Report

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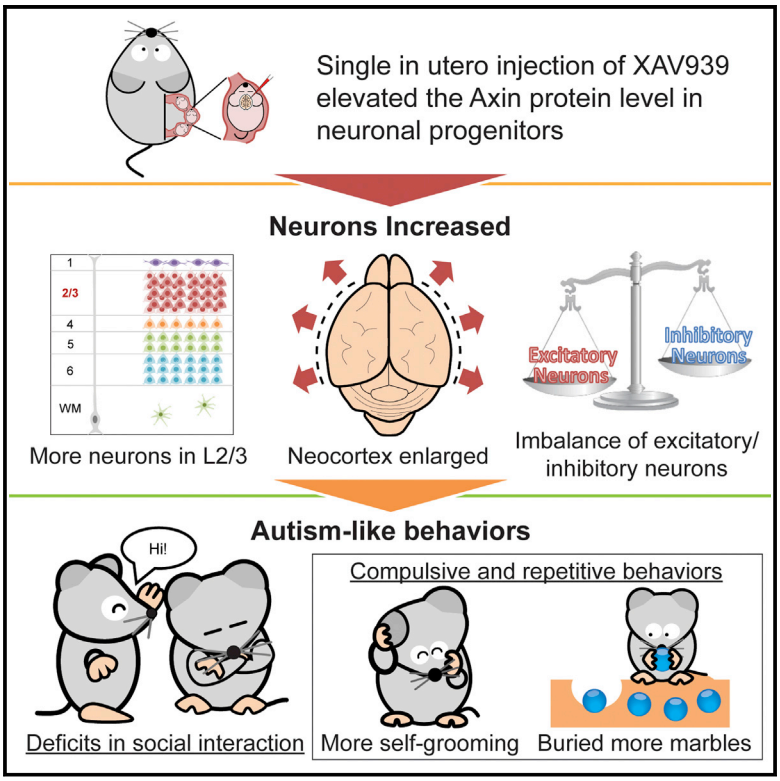
Overproduction of Upper-Layer Neurons in the Neocortex Leads to Autism-like Features in Mice

Graphical Abstract



Highlights

Enhanced embryonic neurogenesis leads to overproduction of excitatory neurons

Excess excitatory neurons perturb excitatory and inhibitory neuronal development

Excess excitatory neurons alter excitatory and inhibitory synaptic connections

Increased cortical neuron number is associated with autism-like behavioral deficits

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In Brief

Fang et al. generated a mouse model with excessive excitatory neurons in the neocortex by manipulating embryonic neurogenesis. Overproduction of neurons results in autism-like anatomical and behavioral features. These findings suggest a causal relationship between overproduction of neurons and cortical malfunction and provide developmental insights into the etiology of autism.

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Report

Overproduction of Upper-Layer Neurons in the Neocortex Leads to Autism-like Features in Mice

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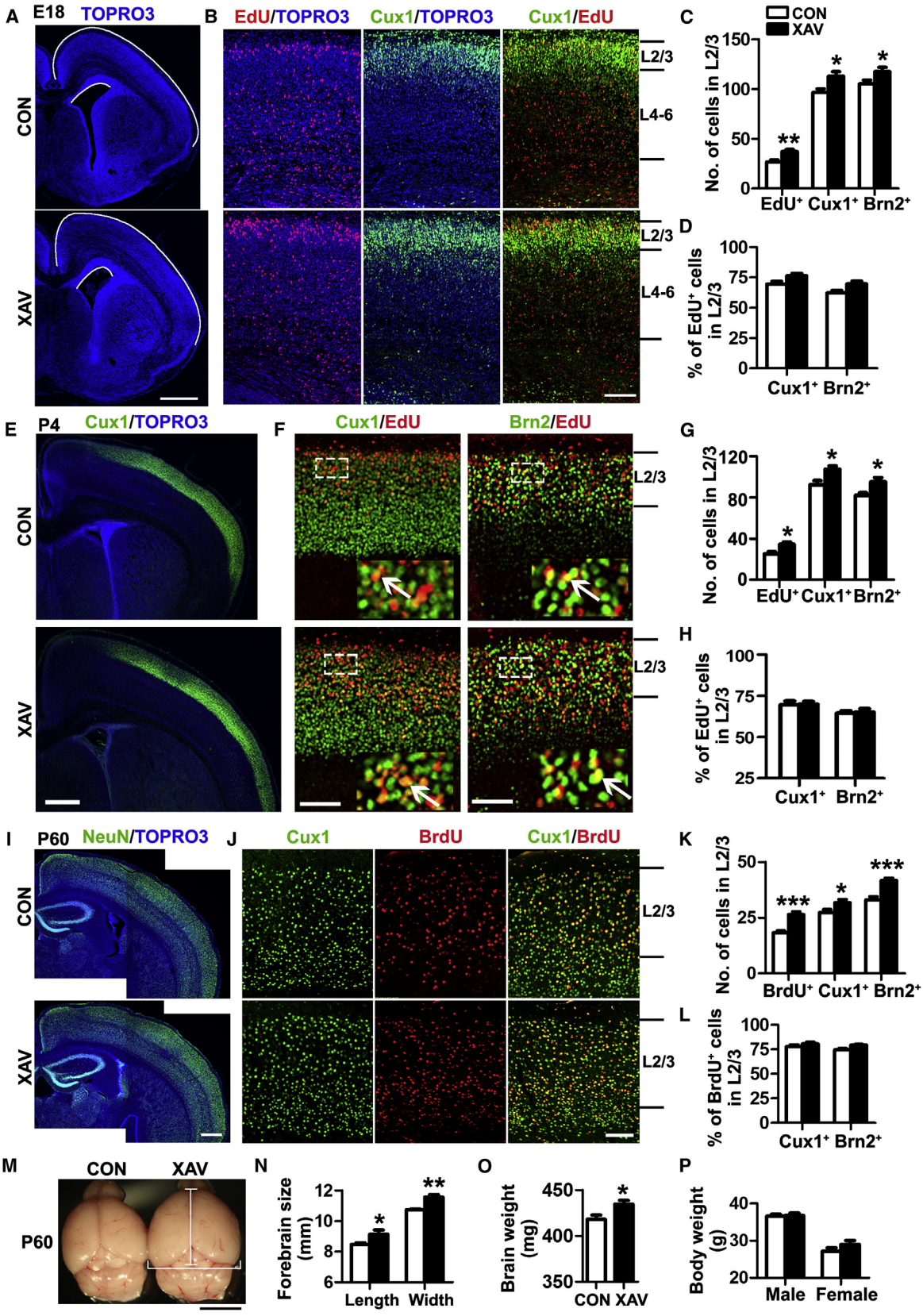
SUMMARY

