Loss of COUP-TFI Alters the Balance between Caudal Ganglionic Eminence- and Medial Ganglionic Eminence-Derived Cortical Interneurons and Results in Resistance to Epilepsy

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In rodents, cortical interneurons originate from the medial ganglionic eminence (MGE) and caudal ganglionic eminence (CGE) according to precise temporal schedules. The mechanisms controlling the specification of CGE-derived interneurons and their role in cortical circuitry are still unknown. Here, we show that COUP-TFI expression becomes restricted to the dorsal MGE and CGE at embryonic day 13.5 in the basal telencephalon. Conditional loss of function of COUP-TFI in subventricular precursors and postmitotic cells leads to a decrease of late-born, CGE-derived, VIP (vasoactive intestinal peptide)- and CR (calretinin)-expressing bipolar cortical neurons, compensated by the concurrent increase of early-born MGE-derived, PV (parvalbumin)-expressing interneurons. Strikingly, COUP-TFI mutants are more resistant to pharmacologically induced seizures, a phenotype that is dependent on GABAergic signaling. Together, our data indicate that COUP-TFI controls the delicate balance between MGE- and CGE-derived cortical interneurons by regulating intermediate progenitor divisions and ultimately affecting the activity of the cortical inhibitory circuitry.

Introduction

In rodents, interneurons are born in basal telencephalic structures, such as the medial ganglionic eminence (MGE), the caudal ganglionic eminence (CGE), and the preoptic area from which they migrate to populate the cortex (Corbin et al., 2001; Marin and Rubenstein, 2001, 2003). Various transplantation and fate-mapping experiments have shown that such a wide diversity is intimately dependent on their birth date and location. Early-born interneurons originate from the MGE and produce mainly the

parvalbumin (PV)- and somatostatin (SST)-expressing subtypes, which contribute primarily to deep cortical layers; late-born interneurons derive predominantly from the CGE, generate calretinin (CR)- and vasoactive intestinal peptide (VIP)-expressing interneurons, and preferentially occupy superficial cortical layers (Nery et al., 2002; Xu et al., 2004; Butt et al., 2005, 2008; Cobos et al., 2006; Fogarty et al., 2007; Miyoshi et al., 2007, 2010).

Understanding the spatial and temporal mechanisms of GABAergic cortical interneuron determination is attracting much interest in the last few years. Transcription factors expressed either broadly within the two eminences or predominantly in one eminence play a fundamental role in the specification and maturation of these cells (Batista-Brito and Fishell, 2009). Whereas loss of *Dlx1/Dlx2* function results in a massive decrease of neocortical GABAergic interneurons at birth (Anderson et al., 1997), mice lacking only the *Dlx1* gene show reduction of CR- and SST-expressing interneurons without affecting the PV-expressing population (Cobos et al., 2005). These mice have cortical dysrhythmia and generalized seizures. Early removal of the transcription factor *Nkx2.1*, restricted to the MGE domain, results in a molecular and cellular switch of MGE-derived cortical interneurons (PV- and SST-positive subpopulations) to CGE-derived neurons (VIP- and CR-expressing cells), leading ultimately to seizure activities (Butt et al., 2008). Down-

Received Dec. 17, 2010; accepted Jan. 17, 2011.

This work was supported by the Italian Telethon Foundation (M.S.), the Spanish Fundación Alicia Koplowitz (L.M.d.I.P.), the Harvard Stem Cell Institute (P.A.), and European Community FP6 STREP Contract 005139 (INTERDEVO) (M.S., L.M.d.I.P.). S.L. is the recipient of a predoctoral fellowship of the European School of Molecular Medicine (Naples, Italy). We are grateful to G. Fishell, O. Marin, M. Ross, and M. Nieto for helpful comments on this manuscript. We thank F. Guillemot for cyclin D1 and cyclin D2 plasmids; V. Pachnis for the VIP plasmid and the *Lhx6* antibody; J. L. R. Rubenstein for the *Nkx2.1*, *Gad67*, and somatostatin plasmids; R. Rusconi for designing the cell counting algorithm developed in Matlab; M. Mancuso, F. Russo, A. I. Merlino for technical assistance; and M. Giordano for animal husbandry. L.M.d.I.P. and M.S. jointly coordinated and designed this work.

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DOI:10.1523/JNEUROSCI.6580-10.2011

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stream of Nkx2.1, the transcription factor Lhx6 is involved in the specification and migration of PV- and SST-expressing interneurons (Liodis et al., 2007), whereas Sox6, acting downstream to Lhx6 (Batista-Brito et al., 2009), is required for the correct balance of PV-, SST-, and neurotransmitter peptide Y (NPY)-expressing subpopulations (Azim et al., 2009; Batista-Brito et al., 2009). Loss of function of Sox6 results in a dramatic reduction of PV- and SST-expressing interneurons, whereas NPY+ interneurons outnumber the normal level without affecting the CGE-derived CR- and VIP-expressing subpopulations. Interestingly, similarly to Dlx1 and Nkx2.1 mutant mice, Sox6 mutants suffer from generalized epileptic seizures (Batista-Brito et al., 2009).

Although many reports have characterized genetic determinants of MGE-derived cortical interneurons, little is known about the regional and cell type specification of CGE-derived interneurons, which comprise ~30% of all cortical interneurons with an unexpected higher diversity than previously anticipated (Miyoshi et al., 2010). The orphan nuclear receptor COUP-TFII shows restricted expression in the CGE (Kanatani et al., 2008; Willi-Monnerat et al., 2008) and, together with COUP-TFI, is required for the caudal migration of cortical interneurons (Tripodi et al., 2004; Kanatani et al., 2008). Moreover, in Nkx2.1 conditional mutant mice, in which a high number of CR- and VIP-expressing cortical interneurons are generated, COUP-TFII is ectopically expressed in the MGE (Butt et al., 2008). Together, these reports strongly suggest that COUP-TF members might be directly involved in the migration and specification of CGE-derived cortical interneurons.

Here, we show that COUP-TFI expression becomes gradually restricted to the caudal basal telencephalon. Conditional inactivation of COUP-TFI using a pan-interneuronal *Cre* mouse line leads to increased PV-expressing interneurons and decreased VIP- and CR-expressing bipolar neurons, without affecting the total number of cortical GABAergic cells. Interestingly, COUP-TFI conditional embryos show increased cellular proliferation predominantly in the MGE as well as enhanced expression of the cell cycle gene *cyclinD2*, known to establish the proper density of PV-expressing interneurons (Glickstein et al., 2007b). Strikingly, we found no discernible electroencephalographic (EEG) abnormalities, but, instead, *COUP-TFI* conditional mice are more resistant to pharmacologically induced seizures, a GABA-dependent property. Together, our data indicate an intrinsic property of COUP-TFI in regulating the balance between MGE- and CGE-derived cortical interneurons, thus contributing to the proper formation of the cortical local inhibitory circuitry.

Materials and Methods

Mice. *COUP-TFI^{flox/+}* mice were generated as previously reported (Armentano et al., 2007) and propagated by backcrossing to C57BL/6 inbred mice. Homozygous *COUP-TFI^{flox/flox}* mice were obtained by intercrossing heterozygous *COUP-TFI^{flox/+}* mice and mated to the *Dlx5/6-Cre-IRES-GFP* transgenic line (Stenman et al., 2003), a kind gift from K. Campbell (Children's Hospital Research Foundation, Cincinnati, OH), to generate conditional *COUP-TFI* *CKO-Dlx5/6* embryos and mice (*COUP-TFI^{flox/flox}* homozygous-*Dlx5/6* *Cre* heterozygous). Throughout the study, we used as controls [referred also as wild type (WT)] either heterozygous (*COUP-TFI^{flox/+}*), homozygous (*COUP-TFI^{flox/flox}*), or *Dlx5/6-Cre*-positive (*Cre+*) mice, since they show no phenotypic abnormalities, as previously described (Stenman et al., 2003; Armentano et al., 2007). We found no phenotypic differences between male and female mutant mice. Genotyping was performed as previously described (Stenman et al., 2003; Armentano et al., 2007). Midday of the day of the vaginal plug was embryonic day 0.5 (E0.5). All experiments were conducted

following guidelines of the Institutional Animal Care and Use Committee, Cardarelli Hospital (Naples, Italy).

