Published in final edited form as:
Neuron. 2011 February 24; 69(4): 763–779. doi:10.1016/j.neuron.2011.01.015.

Excitatory Projection Neuron Subtypes Differentially Control the Distribution of Local Inhibitory Interneurons in the Cerebral Cortex

Simona Lodato[2,5], **Caroline Rouaux**[2,6], **Kathleen B. Quast**[3,6], **Chanati Jantrachotechatchawan**[1,2], **Michèle Studer**[4,5], **Takao K. Hensch**[3], and **Paola Arlotta**[1,2]

[1] Department of Stem Cell and Regenerative Biology and Harvard Stem Cell Institute, Harvard University, Cambridge, MA

[2] Center for Regenerative Medicine and Department of Neurosurgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA

[3] Center for Brain Science, Department of Molecular and Cellular Biology, Harvard University, 52 Oxford St, Cambridge, MA

[4] Inserm U636, and Université de Nice-Sophia Antipolis, Laboratoire de Génétique du Développement Normal et Pathologique, F-06108 Nice, France

[5] European School of Molecular Medicine (SEMM), Naples, Italy

Abstract

In the mammalian cerebral cortex, the developmental events governing the integration of excitatory projection neurons and inhibitory interneurons into balanced local circuitry are poorly understood. We report that different subtypes of projection neurons uniquely and differentially determine the laminar distribution of cortical interneurons. We find that in *Fezf2*$^{-/-}$ cortex, the exclusive absence of subcerebral projection neurons and their replacement by callosal projection neurons cause distinctly abnormal lamination of interneurons and altered GABAergic inhibition. In addition, experimental generation of either corticofugal neurons or callosal neurons below the cortex is sufficient to recruit cortical interneurons to these ectopic locations. Strikingly, the identity of the projection neurons generated, rather than strictly their birthdate, determines the specific types of interneurons recruited. These data demonstrate that in the neocortex individual populations of projection neurons cell-extrinsically control the laminar fate of interneurons and the assembly of local inhibitory circuitry.

Introduction

High-level cortical function including cognition, sensory perception and motor function relies on the coordinated assembly of local microcircuitry between glutamatergic projection neurons and GABAergic interneurons (Hensch, 2005). Excitatory projection neurons represent the largest portion of all cortical neurons. They are born from neural progenitors in

Correspondence should be addressed to: P.A. (paola_arlotta@hms.harvard.edu).
[6]These authors equally contributed to the work.

the dorsal telencephalon and are classified into numerous subtypes based on their location within different cortical layers and areas; their axonal projections to distinct intracortical, subcortical and subcerebral targets; and the combinatorial expression of different neuron type-specific genes (Bayer and Altman, 1991; Molyneaux et al., 2007). Among them, corticofugal projection neurons include corticothalamic projection neurons, which are found in layer VI and project to the thalamus, as well as corticospinal motor neurons (CSMN) and several other types of subcerebral projection neurons, which are located in different areas of layer V and project to the spinal cord, the superior culliculus and other targets in the brainstem and below the brain (Molyneaux et al., 2007). In addition, the cortex includes several types of callosal projection neurons (CPN), which are primarely located in layers II/III and V and connect to targets in the contralateral hemisphere, the striatum and the frontal cortex (Lindwall et al., 2007; Mitchell and Macklis, 2005; Molyneaux et al., 2009; Richards et al., 2004).

The firing activity of these projection neuron types is modulated locally by GABAergic inhibitory interneurons. Cortical interneurons are defined at the intersection of diverse molecular, electrophysiological and morphological properties, and based on their connections to the soma, axonal initial segment or dendritic tree of their projection neuron partners (Ascoli et al., 2008; Markram et al., 2004). However, the classification of cortical interneurons remains an open challenge (Ascoli et al., 2008). Contrary to projection neurons, in rodents, cortical interneurons are mostly generated from neural progenitors located in the medial and caudal ganglionic eminences (MGE and CGE) and in the preoptic area (POA) (Gelman et al., 2009; Wonders and Anderson, 2006). MGE progenitors give rise to Parvalbumin (PV) and Somatostatin (SST) interneurons, which distribute at higher densities in deep cortical layers (Butt et al., 2005; Butt et al., 2008; Cobos et al., 2006; Fogarty et al., 2007). In contrast, Vasoactive Intestinal Peptide (VIP) and Calretinin (CR) interneurons originate in the CGE and preferentially populate the superficial layers II/III (Miyoshi et al., 2007; Miyoshi et al., 2010; Nery et al., 2002; Xu et al., 2004).

Projection neurons and interneurons are born from distal germinal zones, but they ultimately coexist in the cortex, where they assemble into local microcircuitry. This requires coordinated migration and development of these two broad neuronal populations. The migration of interneurons to the cortex is particularly extensive (Corbin et al., 2001; Marin and Rubenstein, 2001). Following tangential migration through the ventral forebrain, interneurons enter the cortex where they disperse along migratory routes in the marginal zone (MZ) and the intermediate zone (IZ)/subventricular zone (SVZ) (Kriegstein and Noctor, 2004; Lavdas et al., 1999). Later, they radially invade the cortical plate (Ang et al., 2003; Polleux et al., 2002; Tanaka et al., 2003), a process that is at least in part timed by CXCL12/CXCR4 signaling (Elias et al., 2008; Li et al., 2008; Liapi et al., 2008; Lopez-Bendito et al., 2008; Tanaka et al., 2010; Tiveron et al., 2006).

The cellular and molecular events that direct interneurons to position precisely within specific cortical layers are poorly understood. Correct cell-autonomous development of cortical interneurons is a critical factor for the radial positioning of GABAergic interneurons, since defective laminar distribution is often observed in mutant mice that have abnormal interneuronal differentiation (Alifragis et al., 2004; Azim et al., 2009; Batista-Brito et al., 2009; Butt et al., 2008; Cobos et al., 2006; Liodis et al., 2007; Wang et al., 2010). In addition, interneurons are cell-intrinsically programmed to modulate expression of the KCC2 co-transporter in order to sense extracellular GABA levels and arrest radial migration (Bortone and Polleux, 2009; Miyoshi and Fishell, 2010).

However, several lines of evidence indicate that the environment of the cortex also plays instructive roles in directing interneuron cortical distribution, pointing at a possible

involvement of projection neurons. For example, synchronically generated GABAergic interneurons and projection neurons preferentially pair, suggesting linked mechanisms of layer distribution (Fairen et al., 1986; Miller, 1985; Peduzzi, 1988; Pla et al., 2006; Valcanis and Tan, 2003). Additionally, interneurons invade the cortical plate only after their projection neuron partners, possibly reflecting a need for signals from appropriately located projection neurons (Lopez-Bendito et al., 2008). Finally, interneurons distribute abnormally in the cortex of the *reeler* mice, in which projection neuron lamination is nearly inverted (Hammond et al., 2006; Hevner et al., 2004; Pla et al., 2006; Yabut et al., 2007).

Here, we demonstrate that projection neurons are required for the laminar distribution of interneurons and, further, that distinct subtypes of projection neurons provide different interneuron populations with specific positional information.

Fezf2 is a critical transcription factor for forebrain development in several species (Molyneaux et al., 2007; Shimizu and Hibi, 2009). Loss of *Fezf2* results in the exclusive absence from the cortex of subcerebral projection neurons, which are replaced by another population of excitatory neurons - callosal projection neurons (Chen et al., 2005a; Chen et al., 2008; Chen et al., 2005b; Molyneaux et al., 2005). We find that GABAergic interneurons distribute abnormally in the *Fezf2*$^{-/-}$ cortex, and that *Fezf2*$^{-/-}$ mice display aberrant cortical activity due to defective GABAergic inhibition. The defect is projection neuron type-specific, since the generation of CPN in place of the missing subcerebral projection neurons cannot compensate for the interneuron abnormalities.

Different types of projection neurons can affect interneuron positioning, as demonstrated by the ectopic presence of cortical interneurons within clusters of either corticofugal projection neurons or layer II/III CPN that are experimentally generated in proximity to the cortex. Interestingly, the types of interneurons recruited are appropriate for the subtype-specific identity of the projection neurons, rather than strictly their day of birth.

We conclude that in the neocortex, individual types of projection neurons play critical roles in governing the radial distribution of GABAergic interneurons, a process that is critically important for the development of balanced cortical circuitry.

Results

Subcerebral projection neurons are absent from the cortex of *Fezf2*$^{-/-}$ mice and are substituted by deep layer callosal projection neurons

Loss of the transcription factor *Fezf2* results in the absence from the cortex of all subcerebral projection neurons, which fail to develop while other projection neuron types across all cortical layers are generated normally (Chen et al., 2005a; Chen et al., 2005b; Molyneaux et al., 2005). Loss of subcerebral neurons in this mutant is accompanied by the expansion of a different population of projection neurons with callosal projection neuron properties (Chen et al., 2008) without overall loss of neurons, defective migration or changes in cortical thickness (Molyneaux et al., 2005). Despite *Fezf2*$^{-/-}$ mice are viable and motile (Molyneaux et al., 2005), they are prone to develop seizures upon handling or exposure to auditory stimuli (unpublished data).