The functional integrity of the neocortex depends upon proper numbers of excitatory and inhibitory neurons; however, the consequences of dysregulated neuronal production during the development of the neocortex are unclear. As excess cortical neurons are linked to the neurodevelopmental disorder autism, we investigated whether the overproduction of neurons leads to neocortical malformation and malfunction in mice. We experimentally increased the number of pyramidal neurons in the upper neocortical layers by using the small molecule XAV939 to expand the intermediate progenitor population. The resultant overpopulation of neurons perturbs development of dendrites and spines of excitatory neurons and alters the laminar distribution of interneurons. Furthermore, these phenotypic changes are accompanied by dysregulated excitatory and inhibitory synaptic connection and balance. Importantly, these mice exhibit behavioral abnormalities resembling those of human autism. Thus, our findings collectively suggest a causal relationship between neuronal overproduction and autism-like features, providing developmental insights into the etiology of autism.

INTRODUCTION

It is unknown whether and how the number of neocortical neurons or brain size affects brain functions. Whereas the evolution of mammalian brains is characterized by the expansion of neocortex, embryonic neurogenesis is strictly controlled to avoid the overproduction of neurons. Overproduction of neurons may increase synaptic connections and alter synaptic strength, both of which may reshape the neuronal wiring patterns and eventually cause atypical brain functions. Indeed, excess neocortical neurons and brain overgrowth are associated with mental disorders, particularly autism. Macrocephaly is observed in approximately 20% of children with autism, and neuronal density and number are elevated in the neocortex of autism patients (Casanova et al., 2006; Courchesne et al., 2011; McCaffery and Deutsch, 2005). Nonetheless, it remains unclear whether unrestrained neuronal production in the neocortex leads to cortical malformation and malfunction characteristic of autism.

Investigating the functional consequence of neuronal overpopulation requires unique strategies to specifically modulate neuron numbers without directly affecting neuronal or glial development. Several mouse models have been reported to generate excess neurons, including *Pten*-knockout mice (Groszer et al., 2001), *Δ90β-catenin*-overexpressing mice (which express a stabilized form of β-catenin; Chenn and Walsh, 2003), and mice intraventricularly microinjected with FGF2 (Vaccarino et al., 2009). However, as Pten and β-catenin play critical roles in neurons and FGF2 affects astrocyte progenitors and microglia, these mouse models exhibit impaired neuronal development, astrogenesis, and/or microglial reactivity. We recently developed a specific approach to induce the overproduction of neurons in the neocortex (Fang et al., 2013). In this model, the small molecule XAV939, which inhibits tankyrase and thus prevents the degradation of the scaffold protein Axin (Huang et al., 2009), is delivered into the developing mouse neocortex. A single in utero injection of XAV939 into the embryonic lateral ventricle transiently increases the Axin protein level in radial glial cells, resulting in the temporary amplification of intermediate progenitors and consequently excessive production of pyramidal neurons in the neocortex (Fang et al., 2013). As layer 2/3 pyramidal neurons are thought to underpin high-level cognitive functions and an increased number of these neurons is implicated in autism (Fame et al., 2011), we examined whether the overproduction of layer 2/3 pyramidal neurons in these mice results in neocortical malformation and malfunction and if this is associated with behavioral deficits similar to those featured in autism.



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RESULTS

Experimental Overproduction of Layer 2/3 Excitatory Neurons in the Murine Neocortex

