LHX2 regulates dendritic morphogenesis in layer II/III of the neocortex via distinct pathways in progenitors and postmitotic neurons.

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Abstract

In the mammalian neocortex, excitatory neurons that send projections via the corpus callosum are critical to integrating information across the two brain hemispheres. The molecular mechanisms governing the development of the dendritic arbours and spines of these callosal neurons are poorly understood, yet these features are critical to their physiological properties. LIM Homeodomain 2 (*Lhx2*), a regulator of fundamental processes in cortical development, is expressed in postmitotic callosal neurons occupying layer II/III of the neocortex and also in their progenitors in the embryonic day (E) 15.5 ventricular zone of the mouse neocortex. We tested whether this factor is essential for dendritic arbour configuration and spine morphogenesis of layer II/III neurons. Here, we report loss of *Lhx2* either in postmitotic callosal neurons or their progenitors, resulting in shrunken dendritic arbours and perturbed spine morphology. In postmitotic neurons, we identified that LHX2 regulates dendritic and spine morphogenesis via the canonical Wnt/β Catenin signalling pathway. Constitutive activation of this pathway in postmitotic neurons recapitulates the *Lhx2* loss-of-function phenotype. In E15.5 progenitors, we identified that bHLH transcription factor *Neurog2* mediates LHX2 function in regulating dendritic and spine morphogenesis. We show that *Neurog2* expression increases upon loss of *Lhx2* and that shRNA-mediated *Neurog2* knockdown rescues the loss of *Lhx2* phenotype. Our study uncovers novel LHX2 functions in cortical circuit assembly consistent with its temporally dynamic and multifunctional roles in development.

Introduction

The functional characteristics of a neuron are critically dependent on its dendritic arborisation and complexity (*1*). Disruptions of dendritic morphogenesis underlie neurodevelopmental disorders, including Autism Spectrum Disorder and schizophrenia (*2*, *3*). In the mammalian cerebral cortex, superficial pyramidal layer neurons, which occupy layers II/III, broadly extend their apical dendrites into layer I and basal dendrites within their layer. They also establish connections with the contralateral hemisphere via the corpus callosum. These neurons are born late in cortical neurogenesis, with their production peaking at embryonic day (E) 15.5 in the mouse (*4*).

Dendritic morphogenesis is regulated by multiple factors, including transcriptional regulators, guidance cues, cell adhesion molecules, cytoskeletal elements, and neuronal activity (*5*, *6*). Among these, transcription factors are known to be the drivers of downstream signalling pathways and gene expression responsible for dendritic development (*7*). In the developing mammalian cerebral cortex, transcription factors like *Cux1*, *Cux2*, *Zfp312*, and *Foxg1* are known to promote dendritic arborisation in specific neuronal subtypes, and the loss of any of these genes leads to reduced complexity (*8*–*12*). On the other hand, factors like *Sox11, Tbr1* and *Neurog2* have been shown to suppress excessive branching, such that the loss of these factors leads to increased complexity (*13*–*15*).

Despite the established knowledge regarding the involvement of various transcription factors in regulating dendritogenesis, the precise stage of differentiation and maturation at which their role is relevant remains to be determined. Some factors, *e.g. Zfp312, Sox11*, *Tbr1,* are known to regulate morphometry exclusively in specific subtypes of postmitotic neurons (*11*, *14*, *15*), while others, such as *Foxg1,* display a broader expression pattern encompassing different progenitors and neuronal classes (*12*, *16*). This complexity prompts an additional question: At which stage of neuronal maturation is dendritic morphometry determined? Is the modulation of dendritic architecture solely a postmitotic event, or does a mechanism for regulating the dendritic structure of a neuron exist in its progenitor? The temporal dynamics of transcription factor engagement in regulating dendritogenesis during the different stages of development, *i.e.*, progenitor and postmitotic, remain poorly explored.

LHX2 is a pleiotropic transcription factor implicated in multiple facets of cortical development, including cortical patterning (*17–21*), progenitor proliferation (*22–24*), cell fate decisions (*25*) and neuron subtype specification (*26*, *27*). Its expression in the cortical primordium is seen as early as E8.5 and is an established regulator of distinct stages of cortical development (*28*). LHX2 function in early cortical progenitors is critical for properly developing the thalamocortical tract and barrel formation (*24*, *27*) and producing the correct numbers of layer VI versus layer V neurons (*26*, *29*). In postmitotic layer IV cortical neurons, *Lhx2* is essential for the activity-dependent orientation of dendrites towards the barrel fields (*30*). A broad cortex-specific loss of *Lhx2* results in a lack of the corpus callosum and hippocampal commissure (*31*). These distinct functions in cortical progenitors and postmitotic neurons motivated the question of whether this factor may regulate a particular process, that of dendritic arborisation and spine maturation, at multiple stages of neuronal differentiation and whether it utilises independent pathways to do so at each stage.

Here, we show that loss of *Lhx2,* either in cortical progenitors or specifically in postmitotic neurons, causes profound alterations of the dendritic and spine morphology of layer II/III neurons while sparing corpus callosum crossing. We demonstrate that the progenitor-specific role is recapitulated by loss of *Lhx2* restricted to basal progenitors. We identify downstream target NEUROG2 as the effector of LHX2 function by utilising shRNA approaches to show that simultaneous knockdown of *Neurog2* can rescue the dendritic arborisation phenotype resulting from loss of *Lhx2.* In postmitotic neurons, we report that LHX2 suppresses the Wnt/β-catenin signalling cascade. We demonstrate that independent stabilisation of β-Catenin in postmitotic neurons recapitulates the effect of loss of *Lhx2* on dendritic arborisation and spine morphology.

In summary, our findings reveal novel insights into the distinct cell-stage-specific roles of transcription factor LHX2 in orchestrating diverse gene networks that govern the dendritic and spine morphogenesis process.

Results

Layer II/III cortical neuronal birth peaks at embryonic day (E) 15.5 in mice (*4*). LHX2 protein is detected in the cortical progenitors at this stage and persists in postmitotic layer II/III neurons (Figure 1A, B). This observation motivated us to compare the effects of loss of *Lhx2,* either in E15.5 progenitors or in newborn postmitotic neurons, on the dendritic morphogenesis of mature layer II/III neurons. All dendritic arbour and spine morphology measurements were performed on postnatal day (P) 30 in the somatosensory cortex layer II/III. To label the dendritic arbours, we used *in utero* electroporation (IUE) to introduce a plasmid expressing membrane-bound EGFP (CAAX-EGFP) in E15.5 progenitors so that the neurons arising from these cells were labelled in perpetuity (Figure 1C, C'). We used an *Lhx2* $^{lox/lox}$ mouse line to effect conditional loss of *Lhx2*. Postmitotic neuron-specific disruption of *Lhx2* was effected by crossing this line to a line expressing *Neurod6-Cre (Nex-Cre; (32))*. IUE of CAAX-EGFP at E15.5 produced EGFP-labelled layer II/III neurons, which then experienced *Lhx2* gene disruption upon becoming postmitotic due to the action of *NexCre*.