Immunocytochemistry and in situ hybridization. Mice were perfused with 4% buffered paraformaldehyde (PFA), and decapitated heads (E12.5–E14.5) or brains [postnatal day 21 (P21)] were postfixed in 4% PFA for 12 h at 4°C. Brains were either sectioned on a vibratome at 50 μ m or cryosectioned in OCT medium (Tissue-Tek) at 20 μ m. Vibratome sections were processed free floating, and standard nonradioactive *in situ* hybridization and immunofluorescence protocols were used. *In situ* hybridization and combined immunohistochemistry were performed as previously described (Tripodi et al., 2004). The following primary antibodies were used: COUP-TFI (rabbit; 1:500) (Tripodi et al., 2004), bromodeoxyuridine (BrdU) (mouse; 1:300; Sigma-Aldrich), Ki67 (rat; 1:250; Dako), GABA (rabbit; 1:1000; Sigma-Aldrich), NPY (rabbit; 1:3000; Diasorin), calretinin (rabbit; 1:5000; Swant), parvalbumin (mouse; 1:1000; Millipore), Lhx6 (rabbit; 1:500; kind gift from V. Pachnis), and green fluorescent protein (GFP) (rabbit; 1:1000; Millipore). The following secondary antibodies were used: 1:400, Alexa Fluor 488 α -rabbit; Alexa Fluor 594 α -rabbit; Alexa Fluor 594 α -mouse (Invitrogen); 1:200, biotinylated goat anti-rabbit (Vector Laboratories). Nonradioactive *in situ* hybridization on 16- to 20- μ m-thick cryostat sections were performed as previously described (Armentano et al., 2007). Antisense RNA probes were labeled using a DIG-RNA labeling kit (Roche). The following probes were used: *COUP-TFI*, *Gad67*, *SST*, *VIP*, *CyclinD2*, and *Nkx2.1*.

BrdU birthdating. Timed-pregnant females received a single intraperitoneal injection of BrdU (50 mg/kg) 1 h before being killed, and embryos were collected at E13.5. WT and *COUP-TFI* *CKO-Dlx5/6* embryos were examined for BrdU-positive cell distribution in the MGE and CGE, as previously described (Tomassy et al., 2010). Sections from rostral to intermediate levels (see Fig. 1B,C), where the MGE is still distinct, and from caudal to caudalmost levels where the CGE (see Fig. 1D) is clearly defined, were photographed at 10 $\times$ magnification on a Leica DM5000B equipped with Leica IM image management software (Leica Microsystems), and then imported into Adobe Photoshop for counting. The percentage of BrdU-positive cells was calculated as the ratio of double BrdU/4',6'-diamidino-2-phenylindole (DAPI)-positive cells divided by the total number of DAPI-positive cells.

Electroencephalographic recordings. Freely moving mice aged between 7 and 13 weeks were tested for spontaneous EEG activity. Animals were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg, i.p.) and placed in a stereotaxic apparatus. Cortical EEG recordings were obtained from 100- μ m-wide tungsten wires implanted over the parietal cortex at 0.2–0.4 mm depth from the cortical surface using bone cement. Two screws were used as a reference and ground at the occipital region. In a group of animals, two separate wires were bilaterally implanted in the dorsal hippocampus to obtain simultaneous cortical and hippocampal EEG activity. After recovering from surgery, EEG was recorded from individual mice over several days, for a maximum of 2 h per day, by using a Grass EEG Neurodata system. Total recording time per mouse ranged from 18 to 36 h over the course of 12–20 d. Animals were simultaneously videotaped for identifying different behavioral states. EEG recordings during awake states were bandpass FIR filtered between 1 and 200 Hz and sampled at 500 Hz. After completing EEG recordings, brains were fixed in 4% PFA for electrode placement verification.

EEG data were analyzed with Spike 2 and routines developed in Matlab. The spectral power was calculated using the fast Fourier transform with Hanning window and 1024 points for artifact-free recording windows of 10 min duration. To study the frequency content independently of amplitude changes, each EEG spectrum was normalized by the total power in the 1–100 Hz band. The mean power spectrum for each group is represented together with the 95% confidence intervals. To look at spectral differences between types of oscillations, four different frequency bands were defined: 1–4 Hz (δ), 4–10 Hz (θ), 10–40 Hz (β), and 40–100 Hz (γ). The power spectrum value for each band was calculated by integrating the normalized power spectrum in the corresponding frequency interval. For statistical comparisons, we first tested data for normal distribution using the Kolmogorov–Smirnov test. Power spectral values in a logarithmic scale were normally distributed

except for the 40–100 Hz gamma band. Thus, to quantify statistical differences, spectra from the two experimental groups were compared using a nonparametric test for multiple comparisons (Mann–Whitney *U* test). To compare the spectral distribution at the delta, theta, and beta bands, a two-way ANOVA was used with the factors group and gender. Differences in the gamma band were compared using the Kruskal–Wallis nonparametric test for the group and for the gender independently.

Pharmacological seizure induction. For pilocarpine-induced seizures, mice were intraperitoneally injected with lithium chloride (423 mg/kg) between 18 and 20 h before 100 mg/kg pilocarpine hydrochloride. Pentylenetetrazole (PTZ) (Sigma-Aldrich) was administered subcutaneously at a dose of 90 mg/kg. We chose a suprathreshold dose of PTZ to induce clonic seizures independently on the gender and with uniform latency within groups (Löscher et al., 1991). In a group of PTZ experiments, we pretreated mice with the GABA_B receptor antagonist (3-aminopropyl)(diethoxymethyl)phosphinic acid hydrate (GCP 35348) (Sigma-Aldrich) at a dose of 136 mg/kg. Mice injected with PTZ and not pretreated with GCP 35348 were divided in two groups, one-half not injected and one-half injected with vehicle. Animals were observed for 45 min after the PTZ injection. Three different stages of seizure activity were scored: stage 1, body and tail rigidity; stage 2, characterized by whole-body clonus; and stage 3, characterized by generalized tonic-clonic hindlimb extensions.

Cell counting. For the quantification of interneuron subpopulations in COUP-TFI *CKO-Dlx5/6* and wild-type cortices, three coronal anatomically matched sections within the sensorimotor area in the rostrocaudal axis (bregma, from 0.50 to −0.34 mm) were selected from littermate mice and processed by immunocytochemistry to detect GABA, PV, NPY, and CR, and *in situ* hybridization to detect SST and VIP transcript ($n = 3$ COUP-TFI *CKO-Dlx5/6*; $n = 3$ wild type, 6 hemispheres per area, for each mouse at P21). Digital boxes of fixed width were superimposed on each coronal section and they were divided into 10 sampling areas (bins) with a dorsoventral extent from the pial surface to the white matter (corpus callosum). Deep layer were assigned to bin 1–5 and superficial layers were assigned to bin 6–10, based on anatomical features. Cell detection and counting were performed using a customized imaging processing software. Bipolar cells were identified manually. The automated algorithm has been developed in Matlab (version 7.6; The MathWorks) by an investigator blinded to the study design and consisted in a series of processing steps. In the first one, original fluorescent or bright-field images, taken at 4× magnification, are corrected for uneven illumination and background noise. Image segmentation is then obtained through edge detection, in which minimum and maximum levels of the intensity gradient are established a priori for each staining analyzed and kept constant throughout the counting analysis. Then, morphological characteristics were added to the filtering process. The eccentricity parameter—where 1 indicates a linear shape and 0 indicates a circular shape—was used to identify cell bodies, by using a threshold values established a priori. In combination with the eccentricity parameter, thresholds for the cell size were established a priori based on the normal distribution of cell area for each staining analyzed. In addition, the area parameter was used to discriminate single cells within clusters. For the quantification of the percentage of interneuron subtypes that express COUP-TFI in P8 and P21 wild-type cortices, three coronal sections spanning within the somatosensory area in the rostrocaudal axis (bregma, −0.34 mm) were selected from littermate mice ($n = 3$, 6 hemispheres per area, for each wild-type mouse at P8 and P21). Digital boxes of fixed width were superimposed on each coronal section and cell counting was performed on images taken at 10× magnification by an investigator blinded to the study design. Counting criteria were established a priori.