During embryonic neocortical development, neuronal differentiation of neural progenitors is strictly controlled in a temporal manner; neurons generated at different stages follow stereotypic migratory routes to occupy specific laminar layers in an inside-out sequence. We previously demonstrated that a single injection of XAV939 into the lateral ventricle at embryonic day 14.5 (E14.5) causes a transient amplification of intermediate progenitors, which in turn results in the overproduction of neurons in layer 2/3 (Fang et al., 2013). In the present study, we characterized how neuronal overproduction in layer 2/3 affects neocortical development and function. The neocortices receiving XAV939 treatment were moderately larger than those injected with vehicle (DMSO; Figure 1A; ratio of pial-to-apical surface area: CON 4.67 ± 0.22; XAV 5.05 ± 0.19; $p = 0.02$), whereas the cortical lamination remained unchanged (Fang et al., 2013). The newly born neurons were labeled with nucleotide analogs 5-ethynyl-2′-deoxyuridine (EdU) or bromodeoxyuridine (BrdU) at E15. As expected, XAV939 increased neuronal production (i.e., EdU^+ cells) in layer 2/3, which were confirmed to be excitatory neurons (i.e., $Cux1^+$ and $Brn2^+$; Figures 1B–1D and S1A). Meanwhile, the number of deeper-layer neurons was not substantially altered (i.e., $Ctip2^+$; cell density: CON 43.7 ± 0.6; XAV 42.1 ± 0.5/100 × 100 μm area at E18; $p = 0.47$; Figure S1B). In addition, XAV939 administration did not drastically alter the generation of interneurons, as shown by the similar densities of EdU^+ cells in the ganglionic eminence (CON 58.3 ± 0.3; XAV 57.1 ± 0.2/100 × 100 μm area; $p = 0.31$; Figures S1C and S1D) and $GABA^+$ cells in the neocortex (Figures S1E–S1G); this is likely because endogenous Axin expression is very low in the neural progenitors of ventral proliferative zones (Fang et al., 2013) where interneurons are generated, thus excluding them as a target of XAV939 treatment. Furthermore, there was no obvious change in the density of $GFAP^+$ astrocytes or $Iba1^+$ microglia in XAV939-treated neocortices (Figures S1H–S1J), suggesting that astrogenesis and microglial activation were unaffected after XAV939 administration. These results collectively indicate that a single in utero injection of XAV939 at E14.5 predominantly leads to an overproduction of excitatory neurons in layer 2/3.

During normal development, neocortical neurons are initially overproduced and subsequently undergo apoptosis, reducing their number throughout the neonatal stage (Buss et al., 2006). Consistently, the experimentally induced overproduction of neurons by XAV939 treatment was accompanied by a moderate increase in cell apoptosis within layer 2/3 (Figures S1K and S1L). Nonetheless, the density of layer 2/3 excitatory neurons remained significantly elevated and the neocortex exhibited a slight lateral expansion by postnatal day (P) 4 after XAV939 injection (Figures 1E–1H), which was maintained until adulthood (Figures 1I–1L). Furthermore, whereas the XAV939-treated adult mouse brains were larger and heavier than the controls, they exhibited normal gross architecture (Figures 1M–1O). In addition, brain regions other than the neocortex were unaffected; for instance, there were no gross changes in the neuronal numbers or densities in XAV939-treated hippocampus (Figures S1M and S1N) or striatum (data not shown). Moreover, XAV939 treatment did not affect the survival, fertility, or body weight (Figure 1P) of the mice. These results indicate that this experimental mouse model exhibits an enlarged neocortex with excessive excitatory neurons in layer 2/3.

Excitatory Neuron Overproduction Affects Dendritic Spine Development and Enrichment of Neighboring Interneurons

To elucidate the functional consequences of excessive excitatory neurons, we examined the development of layer 2/3 neurons in XAV939-treated mice. As the vast majority of layer 2/3 excitatory neurons are callosal projection neurons, we first traced their axons with GFP-expressing adeno-associated virus (AAV-GFP) (Figures 2A and S2A) or fluorescent tracer (micro-Ruby; Figure 2B). The fluorescently labeled axonal tracts crossed the corpus callosum and innervated into the contralateral cortical plate, indicating these neurons had grossly normal axonal projections (Figures 2A, 2B, and S2A). Layer 2/3 excitatory neurons are pyramidal neurons with small and medium somata that extend one apical and several basal dendrites. Infecting layer 2/3 neurons with low-titer AAV-GFP at P1 to P2 (Figures 2C and S2B) allowed us to trace the morphology of individual neurons. Most GFP^+ neurons were pyramidal neurons (Figure 2C) with similar soma size (Figure S2B; CON: 107.7 ± 3.3 μm^2; XAV: 111.7 ± 4.6 μm^2; $p = 0.47$). Whereas apical dendritic branching was not drastically affected, the arborization of basal dendrites was significantly reduced in the layer 2/3 excitatory neurons of young (P18) and older (P60) XAV939-treated mice (Figures 2D–2F, S2C, and S2D). This phenotype is likely attributable to the "dendritic tiling" effect wherein dendrites from neighboring excitatory neurons arrange their branches in a pattern with minimal overlapping (Parrish et al., 2007). Thus, a higher local density may prompt excitatory neurons to reduce their dendritic branching for achieving nonredundant innervation. Despite their simplified dendritic arbors, the dendritic

Figure 1. Overproduction of Layer 2/3 Excitatory Neurons Results in Mouse Brains with Excess Neurons and an Enlarged Neocortex