To disrupt *Lhx2* in progenitors, we co-electroporated a plasmid encoding Cre-TdTomato with CAAX-EGFP at E15.5 (Supplementary Figure S1G–I). As controls, TdTomato (lacking Cre) was used.

Disrupting *Lhx2* in E15.5 progenitors and newly post-mitotic neurons causes dendritic and spine morphology defects in P30 layer II/III neurons.

To analyse the fully developed dendritic arborisation of layer II/III neurons, we examined CAAX-EGFP-labelled cells at P30 when dendritic morphogenesis is complete. Control neurons displayed a typical layer II/III pyramidal morphology (Figure 1C', D).

In contrast, both progenitor-specific (Pg) (Figure 1D') and postmitotic (Pm) (Figure 1D'') displayed shrunken dendritic arbours upon loss of *Lhx2*. The total length of apical dendrites was significantly reduced in Pg mutants. The mean and total length of the basal dendrites exhibited a severe reduction in both Pg and Pm perturbations (Figure 1E–G). Sholl analysis of apical and basal dendrites reflected decreased branching complexity in both Pg and Pm mutant neurons. This decrease in complexity of apical dendrites in Pm mutants was intermediate to that of Pg mutants (Figure 1H, I).

We tested whether loss of *Lhx2* in progenitors affects the subtype identity of the mutant layer II/III neurons since we had previously reported a switch in subtype identity from layer VI to layer V when *Lhx2* was disrupted in deep layer progenitors (*26*). However, preliminary analysis showed that Pg mutant neurons expressed the upper layer marker POU3F2 similar to controls (Supplementary Figure S1A–F) and displayed a characteristic pyramidal morphology (Supplementary Figure S1G–I), suggesting that neuronal subtype identity is broadly preserved.

These results collectively suggest that *Lhx2* plays a crucial role in regulating the dendritic morphogenesis of both apical and basal dendrites in layer II/III neurons, operating in progenitors and postmitotic neurons.

Disrupting *Lhx2* in E15.5 progenitors and postmitotic neurons causes spine development defects in layer II/III neurons.

Dendritic spines serve as the functional units of dendrites and are the sites of synaptic transduction (*33*, *34*). Understandably, the molecular factors influencing dendritic development overlap with those involved in spine development (*34–36*). We investigated whether the loss of *Lhx2* in either progenitor or postmitotic neurons affects the formation of spines in P30 layer II/III neuronal dendrites. Control, Pg, and Pm mutant neurons were analysed for spine morphology in apical and basal dendrites. Spines were categorised into filopodial, thin, stubby, and mushroom types, which correlates with the overall maturation stage of the spines (*34*). We observed a significant increase in the percentage of thin spines and a corresponding decrease in mushroom spines in the apical and basal dendrites of both Pg and Pm mutants. Stubby spines were significantly fewer in Pm but not Pg mutant apical dendrites (Figure 1J–S). These findings indicate a role for LHX2 in the maturation of spines to the mushroom morphology during neurodevelopment.

Corpus Callosum crossing is unaffected upon loss of *Lhx2*.

Axons originating from neurons in layer II/III of the neocortex play a crucial role in corpus callosum formation (*37*, *38*). Previous studies reported that loss of *Lhx2* driven by *Emx1*-Cre or *Nestin*-Cre, which act in cortical progenitors from E11.5, results in corpus callosum agenesis, although inactivation using *Nex-Cre* did not (*31*). We sought to investigate whether the impact of *Lhx2* on

corpus callosum development is cell-autonomous by electroporating Cre-TdTomato in the progenitors of *Lhx2*$^{lox/lox}$*; Ai9/*+ (mutant) and *Ai9/*+ (Control) mice at E15.5. The corpus callosum was examined at P7 and P30.

In contrast to the striking effect on Layer II/III dendrites, the axons of these neurons did not appear to be significantly affected in the mutants. The corpus callosum traversed the midline (Figure 1R, S), reached the contralateral hemisphere, and developed arbours at layer V and layer II/III by P30 (Figure 1T). This finding conclusively shows that the corpus callosum agenesis reported using *Emx1*-Cre or *Nestin*-Cre (*31*) is not due to a cell-autonomous role in the neurons of layer II/III and motivates exploration of additional roles for *Lhx2* in midline guidance mechanisms as suggested in (*31*).

In summary, although LHX2 is a pleiotropic factor, its function in the maturation of layer II/III neurons is confined to their dendritic processes and spines, not their axons. In the following sections, we examined the molecular mechanisms by which LHX2 regulates these processes in postmitotic layer II/III neurons and their progenitors.

Loss of *Lhx2* increases the firing rate and excitability of layer II/III neurons.

We sought to examine the consequences of the morphometric deficits resulting from losing *Lhx2* on the electrophysiological properties of neocortical layer II/III pyramidal neurons. We focused on the Pm *Lhx2* mutant neurons, as the overall dendritic and spine morphogenesis deficits were similar in Pg and Pm mutants. We measured several sub- and supra-threshold intrinsic physiological properties from brain slices of control and Pm *Lhx2* mutant mice using patch-clamp electrophysiology (Figure 2, Supplementary Figure S2–S3). Motivated by dendritic differences in Pm *Lhx2* mutant neurons (Figure 2) and by the established impact of dendritic arborisation on neuronal excitability (*39*, *40*), we measured input resistance, a subthreshold measure of neuronal gain from control and Pm mutant neurons. We found control and Pm *Lhx2* neurons to manifest comparable input resistance values (Figure 2A, B). Several physiological measurements of sub-threshold excitability were consistently comparable between control and Pm *Lhx2* neurons (Supplementary Figure S2).

In striking contrast, we found neurons in Pm *Lhx2* mice to manifest significantly higher supra-threshold excitability than in control mice. Specifically, we found Pm *Lhx2* neurons to manifest higher firing frequencies (Figure 2C, D, Supplementary Figure S3A), a switch to depolarisation-induced block with low current injections (Figure 2C, D), significant reductions in both the latency to first spike (Figure 2E–F) and the first inter-spike interval (Figure 2G, H) and a significant increase in the peak dV/dt associated with the action potentials (Figure 2I, J). Together, differences in supra-threshold measurements between the control and Pm mutant groups (Figure 2C–J, Supplementary Figure S3A) demonstrated a striking increase in supra-threshold excitability upon loss of *Lhx2*.

LHX2 regulates dendritic development in postmitotic neurons by suppressing WNT/ β Catenin signalling cascade genes.

Since the postmitotic loss of *Lhx2* was driven by *NexCre,* which acts in the entire postmitotic cortical plate, we tested whether the role of LHX2 is cell autonomous using prepared primary dissociated cultures. Embryos from control and *NexCre/+; Lhx2*$^{lox/lox}$ embryos were first electroporated *ex vivo* with CAAX-EGFP at E15.5 for labelling purposes and then dissociated and maintained *in vitro* for 15 days. We observed a significant decrease in the total length of both apical and basal dendrites, similar to the phenotype *in vivo* (Figure 3A–D), suggesting that the function of LHX2 in regulating dendritic development is cell-autonomous.