Data analysis. All cell counting data and the graphs were constructed using Microsoft Excel software. For each animal, a mean value was calculated from all the sections counted, and for each genotype, a mean value was obtained by pooling the means of the three sampled animals. EEG data were analyzed using the Mann–Whitney *U* test for comparison of the power spectra between groups and an ANOVA or a Kruskal–Wallis test for comparison of the four different frequency bands between group and gender (see above, Electroencephalographic recordings). All behavioral pharmacological data were analyzed using either one-way ANOVA (genotype, two levels: WT and CKO) or two-way ANOVA [genotype

(two levels: WT and CKO) and pretreatment (two levels: vehicle and CGP 35348)]. *Post hoc* analysis was performed using Duncan's *post hoc* test. All error bars represent the SEM. Statistical significance was determined using two-tailed Student's *t* tests ($*p < 0.05$; $**p < 0.01$).

Results

COUP-TFI is preferentially expressed in the dorsal MGE, in the CGE, and in a subpopulation of mature cortical interneurons

To investigate the function of COUP-TFI in the development of cortical GABAergic interneurons, we first characterized its expression profile in the developing ganglionic eminences. From E10.5 to E12.5, COUP-TFI is expressed in the subpallium, and in the lateral ganglionic eminence (LGE) and MGE, respectively (Armentano et al., 2007; Faedo et al., 2008) (Fig. 1A). However, at E13.5, COUP-TFI expression becomes highly regionalized with a low rostral to high caudal expression gradient (Fig. 1B–D). Whereas COUP-TFI expression is restricted to the corticostriatal boundary and dorsal MGE (dMGE) at rostromedial levels (Fig. 1B), its expression increases at intermediate levels and includes also the preoptic area (Fig. 1C) (Flames et al., 2007). Expression becomes highest in the caudalmost basal telencephalon, particularly in the ventricular zone (VZ) and subventricular zone (SVZ) of the ventral CGE (vCGE), whereas in the LGE and dorsal CGE (dCGE) expression of COUP-TFI is lower (Fig. 1D). Thus, we found that, at E13.5, COUP-TFI becomes restricted in a rostrocaudal and dorsoventral expression gradient in the basal telencephalon, being strongest in the VZ and SVZ of the ventrocaudal ganglionic eminence, the vCGE.

COUP-TFI is also expressed in immature interneurons migrating tangentially from the basal telencephalon to the developing neocortex, as previously described (Tripodi et al., 2004), and in mature cortical interneuron subtypes at postnatal stages (this study) (Fig. 1E–J''). Double labeling for COUP-TFI protein and the glutamate decarboxylase *GAD67* transcript shows a high proportion of postnatal GABAergic interneurons positive for COUP-TFI in the somatosensory cortex ($83.1 \pm 2.2\%$; $n = 3$; P8) (Fig. 1E, E'). To further address whether specific subtypes of cortical interneurons express COUP-TFI, we performed double labeling for COUP-TFI and different molecular markers of mature cortical interneurons including the peptide hormone SST, NPY, and VIP at P8 ($n = 3$) (Fig. 1F–H'), as well as the calcium-binding proteins CR and PV at P21 ($n = 3$) (Fig. 1I, I'') within the somatosensory cortex. At P8, COUP-TFI was found in $55.8 \pm 1.1\%$ of SST-expressing, in $70.0 \pm 2.1\%$ of NPY-expressing, and in $78.1 \pm 5.4\%$ of VIP-expressing interneurons (Fig. 1F–H'). At P21, we found only a $4.0 \pm 0.3\%$ overlapping between COUP-TFI and PV, and $51.6 \pm 5.3\%$ colabeling between COUP-TFI and CR (Fig. 1I, I''). However, detailed morphological analysis indicates that COUP-TFI is highly expressed ($89.2 \pm 5.9\%$) in a subpopulation of CR+ cells that shows a characteristic vertically oriented bipolar morphology (Fig. 1I'–I''). Together, these data indicate that, at postnatal stages, COUP-TFI is expressed in most mature cortical interneuron subtypes, with a preference to CGE-derived interneurons, such as bipolar VIP+ and CR+ cells, and to a subpopulation of NPY+ cells (Karagiannis et al., 2009), whereas it is generally excluded from the MGE-derived PV-expressing cells.

Specific inactivation of COUP-TFI in SVZ intermediate progenitors of the basal telencephalon

To investigate the role of COUP-TFI in interneuron diversity and in the maturation and specification of different cortical interneuron subtypes, we used a conditional genetic approach in which the COUP-TFI *lox/lox* (COUP-TFI^{lox}) line (Armentano et al.,

Figure 1. COUP-TFI becomes gradually restricted to the dorsal MGE and CGE and is highly expressed in CGE-derived cortical interneurons. **A**, Coronal section of E12.5 wild-type embryo at intermediate levels showing localization of COUP-TFI protein in the telencephalon. Note high expression in the LGE and dorsal half of the MGE. **B–D**, Rostral to caudal sequential coronal sections of E13.5 wild-type embryos hybridized with *COUP-TFI* riboprobe indicate a low rostral to high caudal expression gradient, with regionalized expression in the dMGE (**B**, **C**) and preoptic area (POA) (**C**, **D**). Expression is confined to the VZ and SVZ. The arrow in **D** indicates highest COUP-TFI expression levels in vCGE. **E–H**, Double immunostaining for COUP-TFI protein and *in situ* hybridization for *Gad67*, *SST*, and *VIP* (**E**, **F**, **H**) and double immunofluorescence for COUP-TFI and NPY in P8 somatosensory cortices (**G**). The roman numerals denote cortical layers. **E'–H'**, High-magnification views of a representative detail from **E–H** indicate double-positive cells (arrowheads) and the different percentage of *Gad67*+ (83%), *SST*+ (56%), *NPY*+ (70%), and *VIP*+ (78%) interneurons expressing COUP-TFI. **I–J''**, Double immunofluorescence for COUP-TFI and CR (**I**), and COUP-TFI and PV in P21 somatosensory cortices (**J**). High-

2007) was mated to the *Dlx5/6-Cre-IRES-GFP* transgenic line (Stenman et al., 2003), which drives the CRE-mediated activity exclusively within the basal telencephalic SVZ and mantle zone (MZ) (Fig. 2A). Mice homozygous for *COUP-TFI*^{flox} and heterozygous for *Dlx5/6-Cre-IRES-GFP* are viable and fertile, and will be named *COUP-TFI* *CKO-Dlx5/6* throughout this study.

We first assessed whether and at what age COUP-TFI was specifically inactivated in *Dlx5/6*-positive cells by taking advantage of the endogenous GFP fluorescence of the *Dlx5/6-Cre-IRES-GFP* transgenic line (Stenman et al., 2003). In the basal telencephalon of *COUP-TFI* *CKO-Dlx5/6* heterozygotes, only SVZ progenitors are double positive for GFP and COUP-TFI (Fig. 2E,H), whereas VZ progenitors express COUP-TFI (in red) but not GFP (in green) (Fig. 2C,F,D,G). No, or very few, COUP-TFI/GFP double-positive cells are detected in the SVZ or mantle zone of *COUP-TFI* *CKO-Dlx5/6* homozygous embryos at E12.5 and E13.5 (Fig. 2E',H') (data not shown), indicating that, already at E12.5, before COUP-TFI becomes regionalized to dMGE and CGE, COUP-TFI function is specifically abolished in the SVZ (*Dlx5/6*-positive domain), but not in progenitors located in the VZ (*Dlx5/6*-negative domain). Accordingly, at E15.5, COUP-TFI fails to be expressed in postmitotic neurons of the basal telencephalon (Fig. 2I–J'), with the exception of the ventrally migrating stream originating from the *Dlx5/6*-negative interganglionic region, which coexpress COUP-TFI and COUP-TFII (Tripodi et al., 2004). Thus, we generated a conditional COUP-TFI mutant line, in which COUP-TFI is specifically inactivated in SVZ progenitors and postmitotic interneurons, but not in VZ progenitors.

