of epilepsy and schizophrenia, most human studies have indicated that reduced numbers of cortical GABAergic interneurons and decreased inhibitory activity may contribute to these conditions (Levitt, 2005; Sakai et al., 2008). Interestingly, a previous study has suggested a potential linkage of *Gdnf* and *Gfra1* genes to schizophrenia (Moises et al., 2002). On the other hand, the association of autism with reduced inhibitory drive is far more tenuous, mainly derived from an elevated incidence of epilepsy among a subset of autistic patients (Rubenstein and Merzenich, 2003). In fact, recent experimental evidence would appear to indicate the opposite. Mutant mice carrying a point mutation in *neuroligin-3* that has been implicated in several cases of autism showed enhanced inhibitory synaptic transmission in the cerebral cortex (Tabuchi et al., 2007). Interestingly, similar to autistic patients, these animals displayed impaired social behavior. Our observations in *cis*-only mice showing decreased cortical inhibitory activity and enhanced social behavior are in agreement with this relationship. Together, these data suggest that, at least in mice, cortical excitability and social behavior are positively correlated.

In conclusion, the results of this study reveal the GDNF receptor GFR $\alpha$ 1 as an important determinant of cortical GABAergic neuron diversification and allocation of specific interneuron subtypes to distinct cortical areas. The unusual defects of the adult *cis*-only cortex suggest the existence of previously unknown mechanisms of cortical regionalization which remain to be elucidated.

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