To gain insights into the mechanistic role of LHX2 in postmitotic neurons, we isolated control and Pm mutant neurons at P5, when the dendritic arbours are maturing, and performed RNA-Seq. As before, the neurons were labelled by *in utero* electroporation of EGFP in *NexCre/+, Lhx2*$^{lox/lox}$ brains and littermate controls at E15.5. RNA-Seq analysis revealed that several differentially expressed genes were associated with the Wnt signalling pathway, such as *Dkk3, Rspo2, Sfrp1*, and *Wnt5b* (Figure 3E). This was further confirmed in the Gene Ontology analysis of differentially expressed genes (Figure 3F). A typical readout of active canonical Wnt signalling is the stabilisation of cytoplasmic β-Catenin and its translocation to the nucleus (*41*). We validated this by immunostaining for β-CATENIN and discovered a significant increase in its nuclear localisation in layer II/III Pm mutant neurons (Figure 3G, H). These findings suggested that postmitotic loss of *Lhx2* enhances canonical Wnt signalling in layer II/III neurons.

To ascertain whether upregulation of canonical Wnt signalling can regulate dendritic morphogenesis, we examined whether directly stabilising β-catenin can recapitulate the dendritic defects observed upon loss of *Lhx2*.

We used a *Ctnnb1* (β-CATENIN) conditional Gain-of-Function (GOF) mouse line in which exon 3 is floxed. Loss of this exon prevents β-CATENIN from being phosphorylated and tagged for degradation, thus resulting in a constitutively stabilised β-CATENIN protein (*42*). To effect loss of *Ctnnb1* specifically in postmitotic neurons, we electroporated a plasmid encoding Doublecortin (*Dcx*)-driven Cre recombinase together with CAAX-EGFP for labelling purposes. We analysed the outcomes at P30, focusing on EGFP-labelled neurons born at E15.5 from *Dcx*-Cre+CAAX-EGFP electroporated progenitors, which would be expected to display β-catenin GOF upon becoming postmitotic.

Sholl analysis of the apical dendrites of the *Ctnnb1* GOF neurons showed a striking recapitulation of the apical and basal dendritic defects seen in postmitotic *Lhx2* mutant condition (Figure 3I–I'', J, K). *Ctnnb1* GOF neurons also recapitulated the spine defects observed in postmitotic *Lhx2* mutant neurons, *i.e.* an increase in the proportion of thin morphology spines and a concomitant decrease in mushroom morphology spines in both apical and basal dendrites (Figure 3L–U). In summary, these findings indicate that LHX2 suppresses Wnt/β Catenin signalling in postmitotic layer II/III neurons and that this may be the critical pathway via which LHX2 mediates the regulation of dendritic and spine morphogenesis in these cells.

Next, we tested whether LHX2 recruits act via similar or distinct mechanisms in progenitors to regulate these processes.

LHX2 functions in basal progenitors and regulates *Neurog2* expression to regulate layer II/III dendritic morphogenesis.

Although progenitor-specific loss of *Lhx2* using IUE already tests cell-autonomous functions due to the sparse nature of the electroporation we employed, we sought to ascertain whether *Lhx2*-mutant progenitors can recapitulate the *in vivo* dendritic arborisation phenotype (Figure 1) in neurons that are born *in vitro* (Figure

4A, B). We prepared dissociated cultures from *Lhx2*$^{lox/lox}$ cortices electroporated *ex vivo* with either TdTomato+CAAX-EGFP (Control) or Cre-TdTomato+CAAX-EGFP (Progenitor mutant) at E15.5 and cultured the cells *in vitro* for 15 days. Remarkably, the progenitor mutant neurons exhibited similar phenotypes *in vitro*, significantly reducing the total lengths of apical and basal dendrites (Figure 4C, D).

To explore the molecular basis of LHX2 function, we isolated E15.5-electroporated control and progenitor mutant neurons at P5 and performed RNA-Seq (Figure 4E). Loss of *Lhx2* resulted in dysregulation of numerous genes governing dendrite and spine development, including *Ntf3, Xlr4b, Xlr3b, Xlr4c, Sox11*, and *Cdh15* (*8*, *9*, *14*, *43*). The top dysregulated gene was transcription factor *Neurog2,* which was 256-fold upregulated in the *Lhx2* mutant neurons (Figure 4E).

NEUROG2 is a transcriptional regulator previously implicated in neuronal differentiation, migration, and morphogenesis (*13*, *44*). Analysis of the single-cell RNA Seq dataset of E15.5 neocortical cells obtained from (*45*) revealed that while *Lhx2* is enriched in both apical and basal progenitors during neurogenesis, *Neurog2* is specifically enriched in the *Eomes*-positive basal progenitors (Figure 4F–I). This led us to investigate whether the function of *Lhx2* in regulating dendritic morphogenesis is specific to basal progenitors. To test this, we utilised an *Eomes*-driven Cre recombinase (*46*) to effect disruption of *Lhx2* specifically in basal progenitors (Figure 4J–J’’).

Sholl analysis of apical and basal dendrites of *Eomes*-Cre-driven *Lhx2* mutant neurons demonstrated a near-perfect overlap of the two progenitor mutant graphs (Cre and *Eomes*-Cre), indicating that the role of LHX2 in regulating dendrite morphometry is relevant specifically in basal progenitors that go on to produce layer II/III neurons (Figure 4K–L).

Having identified *Neurog2* to be significantly upregulated upon progenitor-specific loss of *Lhx2*, we investigated whether downregulation of *Neurog2* in the *Lhx2* mutant progenitors could rescue the dendritic and spine morphology defects. To test this, we employed a short-hairpin RNA construct to knock down *Neurog2* (*47*; Supplementary Figure S4) in the progenitors together with Cre and CAAX-mCherry in the *Lhx2*$^{lox/lox}$ background, creating a simultaneous knockout of the *Lhx2* gene and a knockdown of *Neurog2* expression. Sholl analysis of these neurons at P30 demonstrated a

significant and complete rescue of the dendritic defect phenotype in the apical dendrites and a significant partial rescue in the basal dendrites (Figure 4M–O). Spine morphology analysis in the apical and basal dendrites of these neurons also revealed substantial amelioration of the spine defects observed in Pg *Lhx2* mutants (Figure 4P–Y).

In summary, these results indicate that LHX2 plays a crucial role in E15.5 basal progenitors and that LHX2-mediated suppression of *Neurog2* expression is vital for the normal development of dendritic arbours and spine morphogenesis in layer II/III cortical neurons.

Discussion

Our results reveal distinct roles of LHX2 in cortical progenitors and postmitotic neurons that govern unique gene regulatory networks critical for dendritic and spine morphometry (Figure 5).