To determine whether the abnormal development of projection neurons in this mutant affects the distribution of cortical interneurons and whether specific subtypes of projection neurons play different roles, we first defined the nature of the CPN that replace subcerebral projection neurons in the *Fezf2*$^{-/-}$ cortex. As expected, CTIP2, a marker of subcerebral projection neurons was not expressed in mutant layer V (Molyneaux et al., 2005). In contrast, TBR1, a marker of layer VI and layer II/III neurons, and SATB2, a protein

important for CPN development, were both expanded (Chen et al., 2008; Molyneaux et al., 2005) and were co-expressed in a new population of projection neurons located in layer Vb (Figure S1G–P). To determine whether these neurons project through the corpus callosum, we retrogradely labeled CPN of the mutant mice with FluoroGold (FG) injections in contralateral hemisphere. Despite the fact that most CPN form Probst bundles (Pb), making their retrograde labeling inefficient, FG-labeled CPN were visible within layer V and co-expressed TBR1 and SATB2 (Figure 1A–E). These data extend prior electrophysiological analysis and characterization of these neurons in chimeric mice by demonstrating that they project through the corpus callosum (Chen et al., 2008). These CPN are distinct from the CPN of layer Va, which expressed SATB2 but not TBR1 (data not shown). To precisely define the subtype-specific identity of CPN in the mutant layer V, we analyzed the expression of three CPN-specific genes among several that were shown to label subpopulations of CPN in different layers (Molyneaux et al., 2009). *Lpl* labels CPN in layers V, VI and in the deeper part of layer II/III; *Inhba* labels CPN of layer II/III; and *Limch1* is expressed in subpopulations of CPN within the upper part of layer II/III. In the *Fezf2*$^{-/-}$ cortex, *Lpl* expression was increased in layer V (Figure 1F,G; arrows), whereas expression of *Inhba* and *Limch1* remained unchanged (Figure 1H–K; arrowheads). This molecular and hodological analysis indicates that CPN from layers Vb/VI, but not from layers Va or II/III, replace subcerebral projection neurons within layer V of the *Fezf2*$^{-/-}$ cortex.

GABAergic interneurons acquire abnormal radial distribution in the *Fezf2*$^{-/-}$ cortex

Fezf2$^{-/-}$ mice represent a unique mutant model in which a single projection neuron population fails to develop and is substituted by projection neurons of a different identity. This motivated us to use this mice to determine whether projection neurons control the laminar distribution of GABAergic interneurons, and, further, whether different types of projection neurons have different roles. (Figure 2A,B). First, we quantified the number of *GAD67*-positive interneurons detected by *in situ* hybridization in motor, somatosensory and visual cortex at P28 in wild type and *Fezf2*$^{-/-}$ littermates (n=3 wt; n=3 *Fezf2*$^{-/-}$). The total number of *GAD67*-positive interneurons did not differ between wild type and mutant corteces (Figure 2C), but the distribution of *GAD67* interneurons was distinctly abnormal in the *Fezf2*$^{-/-}$ mutant (Figure 2A,B).

To precisely determine the radial distribution of *GAD67*-positive interneurons, we divided the cortex into 10 bins of equal size spanning the cortical thickness. Calculation of the percentage of interneurons located in each bin demonstrated a clear reduction of interneurons within lower bins across areas of the *Fezf2*$^{-/-}$ cortex. This difference was particularly prominent for bins 4–5 in the motor and somatosensory areas, and bins 3–4 in the visual area, and it was accompanied by an increase of interneuron percentages in more superficial bins (Figure 2D–F,I–K,N–P). Double staining for *GAD67* and β-galactosidase (labeling subcerebral projection neurons) in *Fezf2*$^{+/-}$ mice showed that bins with reduced percentages of interneurons mostly corresponded to layer V (Figure 2G,L,Q). This anatomical information on layer positioning allowed us to assign bins to three groups spanning layers VI, V and II–III/IV. Analysis of interneuron distribution within each of these layers highlighted acute phenotypic abnormalities within layer V (Figure 2H,M,R). Reduced percentages of interneurons in *Fezf2*$^{-/-}$ layer V were observed across all cortical areas sampled. This was accompanied by increased interneuron percentages in the *Fezf2*$^{-/-}$ superficial layers II–III/IV in somatosensory and visual areas (with a similar trend in the motor area). In contrast, layer VI was unaffected (Figure 2H,M,R).

To understand whether the reduced number of interneurons in layer V of the *Fezf2*$^{-/-}$ cortex might reflect a smaller number of interneurons normally associated with CPN compared to subcerebral projection neurons, we quantified the number of GABA-positive interneurons surrounding these two projection neuron populations within wild type layer V. We found

significantly fewer interneurons associated with SATB2-expressing CPN in layer Va than with CTIP2-positive subcerebral projection neurons in layer Vb (n=3; p value=0.001) (Figure S2). This suggests that the reduced percentages of interneurons observed in layer V of the mutant cortex is in line with a typically lower number of cortical interneurons distributed around deep layer CPN.

Together, these data indicate that upon reaching the cortex, interneurons require projection neurons in order to acquire proper lamination. Furthermore, given that in the *Fezf2*$^{-/-}$ cortex there is a precise replacement of subcerebral projection neurons with CPN, these findings demonstrate that different types of excitatory projection neurons differentially affect the distribution of cortical interneurons.

Fezf2$^{-/-}$ mice exhibit unbalanced cortical activity due to defective GABAergic inhibition

To determine whether the observed changes in interneuron distribution results in unbalanced cortical activity and physiology, we used voltage-sensitive dye imaging (VSDI) (Grinvald and Hildesheim, 2004) to examine spatio-temporal dynamics of functional connections in the *Fezf2*$^{-/-}$ mice. The spread of activity through coronal slices of the visual cortex in response to a current pulse delivered to the white matter was quantified. As expected, wild type and *Fezf2*$^{+/-}$ mice exhibited a strong response that propagated rapidly to the upper layers “on beam” with the stimulating electrode, spreading only weakly along the deep layers even at threshold stimulating strengths (Figure 3B and Movie S1). In contrast, the response in *Fezf2*$^{-/-}$ slices at threshold rarely reached the upper layers, and remained largely confined to the lower layers (Figure 3A,C and Movie S2).

Maximum fluorescence intensity was quantified within two 125 mm^2 regions in-line with the stimulating electrode, one in upper layers (Figure 3B,C; black box) and one in lower layers (Figure 3B,C; white box). Input-output curves revealed an increase in upper layer response with increasing stimulus intensity, however the response failed to reach wild-type levels in the upper layers of *Fezf2*$^{-/-}$ mice across all stimuli (Figure 3D,F). Conversely, lower layer responses were consistently stronger in *Fezf2*$^{-/-}$ mice compared to wild type (Figure 3F). These differences were significant for both upper and lower layers at half maximal stimulation (Figure 3E,G).

Physiological imbalance of excitation across cortical layers in *Fezf2*$^{-/-}$ cortex may be explained by abnormal excitatory networks, altered inhibitory GABAergic interneurons, or both. To distinguish among these possibilities, we performed VSDI measurements in the presence of the $GABA_A$ receptor antagonist bicuculline. Strikingly, the laminar differences between genotypes were eliminated under these conditions (Movies S3 and S4), indicating that the excitatory network scaffold is intact in the *Fezf2*$^{-/-}$ mutant cortex. Taken together, an increased GABAergic tone in the superficial layers of the mutant cortex (without defects in excitatory network function) provides physiological support for our histological findings that interneuron numbers are reduced in layer V and increased in the upper layers II–III/IV of the *Fezf2*$^{-/-}$ cortex.

Fezf2 does not affect the fate specification of cortical interneurons

Defects of interneuron fate specification or tangential migration could account for abnormal lamination of interneurons. Therefore, we investigated whether *Fezf2* plays a cell-autonomous role in the fate specification or migration of GABAergic interneurons in the ventral forebrain. We have previously reported that with the exception of a small area in the developing amygdala, *Fezf2* is absent from the entire ventral telencephalon, and is not expressed in the MGE and CGE, where cortical GABAergic interneurons are born (Rouaux and Arlotta, 2010). In the dorsal telencephalon, *Fezf2* is expressed in progenitors, and within

the cortical plate at high levels in subcerebral projection neurons and at low levels in layer VI projection neurons (Chen et al., 2005a; Chen et al., 2005b; Molyneaux et al., 2005). Here, we found that *Fezf2* was also not expressed in interneurons within the cortex, as assessed by crossing GAD67::GFP mice with *Fezf2*$^{-/+}$ mice that carry a *LacZ* reporter gene at the *Fezf2* locus. GFP-positive interneurons did not colocalize with β-galactosidase- and CTIP2-positive subcerebral neurons (Figure S3A–F).

Accordingly, *Mash1* and *Nkx2.1*, which normally control the specification of early-born interneuron progenitors in the MGE, and *Lhx6*, which labels their postmitotic interneuronal progeny, were unchanged in the mutant (Figure S3G–L). Similarly, *COUPTF-II*, which labels interneuron progenitors within the CGE displayed comparable expression in wild type and mutant mice (Figure S3M,N).

These data demonstrate that in the telencephalon *Fezf2* expression is excluded from GABAergic interneurons and does not affect expression of genes that are critical for interneuron fate specification.

Subcerebral projection neurons are required for the proper distribution of SST- and PV-expressing interneurons, but not CR-expressing interneurons

Despite the fact that cortical interneurons are heterogeneous and it is not currently possible to precisely associate specific interneuron subtypes with strict layer locations, it is known that early-born, SST- and PV-expressing interneurons preferentially populate the deep layers of the cortex (Butt et al., 2005; Cobos et al., 2006; Fogarty et al., 2007), while mostly late-born, CR-expressing interneurons are present in higher numbers in the superficial layers (Miyoshi et al., 2010; Nery et al., 2002; Xu et al., 2004). To define whether the absence of subcerebral projection neurons affects the distribution of all interneurons equally or exerts selective control over interneuronal populations that normally occupy the same deep layers of the cortex, we investigated the distribution of SST-, PV-, and CR-expressing interneurons in the *Fezf2*$^{-/-}$ mutant.

Given that the distribution of *GAD67*-positive interneurons was abnormal in layer V across all areas sampled, we concentrated our analysis on one representative area: the somatosensory cortex. First, we studied the distribution of *SST*-expressing interneurons, which in wild type cortex are present in high numbers within layer V (Figure 4A) (P28; n=3 wt; n=3 *Fezf2*$^{-/-}$). We found that the total number of *SST*-expressing neurons was unchanged in the mutant cortex compared to wild type (Figure 4C). However, the typical laminar distribution of *SST* interneurons was strikingly altered in the mutant (Figure 4A,B), with clear decreased percentages in layer V (bins 4–5) and increased percentages in layers II–III/IV (bins 6–10) (Figure 4D–F).