Conditional loss of COUP-TFI function alters the balance of interneuron subtypes without affecting the total number of GABAergic cortical interneurons

Next, we investigated whether loss of function of COUP-TFI in SVZ progenitors of the basal telencephalon would affect the total number and laminar distribution of cortical interneurons at P21 in

magnification views indicate highest colocalization of COUP-TFI with bipolar CR-positive cells (**I'–I''**, arrowheads), and lowest colocalization of COUP-TFI with PV-positive cells (**J'–J''**). Abbreviations: ctx, Cortex; dMGE, dorsal medial ganglionic eminence; LGE, lateral ganglionic eminence; pir, piriform cortex. Scale bars: **A–D**, 200 μ m; **E–J**, 100 μ m; **E'–J'**, 50 μ m.

Figure 2. Conditional loss of COUP-TFI in cortical interneurons affects SVZ progenitors in the basal telencephalon. **A**, Schematic of the genetic strategy for examining COUP-TFI conditional loss of function by using the *Dlx5/6-Cre-IRES-EGFP* mouse active in the SVZ progenitors and postmitotic interneurons of the basal telencephalon (Stenman et al., 2003). **B**, Schematic of a E13.5 coronal hemisection indicating the position of **C–E'**. Green indicates the expression of the EGFP on Cre-mediated recombination. **C–E'**, Coronal sections of E13.5 *COUP-TFI CKO-Dlx5/6 (CKO-Dlx5/6)* heterozygotes and homozygotes immunostained with anti-GFP (**C, C', F, F'**), anti-COUP-TFI (**D, D', G, G'**), and merge sections (**E, E', H, H'**). Note that GFP is expressed in the SVZ and MZ, but absent in the VZ, whereas COUP-TFI is expressed in the VZ, SVZ, and MZ of the basal telencephalon. **F–H'**, High-magnification views of a region in the basal telencephalon indicated in **E** and **E'**. **E', H'**, In *COUP-TFI CKO-Dlx5/6* homozygotes, no double-labeled cells are detected, confirming loss of COUP-TFI function in SVZ and MZ (*Dlx5/6*-positive domains), but not in progenitors located in VZ (*Dlx5/6*-negative domain). **I–J'**, Immunostaining of anti-COUP-TFI in E15.5 WT and *CKO-Dlx5/6* embryos confirms specific inactivation of COUP-TFI in the SVZ and MZ of the basal telencephalon. **I', J'**, High-magnification views of a region in the basal telencephalon indicated in **I** and **J**. Note that expression of COUP-TFI in the VZ and in the ventrally migrating stream is not affected in *COUP-TFI* conditional mutants (**J'**, arrow). Scale bars: **C–E', I, J**, 200 μ m; **F–H', J'**, 50 μ m; **I', J'**, 100 μ m.

COUP-TFI CKO-Dlx5/6 adult brains compared with control brains. Interestingly, we found no difference in the overall number of cortical interneurons between *COUP-TFI* mutant and WT sensorimotor cortices (Fig. 3*A, A'*) ($p = 0.79$; $n = 3$), and no difference in the radial distribution of GABA-positive cells along the cortical wall of the *COUP-TFI CKO-Dlx5/6* compared with WT (Fig. 3*H*). We next evaluated the total number and the radial distribution of distinct cortical interneuron subtypes by performing immunostaining and *in situ* hybridization using subtype-defining molecular markers for distinct GABAergic interneuron subpopulations (Ascoli et al., 2008) in P21 WT and *COUP-TFI CKO-Dlx5/6* cortices (Fig. 3*B–F'*). As previously reported, PV+ and SST+ interneurons are two MGE early-born subclasses predominantly located in deep layers, whereas VIP+ cells are primarily CGE late-born interneurons located in superficial layers (Kawaguchi and Kubota, 1997; Butt et al., 2005; Wonders and Anderson, 2006; Miyoshi et al., 2007, 2010). By contrast, CR+ and NPY+ interneurons derive from both the MGE and the CGE, and the preoptic area, and are distributed along the radial extent of the cortex, although bipolar CR+ cells are predominantly located in superficial layers (Xu et al., 2004; Fogarty et al., 2007; Caputi et al., 2009).

Interestingly, we found that loss of COUP-TFI function affects the specification of PV+ interneurons and the VIP+ and CR+ bipolar subpopulations in opposite ways (Fig. 3*I*). Whereas the number of PV+ interneurons was increased significantly by 33% (Fig. 3*B, B', I*) ($p = 0.0004$; $n = 3$), the total number of bipolar CR+ and VIP+ interneurons was decreased significantly by 21% (Fig. 3*E, E', I*) ($p = 0.007$; $n = 3$) and by 26% (Fig. 3*F, F', I*) ($p = 0.03$; $n = 3$), respectively. Conversely, there was no significant change in the total number of SST+ (Fig. 3*C, C', I*) ($p = 0.09$; $n = 3$) and NPY+ (Fig. 3*D, D', I*) ($p = 0.19$; $n = 3$) subpopulations, although the number of NPY+ interneurons increased by 25% in deep layers of *COUP-TFI CKO-Dlx5/6* cortices (Fig. 3*J*) ($p = 0.0028$; $n = 3$). Differences in layer distribution were also detected in the MGE-derived PV+ population, which was strikingly increased by >50% in deep layers (Fig. 3*J*) ($p < 0.0001$; $n = 3$), but not affected in superficial layers (Fig. 3*K*) ($p = 0.98$) of *COUP-TFI CKO-Dlx5/6* mice. Regarding the CGE-derived subpopulations, the number of VIP-expressing cells was reduced significantly in all layers (36% reduction in deep layers, $p = 0.02$; and 22% in superficial layers, $p = 0.04$; $n = 3$) (Fig. 3*F, F', I*), whereas the number of CR-expressing cells was decreased by 27% in superficial layers (Fig. 3*K*) ($p = 0.0034$;