We identify an LHX2-NEUROG2 pathway in basal progenitors that modulates dendritic morphometry. NEUROG2 is a well-established factor essential in the differentiation, migration, and maturation of neurons (*13*, *44*, *48*, *49*). Our findings extend these functions to the modulation of dendritic morphometry and spine maturation. Our findings indicate that LHX2 suppresses *Neurog2* expression and regulates its downstream cascade responsible for mediating dendritogenesis. Since NEUROG2 is a pleiotropic transcriptional regulator (*50*) implicated in pioneering chromatin interactions essential for differentiation (*48*), and LHX2 regulates its expression during neurogenesis, understanding how these factors function in tandem to regulate specific developmental programs will be a rich line of future investigation.

These findings also offer novel insights into how LHX2 adapts its roles at different stages of neurogenesis. Previous studies identified an early cortical progenitor-specific role of LHX2 that regulates the electrophysiological properties of the firstborn cells of the cortex, the subplate (*27*). These early (E11.5) progenitors give rise to deep-layer progenitors, in which LHX2 regulates subtype identity via control of layer V fate determinant *Fezf2 (26)*; the deep layer progenitors then give rise to superficial layer progenitors in which the current study reveals yet another role of LHX2, that of regulating dendritic morphogenesis of layer II/III neurons. Together, these studies demonstrate that the role of LHX2 in cortical neurogenesis evolves as progenitors progress through

time, possibly due to temporally unique occupancy patterns of the factor on the genome, resulting in differences in gene regulation. This understanding offers potential for future studies, including a nuanced analysis of such temporally dynamic functions of a single transcription factor.

In Layer II/III postmitotic neurons, we identify that LHX2 regulates Wnt/β Catenin signalling. This pathway is well-established in regulating neuron polarity and dendritogenesis (*50*, *51*). However, our study adds a layer of complexity by showing that enhanced Wnt signalling in postmitotic layer II/III neurons of the lateral somatosensory cortex reduces dendritic complexity, contrary to its function in the medial cortex (*43*, *52*). LHX2, in turn, regulates active Wnt signalling in these postmitotic neurons, contributing to proper dendritogenesis and circuit maturation. This provides insight into region-specific differences in the functionality and responsiveness of signalling cascades and their regulation.

In the developing telencephalon, the cortical hem is a localised medial source of Wnt ligands, and medial cortical structures such as the hippocampus and the retrosplenial cortex display corresponding responsiveness in upregulating target genes (*42*, *43*, *53*). Consequently, the role of Wnts in dendritogenesis in these structures has been well studied and shown to have a positive growth response to enhanced Wnt signalling (*43*, *52*, *54*). However, other members of the Wnt ligand family, such as *Wnt7b,* are expressed widely in the dorsal telencephalon (*55*). This raises the question of whether all cortical regions in the telencephalon interpret Wnt signals similarly, resulting in the same developmental outcomes. Our findings suggest a complex scenario in which enhanced Wnt signalling in postmitotic layer II/III neurons of the lateral somatosensory cortex reduces dendritic complexity, whereas an opposite role has been reported in the medial cortex (*43*, *52*, *54*). The unique molecular context of each neuronal subtype likely influences the overall functional outcomes downstream of the same pathway. LHX2 suppresses Wnt signalling in lateral cortical postmitotic neurons, contributing to proper dendritogenesis and spine maturation, but its role in this process in the medial cortex has not yet been examined. Together, these findings provide valuable insights into region-specific differences in the functionality and responsiveness of signalling cascades and their transcriptional regulation.

We report that loss of *Lhx2* has profound consequences not only on the dendritic arbour of layer II/III neurons but also selectively on their action potential firing properties, with no detectable

changes in subthreshold measurements. These findings motivate new lines of inquiry into how LHX2 regulates specific biophysical properties of neurons.

In summary, our study offers novel insights into cell-type-specific and region-specific gene regulatory networks modulated by LHX2, contributing to neuronal maturation, function, and circuit development. LHX2 haploinsufficiency in humans leads to neurodevelopmental disorders, including autism spectrum disorder, intellectual disability and microcephaly (*56*). In this context, our findings provide a biological foundation for the molecular logic of how this pleiotropic transcription factor affects multiple processes in distinct stages and structures during development. This is an essential step in uncovering the basis of such neurodevelopmental disorders.