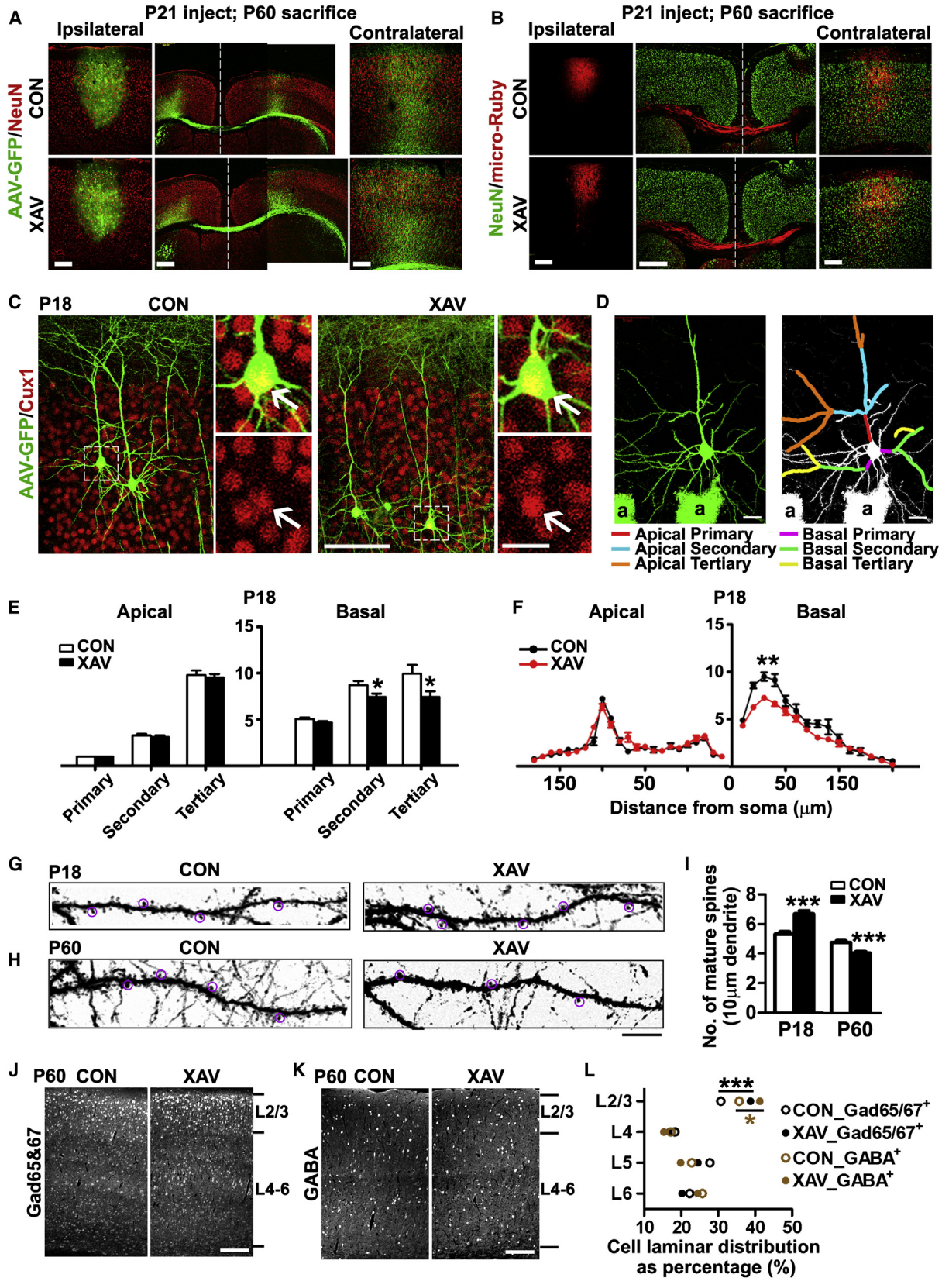
(A–D) XAV939 injection (XAV) enlarged the embryonic mouse neocortex (A) and overproduced layer 2/3 excitatory neurons (B and C). White lines illustrate pial and apical surfaces (A). L2/3, layer 2/3.

(E–H) Overproduction of excitatory neurons resulted in excess excitatory neurons in the neonatal cortex. XAV939-treated mouse neocortices exhibited lateral expansion of the cortical surface (E) and increased density of layer 2/3 excitatory neurons (F and G). (H) Most EdU^+ cells in layer 2/3 were $Cux1^+$ or $Brn2^+$ excitatory pyramidal neurons (arrows in inset images of F).

(I–L) XAV939-injected mice exhibited a higher density of layer 2/3 excitatory neurons in the neocortex at P60.

(M–P) XAV939-injected mature mice had greater brain size and weight, with comparable body weight.

The scale bars represent 500 μm (A, E, and I), 100 μm (B, F, and J), and 5 mm (M). Average cell densities in (C), (G), and (K) are expressed as the average numbers of cells in a 100 × 100 μm area of layer 2/3. Error bars indicate SEM; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ versus control (CON); Student's t test. See also Figure S1.



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spines, in particular, the mushroom-shaped mature spines, had higher densities at P18 (Figures 2G and 2I); this indicates increased excitatory synaptic connections, which is concordant with the higher density of local excitatory neurons within layer 2/3 (Figure 1). Interestingly, the spine density of individual layer 2/3 pyramidal neurons in XAV939-treated brains was reduced to a greater extent than that in the control brains at P60 (Figures 2H and 2I), suggesting that an excess of local excitatory neurons may eventually disrupt spine stability.