Figure 3. Altered balance between PV- and bipolar VIP- and CR-expressing cortical interneurons in the absence of COUP-TFI function. Representative coronal sections of P21 WT and *CKO-Dlx5/6* cortices within the P21 sensorimotor cortex of WT (**A–F**) and *CKO-Dlx5/6* (*COUP-TFI* *CKO-Dlx5/6*) (**A'–F'**) immunostained with anti-GABA (**A–A'**) and with different cortical interneuron subtypes, as indicated (**B–F'**). **C, C', F, F'**, *In situ* hybridization of *SST* and *VIP* on adjacent sections. **G, H**, The number and distribution of GABA+ cells were quantified by subdividing the cortex into 10 bins along the ventricular (bin 1) to pial (bin 10) axis of equivalent areas of the sensorimotor cortex from WT and *CKO-Dlx5/6* animals. No significant difference in the total amount of GABA+ cells (**G**) and in the distribution of cells in each bin (**H**) was detected between WT and mutant cortices. The arrowheads in **B'** and **D'** point to an increased number of PV- and NPY-expressing cells, respectively, in deep layers. The asterisk in **E'** denotes a reduction of CR-expressing cells in superficial layers, whereas asterisks in **F'** indicate a reduction of VIP-expressing cells in both superficial and deep layers. **I–L**, Graphical representation of the percentage of density of the different cortical interneuron subpopulations in the *COUP-TFI* *CKO* relative to WT, as indicated. **I**, Along the whole radial extent of the cortex, the number of the PV+ subpopulation increased significantly by 33% (WT, 165.3 ± 6.7 ; CKO, 219 ± 12.4 ; $p < 0.01$; $n = 3$), whereas the total number of bipolar VIP+ and CR+ interneurons decreased significantly by 26% (VIP; WT, 208.2 ± 28.6 ; CKO, 153.1 ± 9.8 ; $p = 0.03$; $n = 3$) and by 21% (CR; WT, 130.9 ± 5.4 ; CKO, 103.5 ± 7.1 ; $p = 0.01$; $n = 3$), respectively. **J**, In deep layers, the PV+ (WT, 85.9 ± 4.5 ; CKO, 130.1 ± 8.4 ; $p < 0.001$; $n = 3$) and NPY+ (WT, 42.8 ± 1.4 ; CKO, 53.71 ± 2.3 ; $p = 0.003$; $n = 3$) cohorts increased significantly, whereas the VIP+ subpopulation decreased significantly (WT, 64.1 ± 13.3 ; CKO, 40.8 ± 3.3 ; $p = 0.02$; $n = 3$) in *CKO-Dlx5/6* mutants. **K**, In superficial layers, the CR+ (WT, 94.7 ± 5.1 ; CKO, 69.2 ± 5.8 ; $p = 0.003$; $n = 3$) and VIP+ (WT, 144.1 ± 16.8 ; CKO, 112.3 ± 6.9 ; $p = 0.04$; $n = 3$) subpopulations are decreased significantly in *CKO-Dlx5/6* mice. **L**, Graphical representation of the percentage of bipolar-shaped CR+ interneurons indicates a statistically significant decrease of 25% in *CKO-Dlx5/6* cortices (WT, 21.8 ± 1.2 ; CKO, 16.3 ± 1.4 ; $p = 0.008$; $n = 3$). Scale bars: **A–F'**, 100 μ m. Error bars indicate SEM.

$n = 3$), but not altered in deep layers (Fig. 3J) ($p = 0.57$; $n = 3$). Detailed morphological characterization of the CR+ subpopulation demonstrated a 25% decrease (Fig. 3L) ($p = 0.007$; $n = 3$) of bipolar-shaped cells, strongly indicating that the CR+ cohort altered in *COUP-TFI* *CKO-Dlx5/6* mice was mainly composed of bipolar CR+ cells (Caputi et al., 2009). Together, these results indicate that loss of COUP-TFI function alters the balance between PV+ (MGE-derived) and bipolar VIP+, CR+ (CGE-derived) interneuron subpopulations, without affecting the overall cortical interneuron number.

COUP-TFI does not act on cell identity but regulates cell cycle divisions of SVZ precursors in the basal telencephalon

An increase of MGE-derived PV+ interneurons at the expense of CGE-derived CR+ and VIP+ interneurons might suggest either a molecular switch between MGE and CGE identity or an imbalance in the rate of proliferation/differentiation between MGE- and CGE-derived cortical interneurons. To discern between these two hypotheses, we first investigated whether developmental regulators of MGE interneuron cell fate were abnormally expressed in *COUP-TFI* *CKO-Dlx5/6* embryos. We looked at the expression of *Nkx2.1*,

known to determine the identity of progenitor cells in the MGE (Sussel et al., 1999; Butt et al., 2008; Nóbrega-Pereira et al., 2008), and its downstream target *Lhx6* (Du et al., 2008), which starts to be expressed as soon as MGE-derived progenitors leave the VZ (Liodis et al., 2007). No obvious changes in the expression pattern of *Nkx2.1* and *Lhx6* at rostromedial and caudal levels were observed in E13.5 *COUP-TFI* *CKO-Dlx5/6* embryos (Fig. 4*A–D'*), indicating that loss of COUP-TFI in the SVZ does not induce a molecular fate change between CGE- and MGE-derived interneurons.

To further assess whether inactivation of COUP-TFI would have an effect on cell cycle divisions, similarly to what observed in the neocortex (Faedo et al., 2008; Tomassy et al., 2010), we performed acute injection of BrdU in pregnant females and compared the percentage of BrdU-positive cells in the MGE and CGE of WT and E13.5 *COUP-TFI* *CKO-Dlx5/6* embryos (Fig. 4*E–F'*). Interestingly, the ratio of proliferating (BrdU+) cells was significantly higher in the MGE, and tends to increase in the CGE without, however, reaching statistical significance (Fig. 4*G*) [WT: MGE, $25.7 \pm 2.2\%$; CGE, $30.2 \pm 2.0\%$; CKO: MGE, $47.8 \pm 3.9\%$; CGE, $45.0 \pm 4.2\%$; $p(\text{MGE}) = 0.040$; $p(\text{CGE}) = 0.097$]. Similar results were obtained after immunofluorescence of the proliferation marker Ki67, in which the thickness of the Ki67-positive region, relative to the total ventricular-to-pial thickness of the basal telencephalon, results larger in the MGE than CGE of mutant embryos (Fig. 4*H,I*) [WT: MGE, $34.2 \pm 4.1\%$; CGE, $27.0 \pm 2.0\%$; CKO: MGE, $47.3 \pm 0.4\%$; CGE, $33.8 \pm 1.8\%$; $p(\text{MGE}) = 0.033$; $p(\text{CGE}) = 0.062$]. Thus, increased proliferation, particularly in the MGE region, was detected in *COUP-TFI* *CKO-Dlx5/6* embryos. To further investigate whether cell cycle progression was affected in the absence of COUP-TFI in cortical interneurons, we assessed expression of the G_1 -active cell cycle marker cyclinD2, known to promote SVZ divisions in the telencephalon (Glickstein et al., 2007b). Strikingly, we found that expression of *cyclinD2* was increased in SVZ progenitors of E13.5 and E15.5 *COUP-TFI* *CKO-Dlx5/6* embryos, predominantly at rostromedial levels (Fig. 4*J–K'*). Since COUP-TFI is maintained in VZ progenitors of mutant embryos, we found accordingly no differences in the expression of *cyclinD1* (data not shown) (Glickstein et al., 2007a). Together, these data strongly suggest that COUP-TFI regulates cell cycle progression by modulating

Figure 4. Normal molecular identity but increased rates of proliferation in *COUP-TFI* conditional mutants. *A–B'*, *C–D'*, *In situ* hybridization of *Nkx2.1* (*A–B'*) and immunohistochemistry of *Lhx6* (*C–D'*) on E13.5 coronal sections on intermediate (*A, A'*, *C, C'*) and caudal (*B, B'*, *D, D'*) levels indicate no relevant changes in the expression profiles of *Nkx2.1* and *Lhx6* in *CKO-Dlx5/6* embryos. *E, F*, BrdU immunofluorescence on DAPI-stained intermediate coronal sections indicate increased proliferation in the MGE of E13.5 *CKO-Dlx5/6* embryos. *E'*, *F'*, High-magnification views on the boxes depicted on *E* and *F*. *G*, Graphical representation demonstrates a statistically significant increase of BrdU-positive cells in the MGE (* $p = 0.040$), but not CGE ($p = 0.097$), although the percentage of BrdU-positive cells tends to increase in the CGE. *H, H'*, Ki67 immunofluorescence labels highly proliferating cells in intermediate coronal sections of E13.5 of WT and *CKO-Dlx5/6* embryos. *I*, Graphical representation indicates a statistically significant increase in the size of the Ki67-positive region of the MGE (white brackets) relative to the total ventricular-to-pial thickness of mutant embryos (yellow brackets) (* $p = 0.03$). Error bars indicate SEM. *J'–K'*, Expression of the cell cycle protein *cyclinD2* is upregulated in the SVZ of E13.5 (*J'*, arrow) and E15.5 *CKO-Dlx5/6* embryos (*K'*, arrow). Abbreviations: dCGE, Dorsal caudal ganglionic eminence; vCGE, ventral caudal ganglionic eminence; ctx, cortex. Scale bars, 200 μm .