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Figures

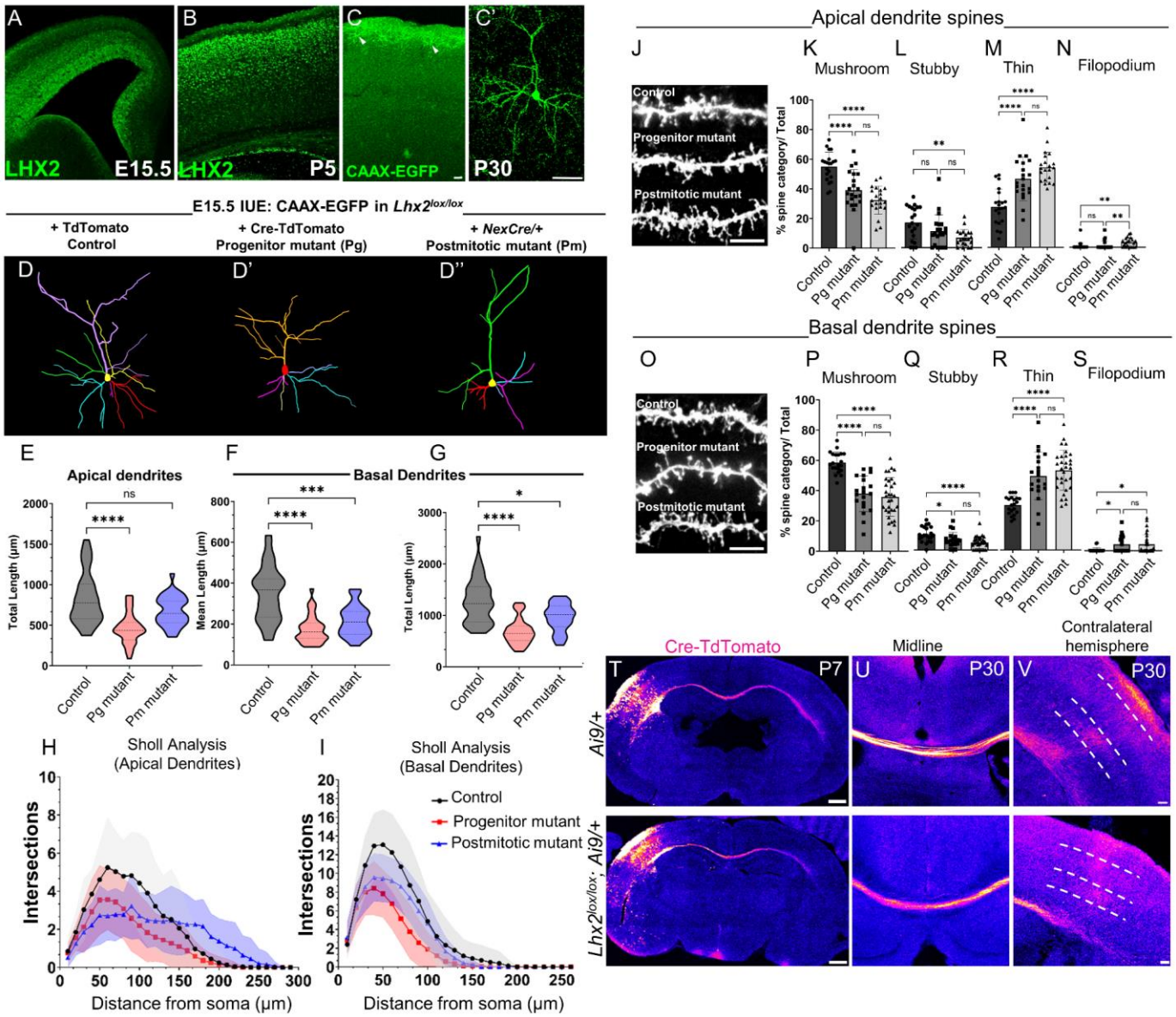

Figure 1: Loss of *Lhx2* either in progenitors or newborn postmitotic neurons induces dendritic morphology and dendritic spine defects in layer II/III neocortical neurons. (A, B) LHX2 in progenitors at E15.5 (A) and layer II/III cortical neurons at P5 (B). (C, C') A P30 layer II/III neuron labelled by CAAX-EGFP IUE in E15.5 progenitors (D–D'') Neurolucida tracings of Control, Progenitor (Pg), and Postmitotic (Pm) Lhx2 mutant neurons at P30. (E–G) The total length of apical dendrites (E) is significantly reduced in Pg mutant neurons (Control: 831.3 ± 316.4µm, Pg mutant: 460.2 ± 185µm; Pm mutant: 677 ± 181.9µm). The mean length (F) and total length (G) of basal dendrites (F) are significantly reduced for Pg and Pm mutant neurons (F: Control: 348.6 ± 131.2µm; Pg mutant: 174.0 ± 66.7µm; Pm mutant: 215.2 ± 74.4µm) ;(G: Control: 1285 ± 438.7µm; Pg mutant: 687 ± 249.7µm; Pm mutant: 956 ± 265.4µm). (H, I) Sholl analysis of apical dendrites (H) and basal dendrites (I) shows a significant decrease in complexity in Pg and Pm mutants. (35 to 40 neurons were scored for each condition obtained from 4 biologically independent replicates; *Statistical test: Two-way ANOVA-multiple comparisons with Tukey's correction for Sholl analysis and Kruskal-Wallis Test for total/mean length analysis; detailed statistical significance values per intersection row provided in Supplementary Data S1*). (J–S) Dendritic spine morphology is affected in Pg and Pm mutant neurons. In apical and basal dendrites, the percentage of spines with mushroom morphology was significantly reduced (K, P) with a

concomitant increase in thin morphology (M, R). The percentage of stubby spines was also reduced in apical dendrites of Pm mutants (L) and basal dendrites of Pg and Pm mutants (Q). The percentage of filopodium spines increased in Pm apical dendrites (N) and Pg basal dendrites (S). (20 dendrites with 25 spines each were analysed from each category. *Statistical Test: Kruskal-Wallis Test*). (T–V) Axons of Control and Lhx2 Pg mutant neurons are seen in the corpus callosum at P7 (T) and arborise in the contralateral hemisphere by P30 (U, V). **(p<0.05), **(p<0.01), ***(p<0.001), ****(p<0.0001). Scale bars in (C, C', T, V) are 50 µm and in (J, O) are 5 µm.*