In addition to the morphological changes in excitatory neurons, the laminar distribution of interneurons (i.e., Gad65$^+$/Gad67$^+$ or GABA$^+$; Figures 2J and 2K) was altered, with a greater proportion of interneurons in layer 2/3 of XAV939-injected cortices in young (i.e., P21; data not shown) and older mice (i.e., P60; Figure 2L). This effect cannot be attributable to the increased production or disturbed migration of interneurons, because the total numbers of interneurons in the cortical plate did not differ significantly between the XAV939-injected and control groups (Gad65$^+$/Gad67$^+$: CON: 75 ± 5; XAV: 71 ± 6/100 μm wide section; $p = 0.73$; GABA$^+$: CON: 25 ± 3; XAV: 23 ± 2/100 μm wide section; $p = 0.26$) nor did the densities of interneurons in each cortical layer (Figure S2E). Instead, the slight thickening of layer 2/3 (Figure S2G) in the XAV939-injected cortices may account for the higher proportion of interneurons in this layer. Furthermore, whereas the densities of major interneuron subtypes (i.e., calretinin$^+$, parvalbumin$^+$, and somatostatin$^+$; Figures S2H–S2J) were similar in XAV939-injected and control cortices (Figure S2K), the number and proportion of calretinin$^+$ interneurons in layer 2/3 were greater in the XAV939-treated neocortices (Figures S2L and S2M), probably owing to the thicker layer 2/3, where most calretinin$^+$ interneurons reside. These findings collectively suggest that the excess of excitatory neurons in layer 2/3 perturbs dendrite and spine development and alters the laminar distribution of interneurons.

Excitatory Neuron Overproduction Shifts the Balance between Excitatory and Inhibitory Synapses

The increased number of excitatory neurons in layer 2/3 may affect the assembly of local excitatory and inhibitory circuitry, resulting in altered balance between cortical excitation and inhibition. To evaluate this possibility, we examined the synaptic density and strength in layer 2/3. Excitatory and inhibitory synapses can be classified according to their morphology as either asymmetric or symmetric synapses, respectively (Figures 3A and S3A). Consistent with the age-dependent decrease in the number of spines (Yuste and Bonhoeffer, 2004) where excitatory synapses reside, there were fewer asymmetric synapses in layer 2/3 in the control cortices at P60 than at P18 (Figure 3B). In concordance with the fact that spine density was reduced to a greater extent in older XAV939-injected brains (Figure 2I), the reduction of asymmetric synapses in the XAV939-injected brains was more drastic than that in the control brains upon aging (Figure 3B). Interestingly, whereas the density of symmetric synapses in the control brains was reduced at P60 compared to that at P18, similar decrease was not observed in the XAV939-injected brains (Figure 3C). This effect is probably due to the increased number of interneurons in layer2/layer 3 of XAV939-injected cortices (Figures 2J–2L), which may enhance the local release of GABA and thus promote the formation and maturation of inhibitory synapses (Huang and Scheiffele, 2008). Consequently, the ratio of excitatory to inhibitory synapses in layer 2/3 of the XAV939-treated neocortices increased at P18 but decreased at P60 (Figure 3D). These changes in synapse number and ratio are most likely attributable to the specific enrichment of excitatory and inhibitory neurons in layer 2/3, because similar effects were not observed in deeper cortical layers such as layer 5 (Figures S3B–S3E).

As altered synapse number and ratio may affect synaptic strength and excitatory/inhibitory balance, we measured the spontaneous excitatory and inhibitory postsynaptic currents (sEPSCs and sIPSCs, respectively) of individual layer 2/3 pyramidal neurons using whole-cell recordings (Figures 3E–3L). Whereas the amplitudes remained unchanged (Figures 3H and 3L), the frequencies of sEPSCs and sIPSCs increased in the layer 2/3 excitatory neurons of the XAV939-injected neocortices (Figures 3F, 3G, 3J, and 3K), concordant with the higher densities of excitatory and inhibitory neurons as well as synaptic connections within layer 2/3 (Figures 1G, 2L, 3B, and 3C). Synaptic strength was further assessed by examining the basal-evoked synaptic activities of neuronal ensembles in layer 2/3. To do so, the input/output curves of evoked field excitatory postsynaptic potentials (fEPSPs) were measured in layer 2/3 in response to the stimulation at layer 4 (Yashiro et al., 2009). Consistent with the differential alteration in the numbers and ratios of synapses (Figures 3B–3D), the evoked fEPSPs were augmented in young (P18) but diminished in older (P60) XAV939-injected neocortices (Figures 3M–3P, S3F, and S3G). Thus, overproduction of excitatory neurons in layer 2/3 dysregulates synaptic connections and may impair the balance between synaptic excitation and inhibition.

Figure 2. Excess Excitatory Neurons Disturb the Dendrite and Spine Development of Excitatory Neurons and Alter Interneuron Distribution

(A and B) The callosal axonal projections of XAV939-injected neocortex were grossly normal.