PV-expressing interneurons also showed comparable, abnormal radial distribution (n=3 wt; n=3 *Fezf2*$^{-/-}$). The percentage of PV-expressing interneurons in the somatosensory cortex at P28 was decreased in layer V and increased in the upper layers II–III/IV, without a change in the total number of PV-positive interneurons (Figure 4G–L). In stark contrast to the abnormal distribution of *SST*- and PV-expressing interneurons, CR-expressing interneurons were not affected by the absence of subcerebral projection neurons and were distributed normally in the P28 *Fezf2*$^{-/-}$ cortex (n=3 wt; n=3 *Fezf2*$^{-/-}$) (Figure S4A–F).

These findings strongly suggest that distinct projection neuron subpopulations selectively and preferentially affect the distribution of cortical interneuron subtypes that are normally destined to populate the same cortical layers.

Interneuron reduction in layer V of *Fezf2*$^{-/-}$ cortex is not due to abnormal connectivity by CPN or interneuron cell death

The reduced percentages of cortical interneurons in *Fezf2*$^{-/-}$ layer V suggest that CPN affect interneurons differently from the subcerebral projection neurons that they replace. However, it is possible that the presence of Probst bundles (Pb) and thus the altered connectivity by CPN across the corpus callosum may have affected their ability to interact with interneurons in the *Fezf2*$^{-/-}$ cortex. In order to understand whether development of Pb *per se* could cause the observed abnormalities, we analyzed the distribution of *SST*-expressing interneurons in SV129S1/SvImJ wild type mice, a strain where females sporadically develop Pb without changes in other cerebral commisures (Wahlsten et al., 2003 and data not shown). The total number and dorso-ventral binned distribution of *SST*-expressing interneurons in somatosensory areas of SV129S1/SvImJ female mice with Pb compared to female littermates with a corpus callosum (cc), showed no differences (total interneuron numbers, Pb mice: 125.93+/−6.23, n=3; control mice: 135.08+/−10.96, n=3; p value= 0.51) (Figure S5A–E). This strongly suggests that abnormal connectivity by CPN to contralateral targets is not sufficient to influence interneuron layer positioning. In addition, our VSDI data show that projection neurons in the mutant cortex were excitable, a finding supported by prior electrophysiological recordings (Chen et al., 2008).

Increased interneuron cell death could have also accounted for the reduced percentage of interneurons in *Fezf2*$^{-/-}$ layer V (Cobos et al., 2006). To investigate this possibility, we performed staining for FluoroJade-C, which broadly labels dying neurons, in wild type (n=3) and *Fezf2*$^{-/-}$ (n=3) cortex at P0, P7 and P14, by which time interneuronal abnormalities are distinctly evident (data not shown). There was no difference in the number of FluoroJade-C positive neurons in wild type and mutant somatosensory areas at any of the ages sampled (Figure S5F–J). In addition, we did not detect any increase in Caspase-3 staining in *GAD67*-expressing interneurons (Figure S5K–N). Together with the finding that the total number of interneurons is unchanged in the *Fezf2*$^{-/-}$ cortex at P28, these data demonstrate that increased cell death did not account for the abnormal distribution of cortical interneurons in this mutant.

De novo generation of corticofugal projection neurons is sufficient to recruit deep layer cortical interneurons to ectopic locations

Our loss-of-function data demonstrate that a fate switch between two populations of projection neurons leads to a change in the distribution of specific subtypes of interneurons. This suggests that signaling between projection neuron and interneuron subtypes may be a mechanism by which projection neuron classes instruct the recruitment of specific interneuron partners.

To investigate this possibility, we overexpressed *Fezf2* by *in utero* electroporation in cortical progenitors at E14.5, a stage at which projection neurons of the superficial layers, mostly CPN, are generated. Elevated levels of *Fezf2* in these late-stage neural progenitors instruct the heterochronic generation of virtually pure corticofugal projection neurons (including subcerebral projection neurons). As previously reported, *Fezf2*-induced corticofugal neurons did not migrate into the cortex, rather they developed in ectopic clusters below the corpus callosum and extended corticofugal axonal projections to the thalamus and the pons (Figure S6C, arrow) (Molyneaux et al., 2005). They also expressed TBR1, TLE4 and SOX5, markers of corticofugal projection neurons and CTIP2, which is expressed at high levels in subcerebral projection neurons (Figure S6B–E,G–P). None of the *Fezf2*-induced neurons expressed SATB2 or CUX1, molecular markers of CPN (Figure S6D,F,G,Q–S). Therefore, overexpression of *Fezf2* results in the generation of a new population of corticofugal projection neurons outside of (yet in close proximity to) the cortex.

We examined expression of GABA in these ectopic corticofugal neuron clusters to determine if interneurons might be present within them. In contrast to contralateral matched locations, we found that many GABAergic interneurons invaded these aggregates (Figure 5B–F). This finding was confirmed using *in situ* hybridization to detect ectopic *GAD67*-positive cells (Figure 5G–H′). We quantified the number of GABA-positive interneurons within each aggregate and normalized it to the area covered by the GFP-positive corticofugal neurons. Interestingly, the number of interneurons present directly correlated with the size of the aggregate (Figure S7), suggesting that projection neurons recruit cortical interneurons in proportion to their own numbers.

Strikingly, ectopic corticofugal projection neurons appear to play an attractive role upon cortical interneurons in an *in vitro* migration assay (Figure S8). *Fezf2* was electroporated *in utero* in cortical progenitors at E14.5 as described above, and the induced clusters of corticofugal neurons were microdissected at E18.5 and cultured in proximity to explants of MGE from *Lhx6*-GFP positive, E12.5 embryos (Figure S8D). As controls, we cultured E12.5 MGE explants alone (Figure S8A). As expected, control MGE explants displayed unbiased outgrowth of GFP-positive interneurons in all directions around the explant (Figure S8B,C). In contrast, interneurons showed polarized migration towards the cluster of *Fezf2*-expressing corticofugal neurons (Figure S8E,F). This indicates that experimentally generated corticofugal neurons can attract cortical interneurons, a finding in agreement with the demonstration that cortical plate neurons attract interneurons in a similar *in vitro* assay (Lopez-Bendito et al., 2008).

We then asked whether the ectopic clusters of corticofugal neurons recruit interneurons that are characteristic of the deep layers of the cortex. Remarkably, many *Lhx6*-positive and *SST*-positive interneurons, which normally populate primarily the deep cortical layers, were found within the corticofugal neuron aggregates (Figure 6A–D′). In contrast, NPY-expressing interneurons, normally restricted to the superficial layers were not present in these clusters (Figure 6G–J). Similarly, *VIP*-expressing interneurons, which show a less restricted, yet still preferential distribution in the upper layers, were only present in very low numbers, likely corresponding to those normally located in the deep layers (Figure 6E–F′). Remarkably, Reelin, a gene that among interneurons labels those located in layers II/III and Va, (Alcantara et al., 1998) was absent from the corticofugal neuron aggregates (Figure 6K–O).

Together with our loss-of-function data, these gain-of-function results support a model by which deep layer corticofugal projection neurons selectively affect the distribution of interneuron subtypes that normally localize to the same layers.

Ectopically positioned upper layer II/III callosal projection neurons can recruit cortical interneurons

The ability to recruit interneurons could be a unique property of deep layer corticofugal projection neurons or might be applicable, more broadly, to other types of projection neurons. To investigate this possibility directly, we experimentally induced ectopic clusters of layer II/III callosal neurons and tested their ability to affect interneuron positioning. It has previously been shown that knock-down of the β-Amyloid Precursor Protein (*APP*) gene in rat cortical progenitors results in arrested migration of projection neurons (Young-Pearse et al., 2007). We took advantage of this system to focally arrest layer II/III CPN below the corpus callosum (Figure 7).

A construct carrying an *APP* shRNA (referred to as U6-*APP* shRNA-2) was electroporated *in utero* together with a reporter CAG-*GFP* construct at E14.5, when upper layer CPN are generated. Mice were sacrificed at P6, by which time distinct clusters of neurons were

visible below the corpus callosum (Figure 7C). The location of these clusters corresponded precisely to that of *Fezf2*-induced corticofugal neuron aggregates. As expected, the ectopic neurons expressed SATB2 and CUX1, and did not express CTIP2 (Figure 7C–E and data not shown), confirming that they differentiated appropriately into upper layer CPN. GABA immunocytochemistry on these CPN clusters (highlighted by the expression of SATB2) showed the distinct presence of GABA-positive interneurons within the aggregates, compared to matched positions in the contralateral hemisphere (n=9; Figure 7B–F). The experiment was also performed in the rat with identical results (n=3; data not shown).

These results demonstrate that layer II/III CPN are also able to recruit interneurons and further support a model by which projection neurons are generally able to affect interneuron lamination.

Projection neurons recruit interneurons that are appropriate for their projection neuron subtype-specific identity and not strictly their birthdate

It is intriguing that cortical projection neurons and interneurons that are synchronically born preferentially populate the same cortical layers (Fairen et al., 1986; Miller, 1985; Peduzzi, 1988; Pla et al., 2006; Valcanis and Tan, 2003). Early-born (peak at E13), largely MGE-derived interneurons localize in deep layers, whereas late-born (peak at E15), largely CGE-derived interneurons occupy the superficial layers (Fairen et al., 1986; Peduzzi, 1988; Pla et al., 2006). Our finding that ectopic corticofugal projection neurons specifically recruit large numbers of *Lhx6*- and *SST*-expressing deep layer interneurons despite being born synchronically with superficial layer CPN led us to investigate whether the projection neuron-type identity, rather than strictly the projection neuron birth date, affects the choice of interneurons recruited.

We examined whether the *Fezf2*-induced corticofugal projection neurons heterochronically generated two days after the bulk production of endogeneous corticofugal neurons has ended preferentially recruit interneurons that are appropriate for their projection neuron-type identity (i.e. interneurons born at E12–E13) or, rather, interneurons appropriate for their heterochronic day of birth (i.e. interneurons born the day of the electroporation, at E14.5). In complementary experiments, we investigated whether upper layer II/III CPN that were synchronically born with *Fezf2*-induced corticofugal neurons at E14.5 and that were similarly positioned below the corpus callosum (due to *APP* knock-down), recruited interneurons born at the same time.