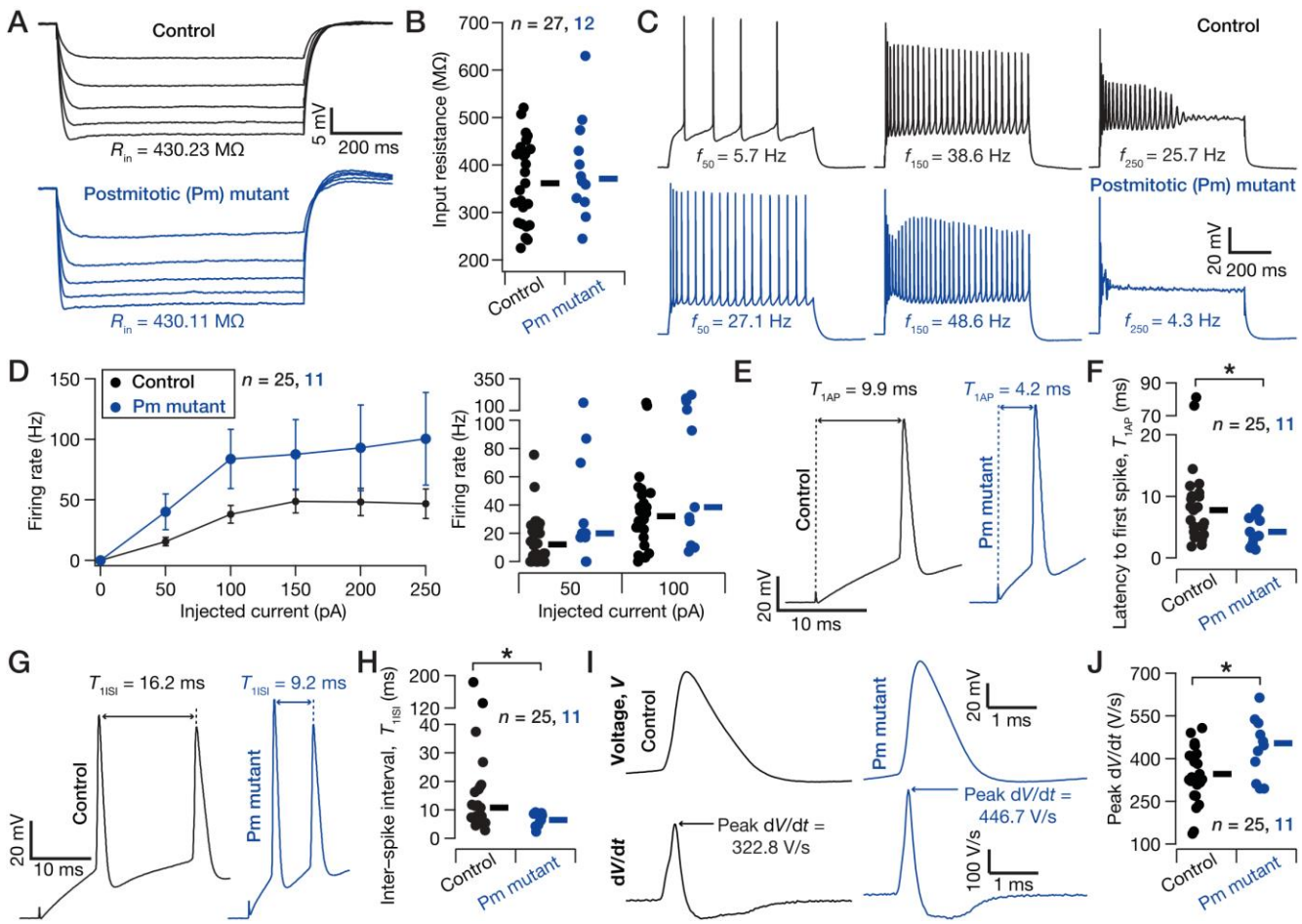

Figure 2: Enhanced intrinsic excitability of neocortical layer II/III pyramidal neurons in postmitotic *Lhx2* mutants. (A) Example voltage responses of neurons to pulse current injection from –250 pA to 0 pA in steps of 50 pA, used for computing input resistance (R_{in}). (B) Beeswarm plots of input resistance (R_{in}) obtained from control and Pm mutant neurons. (C) Voltage traces recorded from example pyramidal neurons in response to 250 pA (left), 750 pA (middle) and 1250 pA (right) from controls (top panels) and Pm mutant (bottom panels) mice. Note the transition of neurons to depolarization-induced block with higher values of current injections. The firing rate associated with each trace is provided at the bottom of the trace. (D) *Left*, mean-SEM plot showing firing rates of neurons from control and Pm mutant mice, plotted against current injection amplitudes. *Right*, Beeswarm plot of action potential firing frequency for 250 pA and 500 pA current injections for the control and Pm mutant groups. (E) The first action potential in a spike train was recorded in response to a 500-pA current injection into neurons from control (left) and Pm mutant (right) groups. Arrows mark the latency to the first spike (T_{1AP}), measured from the start time of the current injection. (F) Beeswarm plot of T_{1AP} recorded from all control and Pm mutant neurons. (G) The first two action potentials in a spike train recorded in response to a 500-pA current injection into neurons from the control (left) and Pm mutant (right) groups. Arrows mark the first inter-spike interval (T_{1ISI}). (H) Beeswarm plot of T_{1ISI} recorded from all control and Pm mutant neurons. (I) The first action potential (top) in a spike train was recorded in response to a 500-pA current injection into neurons from control (left) and Pm mutant (right) groups. The respective temporal derivatives (dV/dt) are shown below. Arrows mark the peak dV/dt associated with the action potential. (J) Beeswarm plot of peak dV/dt associated with action potentials measured from all control and Pm mutant neurons. *Statistical test: Wilcoxon rank sum test: *(p<0.05). n represents the number of total neurons recorded in each group. The thick line on the right of each beeswarm plot depicts respective median values.* All illustrative example traces were taken from a single control and a single Pm mutant neuron.