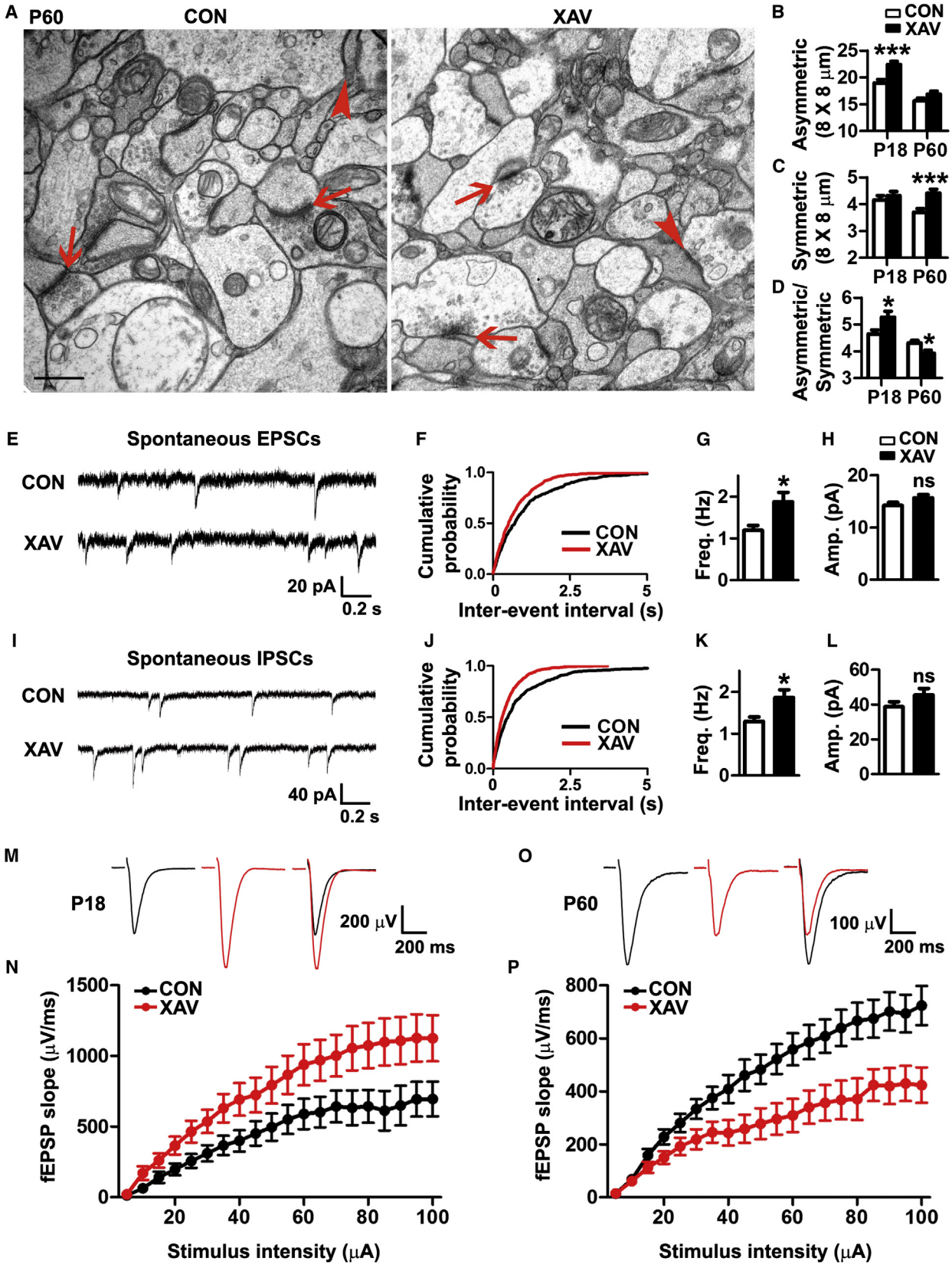
(C) Most GFP$^+$ neurons were typical layer 2/3 excitatory pyramidal neurons. Arrows indicate GFP and Cux1 costaining.

(D–F) Basal dendrite arborization was simplified in layer 2/3 GFP$^+$ neurons in the XAV939-injected neocortices. (D) Representative image of an individual layer 2/3 excitatory pyramidal neuron. The "skeleton outline" of dendritic branches is shown. Some astrocytes were also labeled by GFP (indicated by "a"). (E) Numbers of secondary and tertiary, but not primary, branches were reduced. (F) Sholl analysis revealed a significant decrease in basal dendritic complexity.

(G–I) The density of mature spines along the basal dendrites was increased and decreased in the layer 2/3 pyramidal neurons of young and older XAV939-injected cortices, respectively. Note that only the mushroom-shaped spines were counted (I); examples are indicated by magenta circles (G and H).

(J–L) The overpopulation of layer 2/3 excitatory neurons increased the proportion of interneurons in layer 2/3. (L) The proportions of Gad65$^+$/Gad67$^+$ and GABA$^+$ interneurons at each layer over their total numbers in the whole cortical plate.

The scale bars represent 500 μm (low-magnification images in A), 200 μm (high-magnification images in A, B, J, and K), 100 μm (low-magnification images in C), 20 μm (high-magnification images in C and D), and 10 μm (G and H). Error bars indicate SEM; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ versus control (CON); Student's t test. See also Figure S2.



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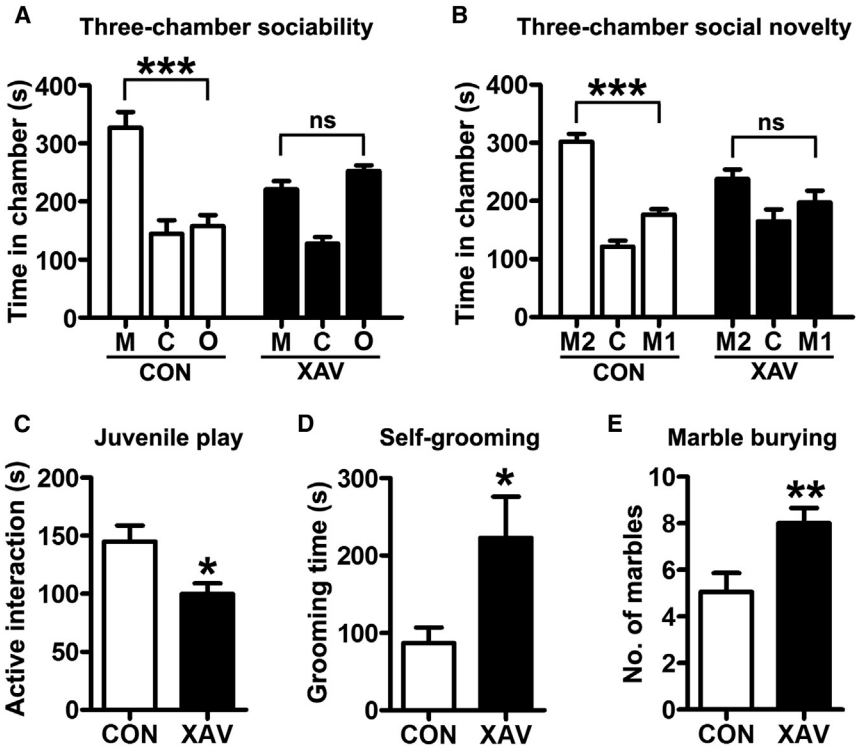