In separate animals, we administered BrdU to timed-pregnant females at either E12.5 or E14.5, combined with either *Fezf2* overexpression or *APP* downregulation in cortical progenitors at E14.5 (Figure 8A). Mice were sacrificed at P6. Quantification of BrdU-labeled, GABA-positive interneurons showed that heterochronic, late-born corticofugal projection neurons preferentially recruited interneurons that were born early, at E12.5 (Figure 8A, C–H). These are appropriate for the deep-layer identity of the corticofugal projection neurons generated, but not for their heterochronic experimental birthdate (E14.5). In agreement with the E14.5 experimental birthdate of the corticofugal projection neurons, neuronal aggregates contained high numbers of E14.5 Brdu-labeled, GABA-negative projection neurons (Figure 8H). Confirming the specificity of this effect, we found that upper layer II/III CPN populations born the same day as the experimentally generated corticofugal neurons specifically attracted late-born interneurons (Figure 8A,I–N). This is noteworthy, since in this experimental system, corticofugal neurons and layer II/III CPN shared the same day of birth and were similarly located below the cortex, giving them access to comparable pools of cortical interneurons.

To determine whether the relative distribution of early- and late-born interneurons found in these aggregates corresponded to the distribution normally present in the deep or superficial layers of the cortex, we quantified the number of E12.5- and E14.5-born interneurons within the deep and superficial layers overlying the aggregates. Strikingly, *Fezf2*-corticofugal aggregates had similar relative proportions of early- and late-born interneurons as found in the deep layers of the cortex (Figure 8A,B). In a complementary trend, *APP* shRNA-layer II/III CPN showed the same distribution of early- and late-born interneurons as present in the superficial layers (Figure 8A,B). We conclude that cortical projection neurons select interneuron partners that are appropriate for their specific projection neuron identity and not strictly based on synchronic birthdates.

Discussion

A prominent trait of the mammalian cerebral cortex is its complex cellular architecture, which relies on the development of a diversity of neurons extensively interconnected into functional networks. Cortical excitation and inhibition are executed by highly heterogeneous populations of glutamatergic excitatory projection neurons and GABAergic inhibitory interneurons, respectively. The establishment of correct reciprocal positioning and interactions between these two broad neuronal classes is critical for balanced electrical activity and normal cortical function. Here, we demonstrate that projection neuron subtypes are necessary to determine the laminar location of their selected interneuron partners, a process that is critical for the development of the local inhibitory microcircuitry.

Projection neurons control interneuron positioning in the cerebral cortex

Many factors have the potential to affect the laminar localization of interneurons, including interneuron fate-specification and tangential and radial migration. The difficulty of uncoupling cell-intrinsic determinants of interneuron development from cell-extrinsic guidance by the environment of the cortex has constrained studies aimed at defining the roles of projection neurons in this process. Our finding that loss of *Fezf2* does not affect the birth or ventral migration of interneurons indicate that projection neurons are cell-extrinsically required for the normal lamination of cortical interneurons.

These findings are consistent with observations made for the *reeler* mouse, in which cortical projection neurons are abnormally distributed in the cortex and interneurons have defective radial distribution (Hevner et al., 2004; Pla et al., 2006; Yabut et al., 2007). This conclusion is also supported by our gain-of-function experiments, showing that projection neurons are sufficient to recruit appropriate interneurons to ectopic locations. It is remarkable that both clusters of corticofugal and callosal projection neurons that were generated ectopically are populated by GABAergic interneurons, indicating that the ability to recruit GABAergic interneurons may be a general property of all projection neuron subpopulations of the cortex.

Mechanistically, projection neurons might affect interneuron positioning in different ways, by attracting, arresting or stabilizing them at specific locations, for example. In addition, the role of projection neurons over interneuron lamination could be strictly local or also rely on population-level contributions. In the future, it will be important to investigate these mechanistic questions.

Distinct types of projection neurons uniquely affect interneuron lamination

Within the broader role of projection neurons in cortical interneuron lamination, it is an open question whether individual subtypes of projection neurons provide interneurons with different signals. The *Fezf2* mutant cortex provides a first model with which to directly

address the function of projection neuron subtypes. In this mutant, CPN cannot compensate for subcerebral projection neurons in the recruitment of appropriate numbers of *SST*- and PV- interneurons despite the fact that CPN are normally present within wild type layer V, where both SST- and PV-interneurons are located.

This could be due to different mechanisms of interneuron sorting used by subcerebral projection neurons compared to CPN, a possibility supported by our finding that CPN of layer V are normally surrounded by fewer interneurons. It is also possible that they interact with different subpopulations of *SST*- and PV-interneurons. Indeed it is well known that both *SST* and PV populations are very diverse at the cytological, electrophyisiological and molecular levels (Batista-Brito and Fishell, 2009; Markram et al., 2004). The implications of these findings therefore extend beyond the conclusion that different layers produce different signals, and argue that even within the same layer not all projection neuron subtypes act identically.

SST- and PV-positive interneurons are specifically affected in the *Fezf2* null-mutant

The classification of cortical interneuron subtypes is extremely complex (Ascoli et al., 2008). Within the limits of their current classification, it is known that MGE-derived, early-born SST-positive and PV-positive interneurons preferentially populate the deep layers of the cortex, whereas CR-positive, late-born, interneurons are mostly found in the superficial layers. Here, we find that *SST*- and PV-positive populations are sensitive to the absence of subcerebral projection neurons, but CR-positive interneurons are unaffected.

Given that cell-cell interactions typically rely on the expression of complementary sets of molecules by the cells involved, it follows that subtypes of interneurons might express different surface molecules, which enable them to respond differently to projection neurons. Intriguingly, this may explain why a recently identified population of Reelin-positive CGE-derived interneurons preferentially locate within the upper layers even if they are born early, at E12.5 (Miyoshi et al., 2010). These early-born CGE-derived interneurons might not be molecularly capable of responding to arrest signals provided by deep layer projection neurons and are exclusively sensitive to cues presented by superficial layer projection neurons. Indeed, our ectopic, deep layer corticofugal neurons do not recruit these early-born Reelin-positive interneurons.

The data support a model of selective interaction among projection neuron and interneuron subtypes, a conclusion in agreement with our gain-of-function results that *Lhx6*- and *SST*-positive interneurons are recruited by corticofugal neurons, while *VIP*-, NPY- and Reelin-positive interneurons of the superficial layers are not. Taken together, these data offer a picture of the striking precision with which different subtypes of projection neurons can affect the radial distribution of selected classes of interneurons.

Cortical projection neurons selectively recruit local interneurons based on subtype-specific identity rather than strictly by birthdate

Projection neurons and interneurons that share similar birthdates preferentially populate the same cortical layers (Fairen et al., 1986; Miller, 1985; Pla et al., 2006; Valcanis and Tan, 2003). This may be explained by different mechanisms, including the possibility that pairing of synchronically-born projection neurons and interneurons relies on the expression of complementary molecules, which may have evolved to ensure the coordinated positioning of populations born at the same time. Should this model be correct, the “molecular identity” of projection neurons would affect interactions with the correct types of interneurons.

We tested this hypothesis by changing the birth date of a projection neuron subpopulation, without affecting its molecular identity. We demonstrate that the ectopic generation of deep

layer corticofugal projection neurons at the wrong time (when superficial layer neurons are normally born) is sufficient to preferentially recruit early-born interneurons, which are appropriate for the deep layer identity of the projection neurons and not for their experimentally-imposed time of birth. This finding is reinforced by the fact that the recruited interneurons expressed *Lhx6* and *SST*. Notably, this selection-based mechanism of differential interneuron sorting is supported by the data that layer II/III CPN born at the same time as the *Fezf2*-induced corticofugal neurons (and occupying similar subcortical locations) select late-born interneurons, as appropriate for their upper layer CPN identity.

This implies that the pairing into circuitry of cortical projection neuron and interneuron partners might be "pre-programmed" during early stages of fate-specification of each neuron type, possibly days before interneurons acquire their final position in the appropriate cortical layers. This study provides a new conceptual framework for exploration of the mechanisms controlling subtype-specific interactions between selected populations of projection neurons and interneurons. Future strategies to identify the molecular mechanisms controlling precise laminar positioning of interneurons may require the purification and molecular comparison of the correct subtypes of "interacting" projection neurons and interneurons.

Understanding the developmental events that regulate subtype-specific interactions between projection neurons and interneurons in the cerebral cortex will provide further insights into the basic developmental processes that establish the local cortical microcircuitry (Hensch, 2005) and may inspire tools with which to modulate these circuits in epileptic and psychiatric diseases.

Experimental Procedures

Mice

Fezf2$^{-/-}$ mice were generated by Hirata and colleagues (Hirata et al., 2004). Gad67::GFP mice were generated by Yanagawa and colleagues (Tamamaki et al., 2003). Timed-pregnant CD-1 mice for *in utero* electroporation were from Charles River Laboratories (Wilmington, MA). *Lhx6*-GFP BAC transgenic mice were from GENSAT (Gong et al., 2003). SV129S1/SvImj strain mice were from Jackson Laboratories (Bar Harbor, ME). The day of the vaginal plug was designated embryonic day 0.5 (E0.5). The day of birth was designated postnatal day 0 (P0). All mouse studies were approved by the Massachusetts General Hospital IACUC and were performed in accordance with institutional and federal guidelines.

Retrograde Tracing

Mice were injected with FG in the contralateral cortex at P2 as previously described (Arlotta et al., 2005). Pups were deeply anesthetized at P6 before perfusion and collection of the cortex for immunocytochemistry.