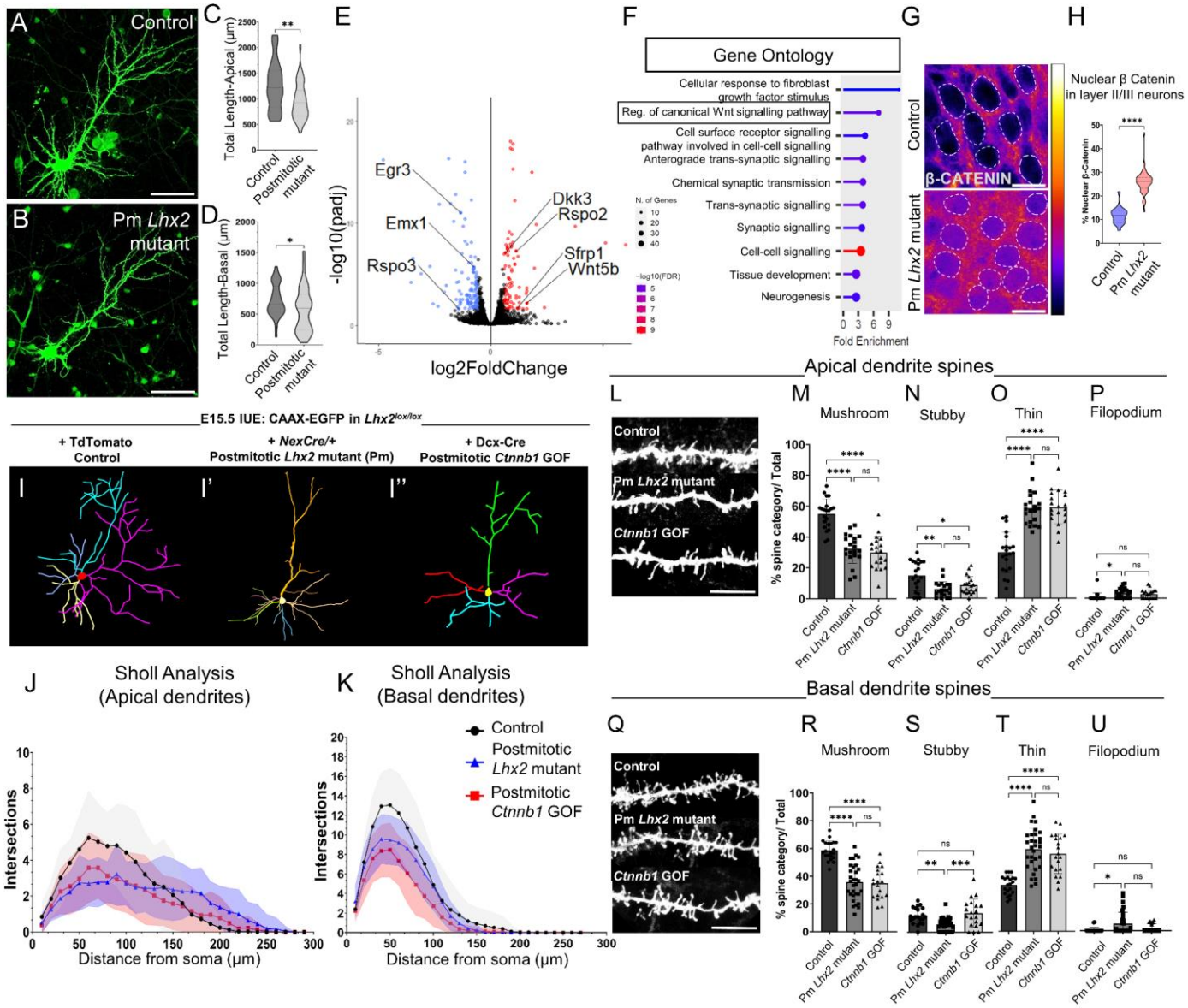

Figure 3: *Lhx2* regulates canonical Wnt signalling in postmitotic neurons. (A–D) *In vitro* neuronal cultures (A, B) display a reduction in the total length of apical (C) and basal (D) dendrites in Pm *Lhx2* mutant neurons. (E, F) RNA Sequencing of control and postmitotic mutant neurons at P5 (E) reveals dysregulated Wnt signalling cascade genes, as confirmed by Gene Ontology (GO) analysis (F). (G, H) Layer II/III Pm *Lhx2* mutant neurons display increased nuclear localisation of the Wnt signalling downstream effector, β CATENIN. (I, I'') Neurolucida tracings of control, Pm *Lhx2* mutant, and Doublecortin-Cre-driven *Ctnnb1* gain-of-function (GOF) neurons at P30. (J, K) Sholl analysis of apical and basal dendrites shows similar dendritic defects in Pm *Lhx2* mutant and *Ctnnb1* GOF neurons (30–40 neurons in each category were analysed from 4 biologically independent experiments. *Statistical test: Two-way ANOVA-multiple comparisons with Tukey's correction; detailed statistical significance values per intersection row provided in Supplementary Data S1)*. (L–U) Dendritic spine morphology defects are comparable between Pm *Lhx2* mutant and *Ctnnb1* GOF neurons. Both apical and basal dendrites exhibit a significant reduction in mushroom and stubby morphology spines (M, R) and a concomitant increase in thin and filopodium morphology spines (O, T; .20 dendrites with 25 spines each were analysed for each category. *Statistical test: Kruskal-Wallis Test for spine morphology analysis*). **(p<0.05), **(p<0.01), ***(p<0.001), ****(p<0.0001). Scale bars are 50 µm in (A, B), 10µm in (G) and 5 µm in (L, Q).*

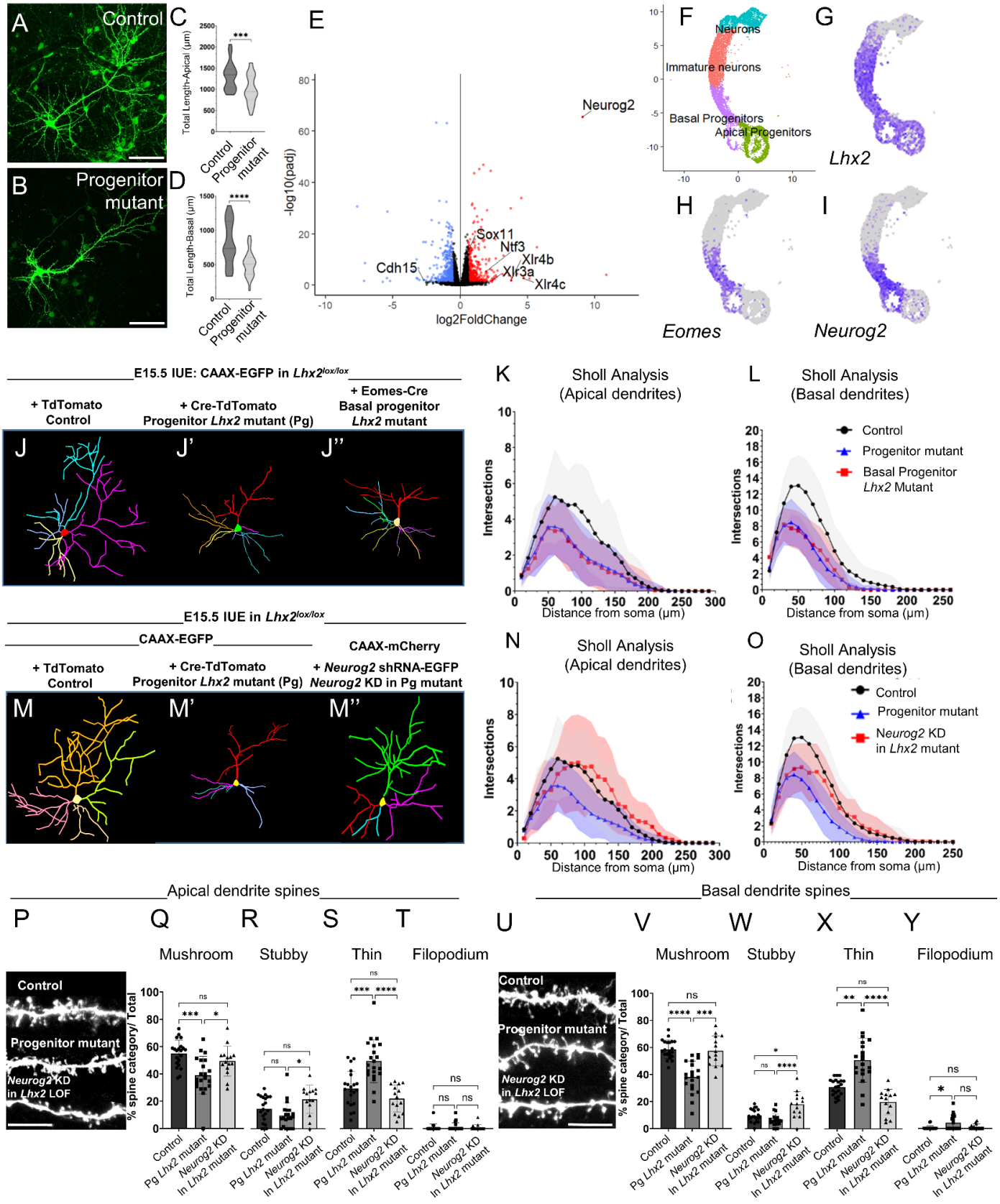
A
Control
B
Progenitor mutant
C
Total Length-Apical (µm)
Control
Progenitor mutant
D
Total Length-Basal (µm)
E
-log10(padj)
log2FoldChange
Neurog2
Sox11
Ntf3
Xlr4b
Xlr3a
Xlr4c
Cdh15
F
Neurons
Immature neurons
Basal Progenitors
Apical Progenitors
G
Lhx2
H
Eomes
I
Neurog2
E15.5 IUE: CAAX-EGFP in Lhx2lox/lox
+ TdTomato Control
+ Cre-TdTomato Progenitor Lhx2 mutant (Pg)
+ Eomes-Cre Basal progenitor Lhx2 mutant
J
J'
J''
K
Sholl Analysis (Apical dendrites)
L
Sholl Analysis (Basal dendrites)
Intersections
Distance from soma (µm)
Control
Progenitor mutant
Basal Progenitor Lhx2 Mutant
E15.5 IUE in Lhx2lox/lox
CAAX-EGFP
CAAX-mCherry
+ TdTomato Control
+ Cre-TdTomato Progenitor Lhx2 mutant (Pg)
+ Neurog2 shRNA-EGFP Neurog2 KD in Pg mutant
M
M'
M''
N
Sholl Analysis (Apical dendrites)
O
Sholl Analysis (Basal dendrites)
Neurog2 KD in Lhx2 mutant
Apical dendrite spines
Basal dendrite spines
P
Q
R
S
T
U
V
W
X
Y
Mushroom
Stubby
Thin
Filopodium
Control
Progenitor mutant
Neurog2 KD in Lhx2 LOF
% spine category/ Total
Control
Pg Lhx2 mutant
Neurog2 KD in Lhx2 mutant