Figure 4. Excess Excitatory Neurons Are Associated with Autism-like Behaviors

(A and B) Three-chamber tests for sociability and social novelty. XAV939-injected mice did not exhibit a preference for unfamiliar mice over novel objects (A) or over familiar mice (B) in contrast to control mice. C, center chamber; M, mouse-housing chamber; M1, chamber with familiar mouse; M2, chamber with unfamiliar mouse; O, object-occupied chamber. ns, not significant.

(C) Reciprocal social interaction test. Juvenile XAV939-injected mice spent less time actively interacting with unfamiliar mice.

(D and E) XAV939-injected mice exhibited repetitive behaviors. They groomed more than the controls in the self-grooming test (D) and buried more marbles in the marble-burying test (E).

Error bars indicate SEM; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ versus control (CON); Student's t test (C–E). One-way ANOVA with a Bonferroni post hoc t test (A and B).

Mice with Excess Excitatory Neurons in the Neocortex Exhibit Autism-like Behaviors

Whereas autistic patients exhibit an excess of neocortical neurons, no functional link has been identified. We reasoned that the XAV939-injected mice described herein would be a good model for evaluating how neuronal overproduction may contribute to autism. As autism is diagnosed on the basis of behavioral characteristics, we assessed the social behaviors and repetition tendencies of these mice (Silverman et al., 2010). In the three-chamber social interaction test, the control mice exhibited a social preference for another mouse (M; Figure 4A) over an inanimate object (O; Figure 4A), spending more time in the chamber that contained a caged and age-matched normal unfamiliar mouse (Figure 4A). Furthermore, control mice interacted longer with unfamiliar mice (M2; Figure 4B) than familiar mice (M1; Figure 4B). However, the XAV939-injected mice did not exhibit these preferences (Figures 4A and 4B). In addition, during the juvenile play test, the XAV939-injected mice spent less time engaged in active interactions (i.e., sniffing, allogrooming, chasing, and playing) with a freely moving unfamiliar mouse (Figure 4C), indicating deficits in social interaction. In addition to impaired social interaction, the XAV939-injected mice exhibited more repetitive behaviors. In the self-grooming test, XAV939-injected mice groomed much more than the control mice (Figure 4D). During the marble-burying test, they exhibited repetitive digging behavior (Figure 4E). The XAV939-injected mice spent a comparable amount of time as the control mice in the center of the arena in the open field test (Figure S4A), did not exhibit a longer latency to begin feeding in the novelty-suppressed feeding test (Figure S4B), and did not show a differential preference for sucrose in the sucrose preference test (Figure S4C). These results collectively indicate that the impaired social interaction and repetitive behaviors of the XAV939-injected mice are unlikely due to increased anxiety or depression. Moreover, the motor function, olfactory function, and overall well-being of these mice did not differ from those of the control mice as evidenced by the moving distance in the open field test (Figure S4D), latency to find food in the buried food test (Figure S4E), and nest-building capability test (Figure S4F), respectively. In summary, the mice with excess excitatory neurons in the neocortical layer 2/3 exhibited autism-like behaviors.

Figure 3. Overproduction of Layer 2/3 Excitatory Neurons Increases Synaptic Connections within Layer 2/3 and Leads to Excitatory/Inhibitory Synaptic Imbalance

(A–D) Overproduction of layer 2/3 excitatory neurons increased synapse number and altered the ratio of excitatory to inhibitory synapses within layer 2/3. (A) Representative electron micrographs of layer 2/3 (arrows, asymmetric/excitatory synapses; arrowheads, symmetric/inhibitory synapses). (B–D) Average number and ratio of asymmetric and symmetric synapses in each fixed-size area (8 × 8 μm).

(E–L) Overproduction of excitatory neurons increased both excitatory and inhibitory synaptic transmission of layer 2/3 pyramidal neurons in young (P18) acute brain slices. The frequencies (G and K), but not amplitudes (H and L), of spontaneous EPSCs and IPSCs increased in the XAV939-injected mouse neocortices.

(M–P) Overproduction of excitatory neurons augmented and suppressed cortical excitation in young and adult mouse cortices, respectively. Representative waveforms (M and O) and input/output curves of the slope fEPSP versus stimulation intensity (N and P).

The scale bars represent 500 nm. Error bars indicate SEM; ***$p < 0.001$; *$p < 0.05$ versus control (CON); Student's t test. See also Figure S3.

DISCUSSION

In the present study, we established a functional link between unrestrained embryonic neurogenesis and autism by generating and characterizing a mouse model involving the overproduction of excitatory neurons in neocortical layer 2/3. The excess of excitatory neurons impaired dendrite and spine development in layer 2/3 excitatory neurons and affected the laminar distribution of interneurons. Dysregulation of neuronal development consequently altered excitatory and inhibitory synaptic connections and strength, causing an imbalance between synaptic excitation and inhibition. Importantly, these mice exhibited behavioral defects resembling those of human autism. Thus, the present findings advance the current understanding of how embryonic neurogenesis shapes the functional neocortex and provide developmental insights into the etiology of autism.