Immunocytochemistry, *in situ* hybridization and Cresyl Violet staining

Brains for immunocytochemistry were processed as previously described (Arlotta et al., 2005; Macklis, 1993). Primary antibodies and dilutions are detailed in the Supplemental Data. Appropriate secondary antibodies were from the Molecular Probes Alexa series. Biotinylated secondary antibodies were used with standard avidin-biotin-diaminobenzidine visualization according to the manufacturer protocol (Vector Laboratories, Burlingame, CA). Cresyl violet staining was processed as previously described (Arlotta et al., 2005; Macklis, 1993). Fluorojade-C (Histo-Chem Incorporation, Jefferson, AR) staining was performed according to (Schmued and Hopkins, 2000). Nonradioactive *in situ* hybridization and combined *in situ* hybridization with immunohistochemistry were performed following

published methods (Tiveron et al., 1996). Riboprobes were generated as previously described (Arlotta et al., 2005). cDNA template clones are detailed in Supplemental Data.

Cell quantification

For quantification of interneurons, anatomically matched sections were processed to detect *Gad67*, *SST*, PV and CR ($n = 3$ *Fezf2*$^{-/-}$; $n = 3$ wild type, 8–10 hemispheres per area, for each mouse at P28). Boxes of 300 pixels in width and spanning the thickness of the cortex were superimposed at matched locations on each section and divided into 10 equally-sized bins. Interneurons were quantified in each bin, and bin-distribution was defined as the percentage of interneurons in each bin relative to the total number of interneurons. *A priori* criteria were defined for analysis. Quantification of interneuron-projection neuron ratio within layer V and of *SST*-positive interneurons in the SV129S1/SvImJ strain was done as detailed in Supplemental Data. For cell death quantification, anatomically matched sections spanning somatosensory cortex from littermates were processed for FluoroJade-C staining (P0, P7 and P14) and for Caspase-3 immunocytochemistry (P14). All counts were performed by an investigator who was blinded to genotype. All results are expressed as the mean ± s.e.m. and the paired, two-tailed *t* test was used for statistical analysis.

Voltage-sensitive dye imaging

Mice were decapitated under brief isoflurane anesthesia, and the brains processed as detailed in Supplemental Data.

A stimulating pulse (1 ms) was delivered through an ACSF-filled patch pipette to the white matter in V1. The resultant change in emitted dye fluorescence, corresponding to a change in membrane potential, was recorded using a MiCam Ultima (Brain Vision, SciMedia) camera (at 1 frame/ms). Changes in fluorescence were averaged across ten 512 ms trials. Regions of interest (125 μm^2) in the upper (150 μm below the pia) and lower layers (300 μm above the white matter) “on beam” with the stimulating electrode were analyzed for maximum change in intensity normalized to the resting intensity (ΔF/F).

In utero electroporation

The *Fezf2*^{GFP} and *Ctl*^{GFP} constructs and conditions for *in utero* electroporation were described previously (Molyneaux et al., 2005; Saito and Nakatsuji, 2001) and are detailed in Supplemental Data. Similar conditions were used to inject and electroporate the U6 *APP* shRNA-2 construct (Young-Pearse et al., 2007). For *APP*-shRNA electroporations, 0.5μg/μl of CAG-*GFP* vector was coeletroporated. Injected embryos were collected for analysis and tissue processing at P5–P6.

In vitro migration assay

E12.5 MGE from *Lhx6*-GFP mice were microdissected and cultured in three-dimensional matrigel matrix (Becton-Dickinson, Billerica, MA) either alone or in close proximity to *Fezf2*-expressing corticofugal neuron aggregates microdissected at E18.5. Migration of interneurons towards the *Fezf2*-expressing explants was quantified as detailed in Supplemental Data.

BrdU birthdating

Timed pregnant CD-1 females received one intraperitoneal injection of BrdU (50 mg/kg) as previously reported (Magavi et al., 2000). Birthdating of interneurons within experimental neuronal aggregates was done by counting the first-generation BrdU-positive cells that co-expressed GABA and were localized within each aggregate. The percentages of first-

generation, Brdu- and GABA-positive interneurons in the deep and superficial layers of the overlying cortex were quantified using the same methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Jeffrey Macklis and Bradley Molyneaux for reagents and insightful advice; Tracy Pearse-Young for sharing the *APP* shRNA construct and for her help with electroporations in the rat. Oscar Marin for critical input and advice during early stages of this study; Roberto Rusconi for support with cell counting; Verne Caviness, Eiman Azim, Giulio Srubek Tomassy, Feng Zhang and Anne Goodwin for their careful reading of the manuscript and comments; Robert Hevner, Carlos Lois, John Rubenstein, Vassilis Pachnis and Andre Goffinet for generous sharing of antibodies, cDNA clones and expression vectors; Yuchio Yanagawa for sharing the GAD67::GFP mice; Alyssa Meleski, Amanda Merlino and Zachary Trayes-Gibson for outstanding technical support; and Claudio Mare for schematic drawings. This work was partially supported by grants from the NIH (NS062849) and the Harvard Stem Cell Institute to P.A.; the NIH Director's Pioneer Award (1DP1 OD 003699-01) and Ellison Medical Foundation to T.K.H. S.L. was partially supported by a predoctoral fellowship from the European School of Molecular Medicine (S.E.M.M.) and C.R. was partially supported by a Milton-Safenowitz postdoctoral fellowship from the ALS Association.

References

Alcantara S, Ruiz M, D'Arcangelo G, Ezan F, de Lecea L, Curran T, Sotelo C, Soriano E. Regional and cellular patterns of reelin mRNA expression in the forebrain of the developing and adult mouse. J Neurosci. 1998; 18:7779–7799. [PubMed: 9742148]

Alifragis P, Liapi A, Parnavelas JG. Lhx6 regulates the migration of cortical interneurons from the ventral telencephalon but does not specify their GABA phenotype. J Neurosci. 2004; 24:5643–5648. [PubMed: 15201337]

Ang ES Jr, Haydar TF, Gluncic V, Rakic P. Four-dimensional migratory coordinates of GABAergic interneurons in the developing mouse cortex. J Neurosci. 2003; 23:5805–5815. [PubMed: 12843285]

Arlotta P, Molyneaux BJ, Chen J, Inoue J, Kominami R, Macklis JD. Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo. Neuron. 2005; 45:207–221. [PubMed: 15664173]

Ascoli GA, Alonso-Nanclares L, Anderson SA, Barrionuevo G, Benavides-Piccione R, Burkhalter A, Buzsaki G, Cauli B, Defelipe J, Fairen A, et al. Petilla terminology: nomenclature of features of GABAergic interneurons of the cerebral cortex. Nat Rev Neurosci. 2008; 9:557–568. [PubMed: 18568015]

Azim E, Jabaudon D, Fame RM, Macklis JD. SOX6 controls dorsal progenitor identity and interneuron diversity during neocortical development. Nat Neurosci. 2009; 12:1238–1247. [PubMed: 19657336]

Batista-Brito R, Fishell G. The developmental integration of cortical interneurons into a functional network. Curr Top Dev Biol. 2009; 87:81–118. [PubMed: 19427517]

Batista-Brito R, Rossignol E, Hjerling-Leffler J, Denaxa M, Wegner M, Lefebvre V, Pachnis V, Fishell G. The cell-intrinsic requirement of Sox6 for cortical interneuron development. Neuron. 2009; 63:466–481. [PubMed: 19709629]

Bayer, SA.; Altman, J. Neocortical Development. New York: Raven Press; 1991.

Bortone D, Polleux F. KCC2 expression promotes the termination of cortical interneuron migration in a voltage-sensitive calcium-dependent manner. Neuron. 2009; 62:53–71. [PubMed: 19376067]

Butt SJ, Fuccillo M, Nery S, Noctor S, Kriegstein A, Corbin JG, Fishell G. The temporal and spatial origins of cortical interneurons predict their physiological subtype. Neuron. 2005; 48:591–604. [PubMed: 16301176]

Butt SJ, Sousa VH, Fuccillo MV, Hjerling-Leffler J, Miyoshi G, Kimura S, Fishell G. The requirement of Nkx2-1 in the temporal specification of cortical interneuron subtypes. Neuron. 2008; 59:722–732. [PubMed: 18786356]

Chen B, Schaevitz LR, McConnell SK. Fezl regulates the differentiation and axon targeting of layer 5 subcortical projection neurons in cerebral cortex. Proc Natl Acad Sci U S A. 2005a; 102:17184–17189. [PubMed: 16284245]

Chen B, Wang SS, Hattox AM, Rayburn H, Nelson SB, McConnell SK. The Fezf2-Ctip2 genetic pathway regulates the fate choice of subcortical projection neurons in the developing cerebral cortex. Proc Natl Acad Sci U S A. 2008; 105:11382–11387. [PubMed: 18678899]

Chen JG, Rasin MR, Kwan KY, Sestan N. Zfp312 is required for subcortical axonal projections and dendritic morphology of deep-layer pyramidal neurons of the cerebral cortex. Proc Natl Acad Sci U S A. 2005b; 102:17792–17797. [PubMed: 16314561]

Cobos I, Long JE, Thwin MT, Rubenstein JL. Cellular patterns of transcription factor expression in developing cortical interneurons. Cereb Cortex. 2006; 16(Suppl 1):i82–88. [PubMed: 16766712]

Corbin JG, Nery S, Fishell G. Telencephalic cells take a tangent: non-radial migration in the mammalian forebrain. Nat Neurosci. 2001; 4(Suppl):1177–1182. [PubMed: 11687827]

Elias LA, Potter GB, Kriegstein AR. A time and a place for nkx2-1 in interneuron specification and migration. Neuron. 2008; 59:679–682. [PubMed: 18786351]

Fairen A, Cobas A, Fonseca M. Times of generation of glutamic acid decarboxylase immunoreactive neurons in mouse somatosensory cortex. J Comp Neurol. 1986; 251:67–83. [PubMed: 3760259]

Fogarty M, Grist M, Gelman D, Marin O, Pachnis V, Kessaris N. Spatial genetic patterning of the embryonic neuroepithelium generates GABAergic interneuron diversity in the adult cortex. J Neurosci. 2007; 27:10935–10946. [PubMed: 17928435]

Gelman DM, Martini FJ, Nobrega-Pereira S, Pierani A, Kessaris N, Marin O. The embryonic preoptic area is a novel source of cortical GABAergic interneurons. J Neurosci. 2009; 29:9380–9389. [PubMed: 19625528]