Figure 5. Normal basal EEG recordings in *COUP-TFI* conditional mutants. **A**, Cortical EEG recorded from awake WT mice shows normal activity at 3–7 Hz with episodes of beta activity at 15–20 Hz. Higher resolution trace shows details from the upper traces. **B**, EEG recordings from awake *COUP-TFI CKO-Dlx5/6* mice exhibit approximately similar features than WT animals. **C**, Normalized power spectra from the representative examples shown in **A** and **B** (left) and for the population means (right). No differences were found between groups. A 95% confidence interval is represented with dotted lines for each group. **D**, Mean spectral power per frequency bands was not different between groups. Error bars indicate SEM. **E**, Age dependence of the spectral content at the different frequency bands.

divisions of SVZ interneuron precursors without affecting the identity of MGE determinant genes.

Basal EEG recordings show no apparent abnormalities in mutant mice

Previous reports have shown that altered balance of interneuronal subtypes correlate with epileptic phenotypes and disruption of cortical EEG rhythms (Powell et al., 2003; Cobos et al., 2005; Glickstein et al., 2007b; Butt et al., 2008; Batista-Brito et al., 2009). We therefore chose to obtain long-lasting recordings of basal EEG activity from adult WT ($n = 6$) and *COUP-TFI CKO-Dlx5/6* mice ($n = 8$). Cortical EEGs from awake WT mice were characterized by rhythmic activity at 3–7 Hz interspersed by episodes of beta activity at 15–20 Hz (Fig. 5A). Strikingly, *COUP-TFI CKO-Dlx5/6* mice showed approximately similar EEG patterns compared with WT (Fig. 5B). Seizures, and other types of epileptic-like activity, were never observed in mutants, and no statistical difference was evident from the normalized mean power spectra from both groups (Fig. 5C) ($U = 25$; $p > 0.491$). In a group of mice ($n = 6$ WT; $n = 10$ CKO), we obtained simultaneous cortical and hippocampal EEG recordings to check for signs of ictal activity confined to the hippocampus with little cortical manifestation. Again, we did not observe signs of hyperexcitability in the hippocampus of *COUP-TFI CKO-Dlx5/6* mice (data not shown), discarding the possibility of nonconvulsive subclinical seizures in these animals.

We also looked for differences in the typical frequency bands of the cortical EEG spectrum [i.e., 1–4 Hz (delta), 4–10 Hz (theta), 10–40 Hz (beta), and 40–100 Hz (gamma)] (Fig. 5D). We found no difference between groups using a two-way

ANOVA comparison for the delta ($F = 1.45$; $p = 0.25$), theta ($F = 0.78$; $p = 0.39$), and beta bands ($F = 0.26$; $p = 0.62$), and the Kruskal–Wallis test for the gamma band ($U = 25$; $p = 0.49$). Finally, we checked for a possible functional compensation in *COUP-TFI* mutants, as reflected in an age dependence of the cortical EEG activity. We found no correlation with age of the cortical EEG power in the different frequency bands over the course of 40 to ~90 d postnatal (Fig. 5E). Furthermore, no gender differences were present in the spectra ($U = 19.5$; $p > 0.203$). Thus, alterations in the distribution of PV+, VIP+, and CR+ interneuron subpopulations result in no obvious abnormalities in the gross cortical EEG of *COUP-TFI* mutant mice.

COUP-TFI mutants are more resistant to seizures induced by lithium-pilocarpine

The preceding data suggest that reduced number of VIP+ and CR+ cortical interneurons has no discernible effect on the basal cortical activity of *COUP-TFI* mutants. This was surprising because a similar reduction in the presence of normal numbers of PV+ interneurons results in dysrhythmia and epilepsy (Cobos et al., 2005). We thus reasoned that the increase of PV+ and possibly NPY+ interneurons observed in our mutants might be functionally compensating the reduction of CGE-derived interneuronal subtypes.

To test this hypothesis, we used the muscarinic receptor agonist pilocarpine to unspecifically increase the excitatory level at doses of 100 mg/kg that induce tonic-clonic seizures and status epilepticus in wild-type mice (see Materials and Methods) (Grötcke et al., 2007). We found that *COUP-TFI CKO-Dlx5/6* mice ($n = 7$) were more resistant to seizures and to developing status epilepticus than WT ($n = 7$), as reflected in both the cortical EEG recordings and clinical manifestations (Fig. 6A,B).

Progression to the status was characterized by the early appearance of body and tail rigidity, and multiple spikes in the cortical EEG between 2 and 3 min after pilocarpine injection (Fig. 6A,B). There was no significant difference between groups in the latency to the first EEG spikes (Fig. 6C). This was followed by whole-body clonic spasms and secondary generalized tonic-clonic seizures with forelimb automatisms, which were associated with ictal EEG patterns. The mean latency to the first electroclinical manifestation of a tonic-clonic seizure was 39.9 ± 6.9 min in mutants versus 14.9 ± 1.6 min in WT animals (Fig. 6C) ($F_{(1,12)} = 12.48$; $p = 0.004$). Similarly, the onset of the status epilepticus, defined from EEG recordings (Fig. 6A,B), was significantly delayed in *COUP-TFI CKO-Dlx5/6* versus WT mice (Fig. 6C) (CKO, 50.4 ± 6.9 min, vs WT, 22.8 ± 3.3 min; $F_{(1,12)} = 13.17$; $p = 0.003$). Together, these data indicate that *COUP-TFI CKO-Dlx5/6* mice are more resistant to seizures induced by pilocarpine, probably reflecting a functional effect of the increased number of PV-, and eventually, NPY-expressing interneurons in counteracting a decreased number of VIP- and CR-positive neurons.

Seizure resistance in mutant mice is mediated by GABA_A and GABA_B signaling

To further confirm that the seizure-resistant character of these mice was GABA dependent, we used PTZ at 90 mg/kg, a GABA_A receptor antagonist that is widely used for testing seizure susceptibility in mice (Powell et al., 2003). Similarly to pilocarpine, we found an early appearance of body and tail rigidity in both WT ($n = 22$) and *COUP-TFI* *CKO-Dlx5/6* ($n = 12$) mice and a larger latency of *COUP-TFI* mutants to the first tonic-clonic seizure (Fig. 6D) (WT, 24 ± 2.7 min, vs CKO, 38 ± 3.3 min; $F_{(1,32)} = 9.59$; $p = 0.004$).

Together, this indicates that *COUP-TFI* *CKO-Dlx5/6* brains are less excitable than WT brains to produce seizures induced by the cholinomimetic convulsant pilocarpine and the GABA_A antagonist pentylenetetrazole. However, latency difference to tonic-clonic seizures can be accounted by other mechanisms, like a different excitability level at the glutamatergic circuits or residual presynaptic and postsynaptic GABA_B receptor-mediated inhibition after PTZ injection. To ascertain that the seizure-resistant feature of these mice are indeed GABA dependent, we preinjected WT ($n = 7$) and mutant animals ($n = 8$) with the GABA_B receptor antagonist CGP 35348 at the nonconvulsive dose of 136 mg/kg intraperitoneally (Karlsson et al., 1992) 30 min before PTZ (Fig. 6D). CGP pretreatment significantly [genotype ($F_{(1,45)} = 1.96$; $p = 0.168$); pretreatment ($F_{(1,45)} = 20.77$; $p < 0.0001$); genotype by pretreatment ($F_{(1,45)} = 6.93$; $p = 0.01$)] reduced the latency to the first tonic-clonic seizure in *COUP-TFI* *CKO-Dlx5/6* mice (CGP 35348 vs vehicle pretreated, $p < 0.0001$) and eliminated latency difference with the WT group ($p = 0.38$) (Fig. 6D). Thus, these data indicate that blockage of GABA_B receptor abolishes resistance to seizures induced by the GABA_A receptor antagonist PTZ, and confirm that the seizure-resistant phenotype observed in *COUP-TFI* *CKO-Dlx5/6* mice is dependent on an altered balance of MGE- versus CGE-derived interneurons acting through both GABA_A and GABA_B receptors.