Autism is primarily considered a neurodevelopmental disorder characterized by aberrant synaptic function (Flavell and Greenberg, 2008) and imbalanced cortical excitation and inhibition (Rubenstein, 2010). Its underlying neurobiological basis includes impairments in neuronal migration, axonal connections, dendritic arborization, synaptic formation, and plasticity of excitatory neurons, as well as abnormal number, distribution, and function of interneurons. Whereas disturbances of these distinct developmental processes may arise independently from various molecular and cellular mechanisms, it is possible that an ''upstream'' process or defect initiates this spectrum of abnormalities. The present results show that neuronal overproduction in layer 2/3 precedes and probably leads to various histological and electrophysiological abnormalities including reduced dendrite arborization, altered synapse number, and synaptic excitation and inhibition imbalance, which are all characteristics of autistic brains. These findings confirm the speculation that layer 2/3 excitatory neurons underpin high-level cognitive functions and are associated with autism (Fame et al., 2011). More importantly, these results not only corroborate previous findings indicating that autism is associated with excess neurons (Casanova et al., 2006; Courchesne et al., 2011; McCaffery and Deutsch, 2005) but also provide experimental evidence that excessive embryonic neurogenesis may contribute to the etiology of autism.

Characterization of the mice with excess neurons in the present study advances our understanding of the association of enhanced embryonic neurogenesis with key anatomical and electrophysiological features of autism. Nonetheless, it remains unclear whether and how these phenotypic abnormalities actually result in behavioral deficits. In addition, interpreting these findings involves some caveats. First, even though the numbers of deep-layer neurons were not dramatically altered, their significance in the etiology of autism should not be underestimated. Second, whereas a global increase of neuronal number in the neocortex leads to autism-like phenotypes, this should not be interpreted to mean that different neocortical regions contribute equally to autism or that brain regions besides the neocortex do not play crucial roles in the etiology of autism. Third, although we have previously characterized how XAV939 injection enhances embryonic neurogenesis (Fang et al., 2013), the possibility that XAV939 causes autistic phenotypes via other cellular processes, e.g., immune challenge or epigenetic dysregulation, which lead to similar behavioral phenotypes, cannot be excluded (Carpentier et al., 2013; Gogolla et al., 2009). Fourth, whereas the cellular and molecular mechanisms of synaptic changes following neuronal overproduction await further investigation, these changes may be the combined consequences of multiple intrinsic and extrinsic factors including competition for trophic support (e.g., neurotrophic factors, afferent inputs, and glial cells) and altered neuronal activity. In conclusion, whereas the specific causes of autism remain to be elucidated, the present study indicates that neuronal overpopulation may be partially responsible for the disease etiology.

EXPERIMENTAL PROCEDURES

Procedures regarding chemicals, reagents, viruses, antibodies, mice, morphological studies, immunohistochemical analyses, electrophysiological experiments, behavioral tests, image acquisition, and quantitative analyses were performed as described previously (Fang et al., 2011) and can be found in the Supplemental Experimental Procedures.

In Utero Intraventricular Microinjection

Timed-pregnant imprinting control region mice at E14.5 were anesthetized with pentobarbital (5 $mg \cdot ml^{-1}$), and the embryos received a one-pulse microinjection of 0.4 μl XAV939 (10 μM; dissolved in DMSO) in the developing lateral ventricle (Fang et al., 2013). Embryos that received DMSO injection were used as control groups. To calculate the number and proportion of neurons generated after XAV939 injection, the pregnant mice were further intraperitoneally injected with one pulse of EdU (5-ethynyl-2′-deoxyuridine; 30 $mg \cdot kg^{-1}$) or BrdU (5-bromo-2′-deoxyuridine; 50 $mg \cdot kg^{-1}$) 12 hr after XAV939 injection. The injected brains were harvested at E18, P4, P14–P21, or P60, intracardially perfused with 4% paraformaldehyde, and subjected to immunostaining or detection of EdU/BrdU. At least five brains from three independent experiments were analyzed for each condition. The mice used in morphological, electrophysiological, and behavioral studies were not injected with EdU/BrdU to eliminate the potential side effects of EdU/BrdU in neuronal development and activity. Only male mice were used for electrophysiological and behavioral studies. All animal procedures were conducted in accordance with the Guidelines of the Animal Care Facility of The Hong Kong University of Science and Technology (HKUST) and were approved by the Animal Ethics Committee at HKUST.

Statistical Analysis

Comparisons between groups were made with a Student's t test, and associations among variables were analyzed by one-way ANOVA. All analyses were performed with GraphPad Prism (GraphPad Software). All data in bar graphs represent mean ± SEM. The level of significance was set at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.11.003.

AUTHOR CONTRIBUTIONS

N.Y.I. supervised the project; W.-Q.F. conceived the project and designed research; W.-Q.F. and W.-W.C. performed most of the experiments; K.L., L.J., and W.-H.Y. performed the axonal tracing, electron microscopy, and electrophysiology experiments, respectively; W.-Q.F., W.-W.C., A.K.Y.F., and N.Y.I. analyzed the data; and W.-Q.F., A.K.Y.F., and N.Y.I. wrote the manuscript.

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