Gong S, Zheng C, Doughty ML, Losos K, Didkovsky N, Schambra UB, Nowak NJ, Joyner A, Leblanc G, Hatten ME, Heintz N. A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. Nature. 2003; 425:917–925. [PubMed: 14586460]

Grinvald A, Hildesheim R. VSDI: a new era in functional imaging of cortical dynamics. Nat Rev Neurosci. 2004; 5:874–885. [PubMed: 15496865]

Hammond V, So E, Gunnersen J, Valcanis H, Kalloniatis M, Tan SS. Layer positioning of late-born cortical interneurons is dependent on Reelin but not p35 signaling. J Neurosci. 2006; 26:1646–1655. [PubMed: 16452688]

Hensch TK. Critical period plasticity in local cortical circuits. Nat Rev Neurosci. 2005; 6:877–888. [PubMed: 16261181]

Hevner RF, Daza RA, Englund C, Kohtz J, Fink A. Postnatal shifts of interneuron position in the neocortex of normal and reeler mice: evidence for inward radial migration. Neuroscience. 2004; 124:605–618. [PubMed: 14980731]

Hirata T, Suda Y, Nakao K, Narimatsu M, Hirano T, Hibi M. Zinc finger gene fez-like functions in the formation of subplate neurons and thalamocortical axons. Dev Dyn. 2004; 230:546–556. [PubMed: 15188439]

Kriegstein AR, Noctor SC. Patterns of neuronal migration in the embryonic cortex. Trends Neurosci. 2004; 27:392–399. [PubMed: 15219738]

Lavdas AA, Grigoriou M, Pachnis V, Parnavelas JG. The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. J Neurosci. 1999; 19:7881–7888. [PubMed: 10479690]

Li G, Adesnik H, Li J, Long J, Nicoll RA, Rubenstein JL, Pleasure SJ. Regional distribution of cortical interneurons and development of inhibitory tone are regulated by Cxcl12/Cxcr4 signaling. J Neurosci. 2008; 28:1085–1098. [PubMed: 18234887]

Liapi A, Pritchett J, Jones O, Fujii N, Parnavelas JG, Nadarajah B. Stromal-derived factor 1 signalling regulates radial and tangential migration in the developing cerebral cortex. Dev Neurosci. 2008; 30:117–131. [PubMed: 18075260]

Lindwall C, Fothergill T, Richards LJ. Commissure formation in the mammalian forebrain. Curr Opin Neurobiol. 2007; 17:3–14. [PubMed: 17275286]

Liodis P, Denaxa M, Grigoriou M, Akufo-Addo C, Yanagawa Y, Pachnis V. Lhx6 activity is required for the normal migration and specification of cortical interneuron subtypes. J Neurosci. 2007; 27:3078–3089. [PubMed: 17376969]

Lopez-Bendito G, Sanchez-Alcaniz JA, Pla R, Borrell V, Pico E, Valdeolmillos M, Marin O. Chemokine signaling controls intracortical migration and final distribution of GABAergic interneurons. J Neurosci. 2008; 28:1613–1624. [PubMed: 18272682]

Magavi SS, Leavitt BR, Macklis JD. Induction of neurogenesis in the neocortex of adult mice. Nature. 2000; 405:951–955. [PubMed: 10879536]

Marin O, Rubenstein JL. A long, remarkable journey: tangential migration in the telencephalon. Nat Rev Neurosci. 2001; 2:780–790. [PubMed: 11715055]

Markram H, Toledo-Rodriguez M, Wang Y, Gupta A, Silberberg G, Wu C. Interneurons of the neocortical inhibitory system. Nat Rev Neurosci. 2004; 5:793–807. [PubMed: 15378039]

Miller MW. Cogeneration of retrogradely labeled corticocortical projection and GABA-immunoreactive local circuit neurons in cerebral cortex. Brain Res. 1985; 355:187–192. [PubMed: 3910166]

Mitchell BD, Macklis JD. Large-scale maintenance of dual projections by callosal and frontal cortical projection neurons in adult mice. J Comp Neurol. 2005; 482:17–32. [PubMed: 15612019]

Miyoshi G, Butt SJ, Takebayashi H, Fishell G. Physiologically distinct temporal cohorts of cortical interneurons arise from telencephalic Olig2-expressing precursors. J Neurosci. 2007; 27:7786–7798. [PubMed: 17634372]

Miyoshi G, Fishell G. GABAergic Interneuron Lineages Selectively Sort into Specific Cortical Layers during Early Postnatal Development. Cereb Cortex.

Miyoshi G, Hjerling-Leffler J, Karayannis T, Sousa VH, Butt SJ, Battiste J, Johnson JE, Machold RP, Fishell G. Genetic fate mapping reveals that the caudal ganglionic eminence produces a large and diverse population of superficial cortical interneurons. J Neurosci. 2010; 30:1582–1594. [PubMed: 20130169]

Molyneaux BJ, Arlotta P, Fame RM, MacDonald JL, MacQuarrie KL, Macklis JD. Novel subtype-specific genes identify distinct subpopulations of callosal projection neurons. J Neurosci. 2009; 29:12343–12354. [PubMed: 19793993]

Molyneaux BJ, Arlotta P, Hirata T, Hibi M, Macklis JD. Fezl is required for the birth and specification of corticospinal motor neurons. Neuron. 2005; 47:817–831. [PubMed: 16157277]

Molyneaux BJ, Arlotta P, Menezes JR, Macklis JD. Neuronal subtype specification in the cerebral cortex. Nat Rev Neurosci. 2007; 8:427–437. [PubMed: 17514196]

Nery S, Fishell G, Corbin JG. The caudal ganglionic eminence is a source of distinct cortical and subcortical cell populations. Nat Neurosci. 2002; 5:1279–1287. [PubMed: 12411960]

Peduzzi JD. Genesis of GABA-immunoreactive neurons in the ferret visual cortex. J Neurosci. 1988; 8:920–931. [PubMed: 3346729]

Pla R, Borrell V, Flames N, Marin O. Layer acquisition by cortical GABAergic interneurons is independent of Reelin signaling. J Neurosci. 2006; 26:6924–6934. [PubMed: 16807322]

Polleux F, Whitford KL, Dijkhuizen PA, Vitalis T, Ghosh A. Control of cortical interneuron migration by neurotrophins and PI3-kinase signaling. Development. 2002; 129:3147–3160. [PubMed: 12070090]

Richards LJ, Plachez C, Ren T. Mechanisms regulating the development of the corpus callosum and its agenesis in mouse and human. Clin Genet. 2004; 66:276–289. [PubMed: 15355427]

Rouaux C, Arlotta P. Fezf2 directs the differentiation of corticofugal neurons from striatal progenitors in vivo. Nat Neurosci. 13:1345–1347. [PubMed: 20953195]

Saito T, Nakatsuji N. Efficient gene transfer into the embryonic mouse brain using in vivo electroporation. Dev Biol. 2001; 240:237–246. [PubMed: 11784059]

Schmued LC, Hopkins KJ. Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res. 2000; 874:123–130. [PubMed: 10960596]

Shimizu T, Hibi M. Formation and patterning of the forebrain and olfactory system by zinc-finger genes Fezf1 and Fezf2. Dev Growth Differ. 2009; 51:221–231. [PubMed: 19222525]

Tamamaki N, Yanagawa Y, Tomioka R, Miyazaki J, Obata K, Kaneko T. Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. J Comp Neurol. 2003; 467:60–79. [PubMed: 14574680]

Tanaka D, Nakaya Y, Yanagawa Y, Obata K, Murakami F. Multimodal tangential migration of neocortical GABAergic neurons independent of GPI-anchored proteins. Development. 2003; 130:5803–5813. [PubMed: 14534141]

Tanaka DH, Mikami S, Nagasawa T, Miyazaki JI, Nakajima K, Murakami F. CXCR4 Is Required for Proper Regional and Laminar Distribution of Cortical Somatostatin-, Calretinin-, and Neuropeptide Y-Expressing GABAergic Interneurons. Cereb Cortex. 2010

Tiveron MC, Hirsch MR, Brunet JF. The expression pattern of the transcription factor Phox2 delineates synaptic pathways of the autonomic nervous system. J Neurosci. 1996; 16:7649–7660. [PubMed: 8922421]

Tiveron MC, Rossel M, Moepps B, Zhang YL, Seidenfaden R, Favor J, Konig N, Cremer H. Molecular interaction between projection neuron precursors and invading interneurons via stromal-derived factor 1 (CXCL12)/CXCR4 signaling in the cortical subventricular zone/ intermediate zone. J Neurosci. 2006; 26:13273–13278. [PubMed: 17182777]

Valcanis H, Tan SS. Layer specification of transplanted interneurons in developing mouse neocortex. J Neurosci. 2003; 23:5113–5122. [PubMed: 12832535]

Wahlsten D, Metten P, Crabbe JC. Survey of 21 inbred mouse strains in two laboratories reveals that BTBR T/+ tf/tf has severely reduced hippocampal commissure and absent corpus callosum. Brain Res. 2003; 971:47–54. [PubMed: 12691836]

Wang Y, Dye CA, Sohal V, Long JE, Estrada RC, Roztocil T, Lufkin T, Deisseroth K, Baraban SC, Rubenstein JL. Dlx5 and Dlx6 regulate the development of parvalbumin-expressing cortical interneurons. J Neurosci. 2010; 30:5334–5345. [PubMed: 20392955]

Wonders CP, Anderson SA. The origin and specification of cortical interneurons. Nat Rev Neurosci. 2006; 7:687–696. [PubMed: 16883309]

Xu Q, Cobos I, De La Cruz E, Rubenstein JL, Anderson SA. Origins of cortical interneuron subtypes. J Neurosci. 2004; 24:2612–2622. [PubMed: 15028753]

Yabut O, Renfro A, Niu S, Swann JW, Marin O, D'Arcangelo G. Abnormal laminar position and dendrite development of interneurons in the reeler forebrain. Brain Res. 2007; 1140:75–83. [PubMed: 16996039]