Discussion

In this study, we show that COUP-TFI regulates the balance between distinct cortical interneuron subtypes in a spatiotemporal controlled manner during corticogenesis, thereby enabling proper cortical microcircuitry and functional activity. Conditional inactivation of COUP-TFI solely in interneuron SVZ progenitors leads to a decreased number of CR- and VIP-bipolar GABAergic cells in superficial cortical layers and a concomitant increase of PV- and NPY-expressing interneurons in deep cortical layers of the mature cortex, without affecting the total number of GABAergic cortical interneurons. Physiologically, this alters the balance of both GABA_A and GABA_B receptor-mediated inhibition so that mutants become more resistant to pharmacologically induced seizures. To our knowledge, this study describes for the first time an epilepsy-resistant phenotype

Figure 6. Seizure resistance of *COUP-TFI* *CKO-Dlx5/6* mice. **A**, Bilateral cortical EEG recordings obtained from a representative WT mouse during the progression to a status epilepticus induced by intraperitoneal injection of the muscarinic receptor agonist pilocarpine. The arrows indicate the timing of pilocarpine injections; the first generalized tonic-clonic seizure (first Sz) and the onset of status epilepticus, as defined by electroclinical information. **A'**, Expanded traces highlight EEG epochs recorded before, after 2–3 min of pilocarpine injection, and at the status onset. **B**, Bilateral cortical EEG from a representative *COUP-TFI* *CKO-Dlx5/6* mouse. Note the long latency to the first generalized tonic-clonic seizure (first Sz) and the onset of the status epilepticus. **B'**, Expanded traces similar to that shown in **A'** for *COUP-TFI* *CKO-Dlx5/6* (*CKO-Dlx5/6*) mice. **C**, Mean latency to first spikes recorded after pilocarpine injections. Note latency difference to the first generalized tonic-clonic seizure (first Sz) and the status epilepticus between groups. **D**, Latency difference to the first tonic-clonic seizure induced by subcutaneous injection of the GABA_A receptor antagonist PTZ. Pretreatment with the GABA_B receptor antagonist CGP 35348 significantly reduces the latency to the first tonic-clonic seizures and eliminates latency difference between WT and mutants. ** $p < 0.01$, *CKO-Dlx5/6* versus WT, within treatment; # $p < 0.0001$, CGP 35348 versus saline pretreatment, within genotype. Error bars indicate SEM.

after genetic manipulation of interneuronal subtypes and suggests a potential role for increased PV+ and NPY+ interneurons in controlling seizures.

COUP-TFI controls the balance between MGE- and CGE-derived interneurons

Until recently, the CGE was not considered a distinct anatomical and molecular entity, mainly because of the absence of a morphologically definite sulcus demarcating the CGE from the MGE and LGE, and to the lack of CGE-restricted molecular markers (Flames et al., 2007). However, a series of experimental evidence including *in vivo* transplantation studies, *in vitro* migratory assays, and fate-mapping analyses have established the CGE as a separate molecular territory and confirmed that CGE-derived cortical interneurons contribute to a subset of interneuron subtypes with distinct morphological and electrophysiological interneurons (Nery et al., 2002; Yozu et al., 2005; Kanatani et al., 2008; Willi-Monnerat et al., 2008; Miyoshi et al., 2010). Still, no functional studies on genes required for the specification of CGE-derived cortical interneurons have been described to date.

Differently from COUP-TFII, COUP-TFI is expressed in precursors of the basal telencephalon along the whole rostrocaudal extent at early stages (Armentano et al., 2007; Faedo et al., 2008), before getting restricted to the dorsal MGE and CGE at E13.5, and to CGE-derived cortical interneurons, such as VIP- and

CR-expressing bipolar interneurons (~80 and 90%, respectively). Furthermore, 56% of COUP-TFI+ express SST, most presumably originating from the dMGE, whereas 70% express NPY+, which might derive from the dMGE, the CGE, and, in a small contribution, the preoptic area (Fogarty et al., 2007; Gelman et al., 2009; Karagiannis et al., 2009; Sousa et al., 2009).

Interestingly, although COUP-TFI is expressed in the MGE at early stages during the production of PV+ interneurons, its expression is not maintained in this cell type at P21. In this study, we demonstrate that loss of COUP-TFI function affects the generation, but not maturation of PV+ interneurons. Differently from *Nkx2.1*, which normally acts as a cell fate switch between PV+ (MGE-derived) and CR+/VIP+ (CGE-derived) interneurons (Butt et al., 2008), COUP-TFI acts in the opposite way by limiting generation of PV+ precursors and promoting specification of CR+ and VIP+ cells. However, the imbalance between MGE- and CGE-derived interneurons induced by loss of COUP-TFI function is not attributable to a change of cell fate, as observed for *Nkx2.1* mutants (Butt et al., 2008), but rather to a control on precursor cell divisions mainly within the MGE, as seen by increased proliferation and expression levels of the cell cycle protein cyclinD2 in SVZ precursors (Glickstein et al., 2007a,b). Null mutant mice for cyclinD2 have a selective deficit in cortical PV-expressing interneurons and increased excitability, without affecting other MGE-derived subtypes, such as SST+ interneurons (Glickstein et al., 2007b). Accordingly, overexpression of *cyclinD2* in COUP-TFI conditional mutants results in an increase of PV+ neurons (Glickstein et al., 2007b), implying that negative control of SVZ divisions by COUP-TFI normally limits the number of PV+ interneurons. Thus, in the absence of COUP-TFI function, this repression is released and an excess of PV+ interneurons is produced. Since COUP-TFI is expressed in the MGE from E10.5 to E12.5 (Armentano et al., 2006, 2007; this study), and production of PV+ interneurons occurs from E9.5 to E15.5 (Miyoshi et al., 2007), it is reasonable to assume that presumptive PV+ cells express COUP-TFI while proliferating and that COUP-TFI normally controls early SVZ progenitor divisions during generation of PV+ interneurons.

However, in addition to the increase of PV+ interneurons, loss of COUP-TFI function also affects correct specification of late-born CR+ and VIP+ interneurons. We hypothesize that overproduction of PV+ (and in part NPY+ cells) at a time when normally VIP+ and CR+ cells are generated (from E14.5 to E18.5) (Miyoshi et al., 2010), depletes the progenitor pool resulting in a decrease of VIP+ and CR+ bipolar interneuron precursors in the CGE. Thus, we propose that COUP-TFI normally regulates the number of PV+ cells during generation of CR+ and VIP+ neurons by controlling sequential cell divisions in SVZ progenitors. Excessive cell divisions during the time of PV+ interneuron generation would affect the sequential production of VIP/CR bipolar interneurons. In support of this mechanism, increased PV+ interneurons are found in deep cortical layers (appropriately for their MGE origin), and decreased CR+ and VIP+ interneurons are still located in superficial layers (appropriate for their CGE origin) in COUP-TFI conditional mutants.

Together, our data support a model by which COUP-TFI regulates the fine balance between MGE- and CGE-derived interneurons by ensuring proper generation and specification of different subsets of cortical interneurons. This is in accordance with the control of pyramidal projection neurons by COUP-TFI, which is required to balance motor and sensory cortical areas by repressing corticospinal motor neuron generation during production of corticofugal pyramidal neurons in an area- and

temporal-specific manner (Armentano et al., 2007; Tomassy et al., 2010). Moreover, both COUP-TFs regulate the switch between neurogenesis (early corticogenesis) and gliogenesis (late corticogenesis), and in their absence, neurogenesis is sustained and the generation of early-born neurons is prolonged (Naka et al., 2008). Finally, the *Drosophila* COUP-TF ortholog, SVP, controls neuroblast diversity in a temporally controlled mode by regulating the balance between early- and late-born neuroblasts during neurogenesis (Kanai et al., 2005). Overall, we propose that in the basal telencephalon COUP-TFI plays a critical temporal and spatial control over the differentiation of different subtypes of cortical interneurons, thereby enabling the temporal and spatial specification of PV-, and bipolar CR- and VIP-expressing interneurons.