Young-Pearse TL, Bai J, Chang R, Zheng JB, LoTurco JJ, Selkoe DJ. A critical function for beta-amyloid precursor protein in neuronal migration revealed by in utero RNA interference. J Neurosci. 2007; 27:14459–14469. [PubMed: 18160654]

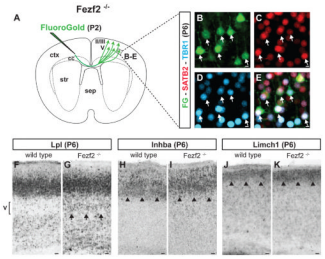

Figure 1. Neurons that replace subcerebral projection neurons in *Fezf2*$^{-/-}$ cortex are CPN of deep layer identity

(A–E) Retrograde labeling of CPN in contralateral *Fezf2*$^{-/-}$ cortex shows colocalization of FG with the expanded population of TBR1- and SATB2 -positive neurons in layer V. (F–K) *In situ* hybridizations at P6 for *Lpl*, *Inhba* and *Limch1* (CPN markers), showing that the expanded population of CPN in *Fezf2*$^{-/-}$ layer V are of deep layer identity. Scale bars, 100 μm (F–K), 20 μm (B–E). (See also Figure S1)

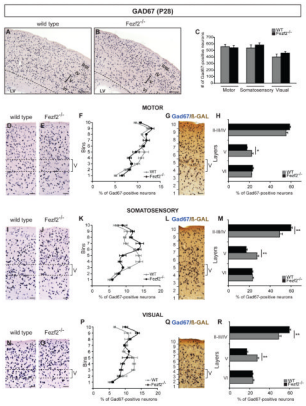

Figure 2. The *Fezf2*$^{-/-}$ cortex has abnormal radial distribution of GABAergic interneurons

(A,B) *In situ* hybridization for *Gad67* in wild type (A) and *Fezf2*$^{-/-}$ (B) cortex at P28 shows reduced numbers of interneurons in layer V and increased numbers in superficial layers of the *Fezf2*$^{-/-}$ cortex. (C) Total number of *Gad67*-interneurons is unchanged between wild type and *Fezf2*$^{-/-}$ cortex. (D–R) Quantification of the layer distribution of *Gad67*-interneurons across motor (D–H), somatosensory (I–M) and visual (N–R) cortex. (D,E,I,J,N,O) Representative sections of wild type and *Fezf2*$^{-/-}$ cortex. (F,K,P) Unbiased binned distribution of *Gad67*-interneuron percentages shows decreased percentages in deep bins and increased percentages in superficial bins of the mutant. (G,L,Q) β-galactosidase immunocytochemistry in *Fezf2*$^{+/-}$ heterozygote mice demonstrates that layer V corresponds to bins 4–5 (in motor and somatosensory cortex) and bins 3–4 (in visual cortex). (H,M,R) Quantification of interneurons within layers demonstrates a specific reduction in interneuron percentages in mutant layer V across all cortical areas, and an increase in interneuron percentages in layers II/III–IV. All results are expressed as the mean ± s.e.m.. LV, lateral ventricle. Scale bars, 500 μm (A,B); 200 μm (D,E,G,I,J,L,N,O,Q). (See also Figures S2 and S3).

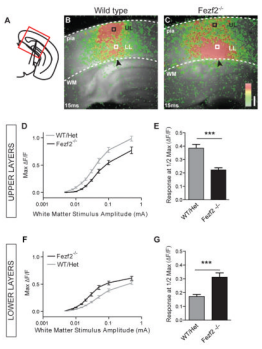

Figure 3. Spread of neuronal activity is restricted to the deep layers in *Fezf2*$^{-/-}$ cortex

(A) Schematic of recording area (red box) indicating the position of the stimulating electrode in the white matter (WM). (B,C) Pseudocolor peak response frames from VSDI movies of wild type (B) and *Fezf2*$^{-/-}$ (C) slices 15 ms after the stimulus (arrowhead), showing that wild type mice exhibit a strong stimulus response that propagates rapidly to the upper layers (B); whereas, the response in *Fezf2*$^{-/-}$ slices rarely reaches the upper layers, remaining largely confined to the lower layers (C). Black and white squares indicate quantified regions of interest in the upper (UL) and lower layers (LL), respectively. Scale bars, 250 μm. (D,F) Stimulus response curve in the regions of interest for upper (D) and lower (F) layers. (E, G) Response at half max. *** $p<0.001$, t-test. (See also Movies S1, S2, S3 and S4).

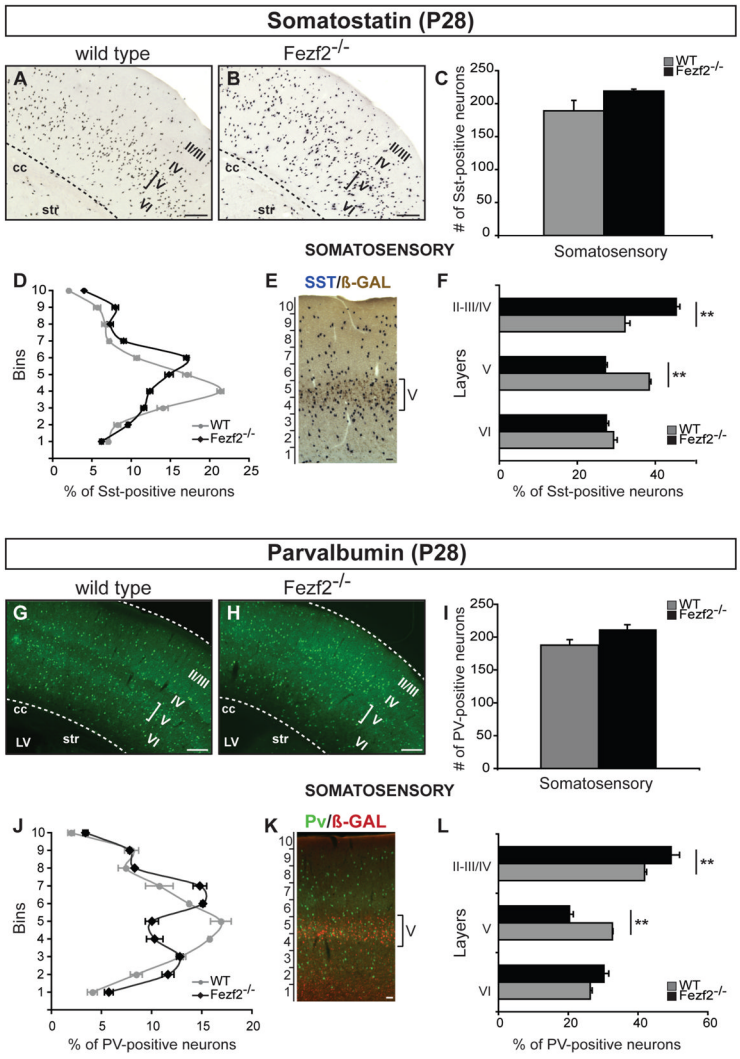

Figure 4. *SST*- and PV-interneuron subtypes are reduced in layer V and increased in the superficial layers of the *Fezf2*$^{-/-}$ cortex

(A,B,G,H) *In situ* hybridization for *SST* (A,B) and immunocytochemistry for PV (G,H) in wild type and *Fezf2*$^{-/-}$ somatosensory cortex at P28 show reduction of both interneuron populations in layer V and increase in upper layers. (C,I) Total number of *SST*- (C) and PV- (I) interneurons is unchanged between wild type and *Fezf2*$^{-/-}$ cortex. (D,J) Unbiased binned distribution of *SST*- and PV-interneuron percentages shows decreased numbers in deep bins and increased numbers in superficial bins of the mutant. (E,K) β-galactosidase immunocytochemistry in *Fezf2*$^{+/-}$ heterozygote mice highlights layer V in bins 4–5. (F,L) Quantification of *SST*- (F) and PV- (L) interneurons within layers demonstrates a reduction in the percentages of both interneuronal subpopulations in mutant layer V and an increase in layers II/III–IV. All results are expressed as the mean ± s.e.m.. LV, lateral ventricle; str, striatum; cc, corpus callosum. Scale bars, 500 μm (A,B,G,H); 100 μm (E,K). (See also Figures S4 and S5).

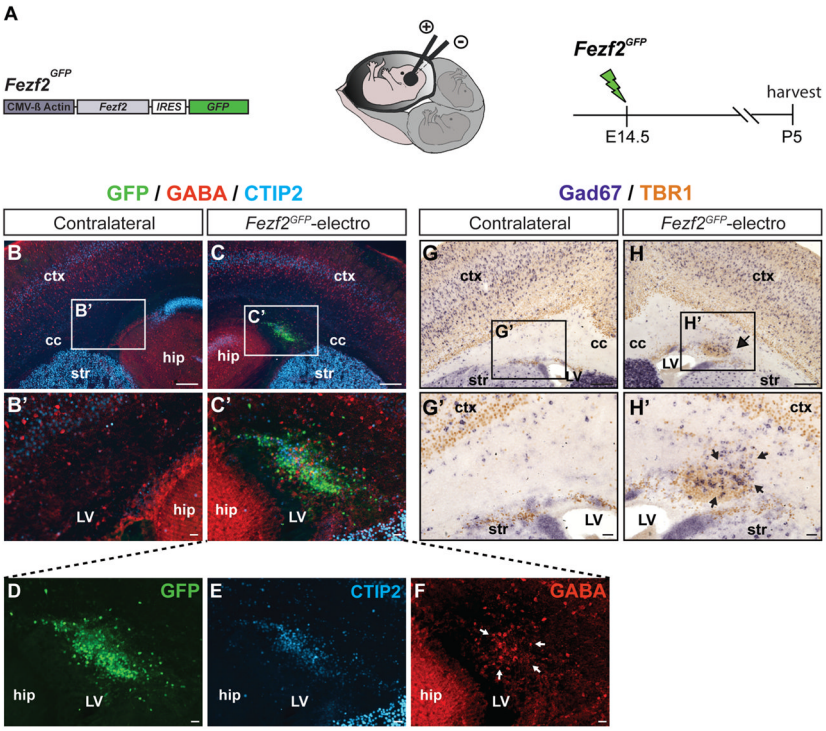

Figure 5. Experimentally-generated aggregates of corticofugal projection neurons recruit cortical interneurons to new ectopic locations

(A) Schematic of experimental approach. (B–C′) *In utero* overexpression of *Fezf2* in cortical progenitors at E14.5 causes a switch of fate to corticofugal projection neurons, which develop as ectopic aggregates below the corpus callosum. (C′–F) GFP- and CTIP2-positive aggregates of corticofugal neurons contain GABA-positive interneurons (arrows). (G–H′) *In situ* hybridization for *Gad67* and immunocytochemistry for TBR1 demonstrates that TBR1-positive aggregates of corticofugal neurons contain *Gad67*-interneurons (arrows). Scale bars, 500 μm (B,C,G,H); 100 μm (B′,C′,D–F,G′,H′). (See also Figures S6, S7 and S8).

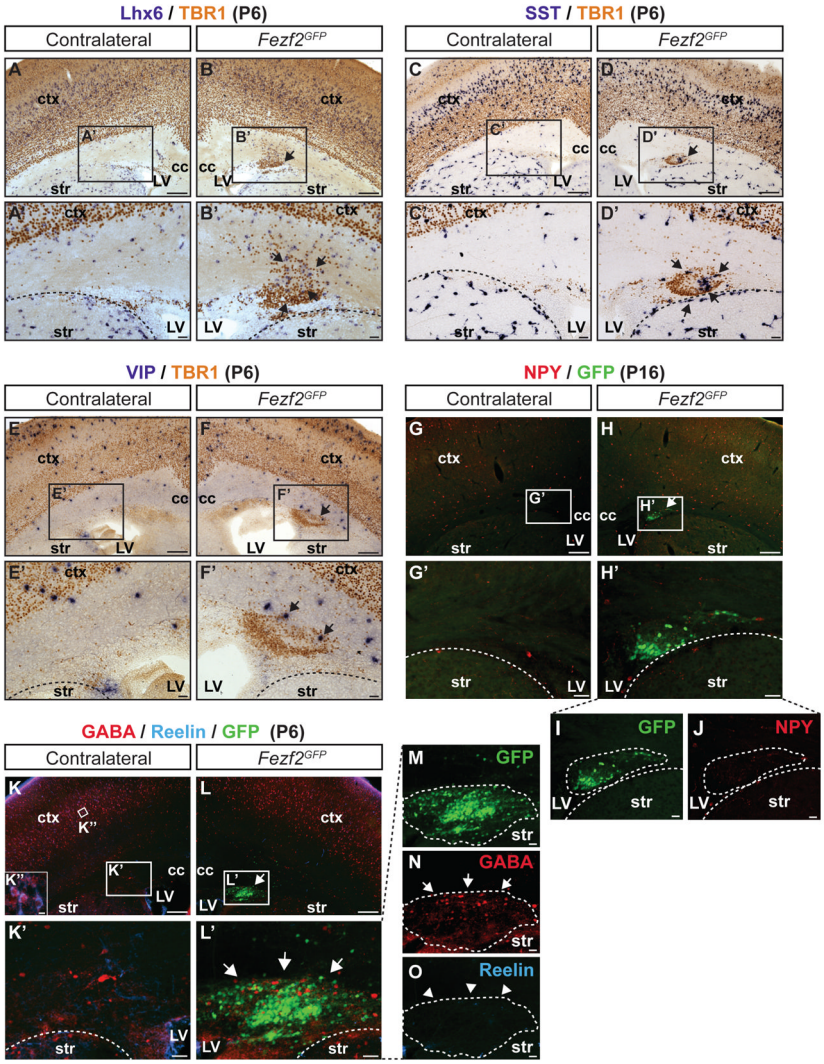

Figure 6. Interneurons characteristic of deep-layers are selectively found in corticofugal neuron aggregates

(A–F′) *In situ* hybridization for *Lhx6* (A–B′), *SST* (C–D′) and *VIP* (E–F′) combined with immunocytochemistry for TBR1 on electroporated brains demonstrates that aggregates of corticofugal neurons (B′,D′,F′, arrows) contain deep layer *Lhx6*-positive (B,B′, arrows) and *SST*-positive (D,D′, arrows) interneurons and very small numbers of *VIP*-positive (F,F′, arrows) interneurons. Aggregates of interneurons are absent from contralateral locations (A′,C′,E′). (GO) Immunocytochemistry for NPY (G–J), GABA, Reelin and GFP (K–O) show that interneuron subpopulations that are mostly found in cortical superficial layers or layer Va are absent from the *Fezf2*-positive aggregates (H′,J,L′,O). (K″) Coexpression of GABA and Reelin in layer Va. Scale bars, 500 μm (A,B,C,D,E,F,G,H,K,L); 100 μm (A′B′,C′,D′,E′,F′,G′H′,I,J,M–O).

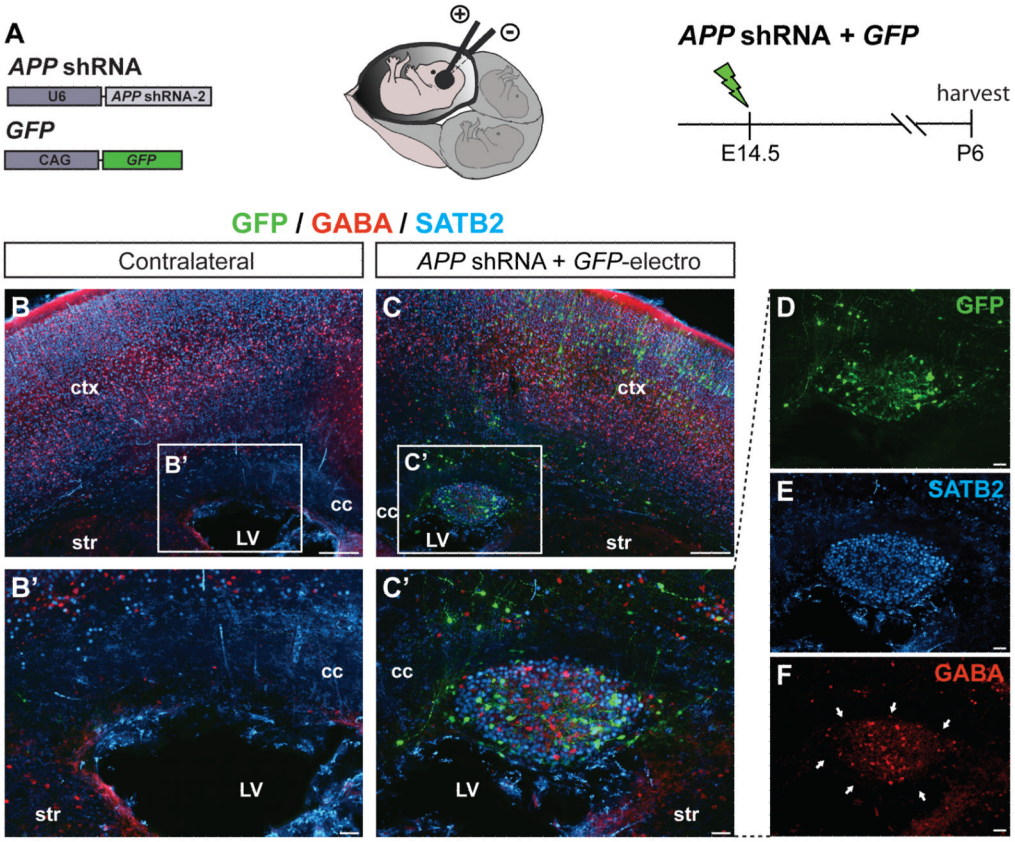

Figure 7. Ectopically-located aggregates of layer II/III callosal projection neurons recruit cortical interneurons

(A) Schematic of experimental approach. (B–C′) *In utero* overexpression of *APP* shRNA combined with CAG-GFP in cortical progenitors at E14.5 blocks the migration of superficial layer projection neurons, which differentiate as ectopic aggregates below the corpus callosum. (C′, D–F) GFP- and SATB2-positive aggregates of upper layer CPN contain GABA-positive interneurons (arrows). Scale bars, 500 μm (B,C); 100 μm (B′,C′,D–F).

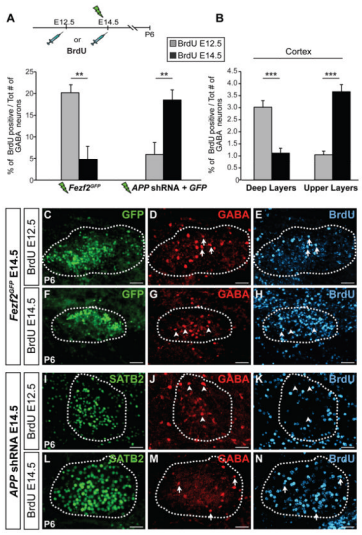

Figure 8. Ectopic aggregates of corticofugal projection neurons recruit interneurons that are appropriate for their projection neuron-type identity

(A) Schematic of experimental design and quantification of first-generation, BrdU-positive and GABA-positive interneurons within aggregates expressing either *Fezf2*^{GFP} or *APP* shRNA. GABAergic interneurons in the corticofugal aggregates (*Fezf2*^{GFP}) are largely early-born, while those in the aggregates of layer II/III CPN (*APP* shRNA and CAG-GFP) are largely late-born. (B) Quantification of first-generation, BrdU-positive and GABA-positive interneurons within the deep and superficial layers of the cortex overlying the aggregates. Late-generated corticofugal neuron aggregates and synchronically generated CPN show distribution of E12.5- and E14.5- interneurons as observed in the deep and superficial cortical layers, respectively. (C–N) GABA-positive interneurons within *Fezf2*^{GFP} aggregates are born at E12.5 (C–E, arrows) and not at E14.5 (F–H, arrowheads), while GABA-positive interneurons within the *APP* shRNA aggregates are born at E14.5 (L–N, arrows) and not at E12.5 (I–K, arrowheads). Scale bars, 100 μm (C–N).