Increased inhibition mediated by altered balance of cortical interneuronal types might account for epilepsy resistance

The role of GABAergic interneurons in the proper operation of cortical circuitry is widely recognized. By means of a precise somatodendritic arrangement of their synaptic contacts, diverse types of interneurons specifically control excitability of principal cells and other neuronal types (Markram et al., 2004; Ascoli et al., 2008), being responsible of setting brain rhythms and local field potential oscillations (Buzsáki and Draguhn, 2004). We know that GABAergic cell dysfunction is associated primarily with epilepsy, one of the most frequent neurological disorders occurring in the young population (Cossart et al., 2005; Baraban, 2007). However, we still lack a comprehensive understanding of what determines the expression of a particular phenotype and whether interneuronal dysfunction is cause or consequence of epileptic seizures.

Recent genetic models aimed to study the development of cortical interneurons have given us additional cues to draw this picture (Fig. 7). They constitute a unique tool because in most cases changes of subtype proportions precede pathological manifestations (Fig. 7B). Mice lacking the transcription factor *Dlx1* suffer from an apoptotic loss of SST+, CR+, and NPY+ subtypes, whereas PV+ interneurons were spared (Cobos et al., 2005). This reduction was associated with decreased GABAergic synaptic activity, distorted theta oscillations, and generalized spontaneous seizures, suggesting that less SST-, CR-, and NPY-expressing subtypes alone can account for seizures when the number of PV+ cells remains constant. Interestingly, *Sox6* mutants, which have a reduction of PV+ and SST+ interneurons and a constant number of the VIP+ and CR+ subtypes, exhibit pathological oscillations in the delta and beta bands and an early epileptic phenotype onset, that is not compensated by increased number of NPY+ interneurons (Azim et al., 2009; Batista-Brito et al., 2009). This critical role of PV+ and SST+ subtypes is confirmed by early loss-of-function experiments of *Nkx2.1* in which an excess of VIP+ and CR+ cells are generated at the expense of PV- and SST-expressing interneuronal populations (Butt et al., 2008). As a consequence, juvenile mice exhibit generalized seizures. A late (E12.5) loss of *Nkx2.1* function leaves SST+ cells unaffected and causes the same interneuronal shift exclusively at the upper layers. Remarkably, although their seizure susceptibility was not directly tested, these mice did not exhibit spontaneous seizures (Butt et al., 2008).

Very interestingly, mice lacking cyclinD2, which have reduced density of PV+ interneurons but normal density of SST+ and other CGE-derived interneurons, display decreased inhibitory synaptic activity and enhanced cortical excitability, although no spontaneous seizures were recorded (Glickstein et al., 2007b). In contrast,

mice with mutation of the gene encoding *uPAR* (urokinase plasminogen activator receptor) display a large reduction of PV+ cells exclusively, and have spontaneous generalized seizures, being also more susceptible to PTZ-induced seizures (Powell et al., 2003).

All these mouse models argue for a differential role of diverse interneuronal types in controlling cortical excitability and seizures. Our study moves forward and demonstrates that, in the presence of a higher number of PV+ and NPY+ interneurons, COUP-TFI mutants show no discernible cortical EEG abnormalities, but on the contrary are more resistant to pharmacologically induced seizures. Such a resistance is dependent on GABAergic signaling because it is abolished by blockage of GABA_A and GABA_B receptors. Surprisingly, we found no changes in the gamma region of the spectrum despite the increased number of PV+ interneurons. However, we must keep in mind that coarse wire EEG recordings are not well suited to look at the local organization of cortical gamma rhythms, and probably our recordings remained too superficial to detect changes caused by increased numbers of PV+ cells at deep layers in mutants. Obviously, the cortical microcircuit is extremely complex, and other factors, such as the layer specificity of interneuronal loss and the total number of affected cells, can constrain the emergence of epileptic phenotypes and rhythm distortion. Future detailed studies of the local field potentials using multisite recordings would help to look at the finest spatial scale of the neocortex.

Another potential source of enhanced inhibition in our *COUP-TFI* *CKO-Dlx5/6* mice is the reduction of the control exerted by fewer CR+ and VIP+ interneurons acting over other superficial layer interneurons. CR+ and VIP+ interneurons are known to preferentially target other interneurons (Staiger et al., 2004; Dávid et al., 2007). Thus, under conditions of reduced number of CR+ and VIP+ interneurons, pyramidal cells would not be relieved from inhibition, being less excitable in *COUP-TFI* mutant mice. However, this is unlikely because (1) similar decrease of CR+ interneurons in the *Dlx1* mutants results in hyperexcitability and seizures, probably aided by concomitant reduction in the number of NPY+ and SST+ cells (Cobos et al., 2005); and (2) increased CR+ and VIP+ cells, in the presence of reduced number of PV+ interneurons, failed to rescue the epileptic phenotype of *Nkx2.1* mutants (Butt et al., 2008). Indeed, synaptic potentials elicited by CR+ multipolar and bipolar interneurons on their few cortical pyramidal targets are weaker than potentials initiated by PV+ interneurons, which dominate thalamocortical and intralaminar feedforward inhibition (Sun et al., 2006; Caputi et al., 2009; Xu and Callaway, 2009).

Figure 7. Summary on the relationship between different interneuronal subpopulations and epileptic phenotypes. **A**, Schematic showing the proportion of the different cortical interneuronal subpopulations classified according to their molecular expression profiles. In the normal mouse neocortex, PV-positive interneurons represent ~40% of the total numbers of interneurons, which together with SST-positive cells (~18%) mainly derive from the MGE. Instead, CR and VIP-expressing interneurons originate from the CGE and together contribute ~25% to the total number of cortical interneurons. Interneurons expressing the neurotransmitter peptide NPY are known to derive mainly from the MGE and the CGE and constitute ~9%. Many interneuronal markers colocalize, with SST/CR, SST/NPY, VIP/CR, and VIP/NPY being the most common. Data are from Gonchar and Burkhalter (1999) and Xu and Callaway (2009). **B**, Schematic showing the percentage of interneuron subtypes affected by different genetic manipulations in the different papers cited at right and the corresponding clinical phenotype. =, Similar proportions compared with control; ↑, an increased proportion; ↓, decrease.

We also detected an increase of NPY+ interneurons in the deep neocortical layers of COUP-TFI mutants. This probably reflects a population of NPY+ interneurons, which derive from the dorsal MGE (Fogarty et al., 2007). NPY+ interneurons constitute a heterogeneous group, as this protein has been detected in a variety of cortical interneurons (Karagiannis et al., 2009), although a more homogeneous fraction appears to be generated from the preoptic area (Gelman et al., 2009). Interestingly, NPY+ interneurons known to coexpress nitric oxide synthase (NOS) can be morphologically and physiologically identified as neurogliaform cells and share mainly an MGE origin, although a subpopulation of NPY+ neurogliaform cells do not express NOS and derive from the CGE (Tricoire et al., 2010). Neurogliaform interneurons are a unique source of GABA_B-mediated inhibition

for pyramidal cells (Tamás et al., 2003). If some of the exceeding NPY+ interneurons detected in the lower cortical layers of COUP-TFI mutants correspond with neurogliaform cells, their possible role in controlling cortical excitability is intriguing. Indeed, we found that removal of GABA_B inhibition in the presence of a GABA_A antagonist was required to abolish differences of seizure onset between *COUP-TFI* mutants and WT mice. Although other sources of GABA_B-mediated inhibition operate at both presynaptic and postsynaptic levels, the specific role of this recently discovered interneuron population remains unknown. Future studies will be essential to overcome the origin, identity, and function of this miscellaneous interneuronal subtype.

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