Figure 4: LHX2 modulates dendritic morphogenesis of neurons by suppression of *Neurog2* in progenitors. (A–D) *In vitro* neuronal cultures display a reduction in the total length of apical (C) and basal (D) dendrites in Pg *Lhx2* mutant neurons. (E) RNA Sequencing of control and Pg *Lhx2* mutant neurons at P5 shows significant upregulation of *Neurog2*. (F–I) Analysis of E15.5 single-cell RNA seq data shows *Lhx2* expression in apical, basal progenitors and immature neurons (G), while *Neurog2* expression (I) specifically in *Eomes*-positive (H) basal progenitors. (J–L) Dendritic morphology analysis of Control, Pg *Lhx2* mutant neurons and *pEomes*-Cre driven basal progenitor *Lhx2* mutant layer II/III neurons. (J–J'') Neurolucida tracings and Sholl analysis of apical (K) and basal (L) dendrites reveal that loss of *Lhx2* in basal progenitors recapitulates the pan-progenitor Pg *Lhx2* mutant phenotype. (M–O) Dendritic morphology analysis of Control, Pg *Lhx2* mutant neurons, and Pg *Lhx2* mutant neurons with *Neurog2* shRNA (M–M'') Neurolucida tracings and Sholl analysis reveals that *Neurog2* knockdown in Pg *Lhx2* mutant neurons leads to a significant and complete rescue in apical dendrites (N) and a significant partial rescue in basal dendrites (O). Spine (30–35 neurons were analysed from 3 biologically independent experiments. *Statistical test: Two-way ANOVA-multiple comparisons with Tukey correction; detailed statistical significance values per intersection row provided in Supplementary Data S1)*.). (P–Y) Spine morphology analysis of apical and basal dendrites of Control, Pg and *Neurog2* KD in *Lhx2* Pg mutant neurons reveals a significant amelioration of spine morphology defects. **(p<0.05), **(p<0.01), ***(p<0.001), ****(p<0.0001). Scale bars are 50 µm in (A, B) and 5 µm in (P, U).*

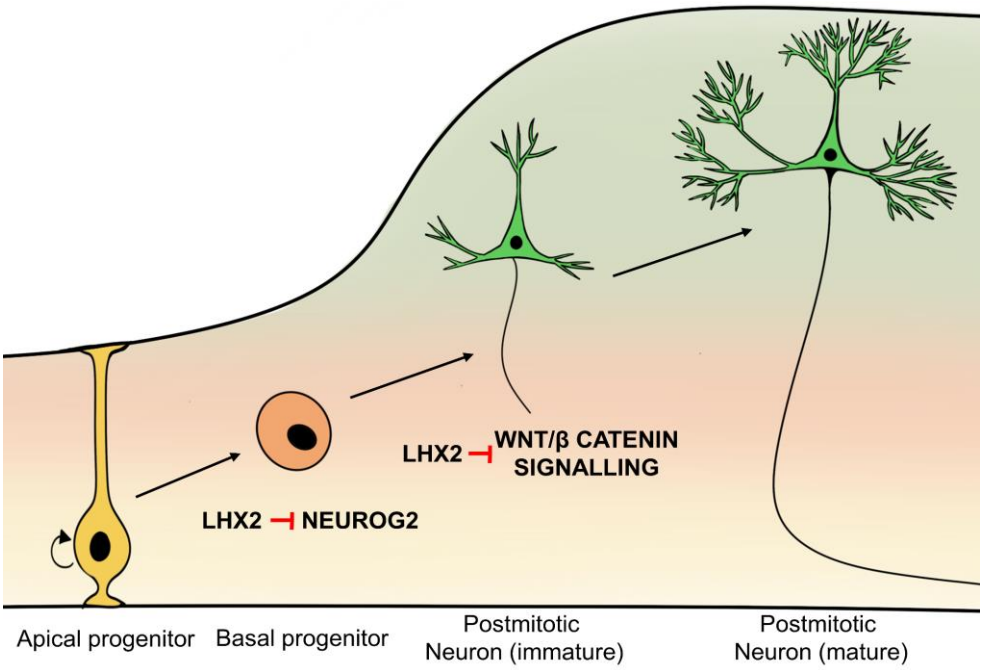

Figure 5: Schematic depicting the mechanism of action of LHX2 during the different stages of neuron development. LHX2 plays distinct roles in regulating gene networks during dendritic morphogenesis in progenitors and postmitotic neurons. LHX2 is crucial for morphogenesis in basal progenitors and controls *Neurog2* expression, facilitating its downregulation as cells differentiate from basal progenitors. Moreover, in postmitotic neurons, LHX2 regulates the Wnt/β-catenin signalling pathway by repressing active Wnt/β Catenin signalling cascade genes, thereby promoting proper dendritic arborisation of layer II/III cortical neurons.

Methods

Mice:

All procedures followed the Tata Institute of Fundamental Research Animal Ethics Committee (TIFR-IAEC) guidelines. The *Lhx2*$^{lox/lox}$ mouse line was a gift from Edwin Monuki (University of California, Irvine, USA). The *Ai9* reporter mouse line (Strain #:007909) was obtained from Jackson Laboratory. The *NexCre/+* mouse line was received from Klaus Nave, Max Planck Institute for Experimental Medicine (*32*). The *Ctnnb1* conditional GOF mouse line was obtained from M. Mark Taketo (Kyoto University, Japan). All animals were kept at an ambient temperature and humidity, with a 12h light-dark cycle and food available ad libitum. Noon of the day of the vaginal plug was designated as embryonic day 0.5 (E0.5). Both male and female animals were used for all experiments.

All the electrophysiology recording experiments were reviewed and approved by the Institute Animal Ethics Committee of the Indian Institute of Science, Bangalore. Experimental procedures were similar to previously established protocols (*57–59*) and are detailed below. 7 (4 males and 3 females) mice belonging to the control group between 7–9 months old and 7 (3 males and 4 females) postmitotic (Pm) *Lhx2* mutant mice between 6-10 months old were used for *in vitro* patch-clamp electrophysiology experiments. Animals were provided *ad libitum* food and water and were housed with an automated 12:12-h light-dark cycle, with the facility temperature maintained at 23° C.

Primers for genotyping were (expected band sizes):
Lhx2 conditional forward: ACCGGTGGAGGAAGACTTTT
Lhx2 conditional reverse: CAGCGGTTAAGTATTGGGACA
(WT: 144 bp, *Lhx2*^{lox}: 188bp, Null: 231 bp)
NexCre Forward: GAGTCCTGGAATCAGTCTTTTTC
NexCre Reverse: AGAATGTGGAGTAGGGTGAC
NexCre Mutant Reverse: CCGCATAACCAGTGAAACAG
(WT: 770 bp, *NexCre*: 525 bp).

In utero electroporation and DNA constructs:

In utero electroporation was performed at E15.5 as previously described (*27*). Embryos were injected with plasmid DNA solution dissolved in nuclease-free water with 0.1% fast green with plasmid DNA into the lateral ventricle through the uterine wall using a fine glass microcapillary (Sutter capillaries #B100-75-10).

The pCAGGS-IRES-TdTomato construct was obtained from Addgene (#83029). pCAGGS-IRES-Cre-TdTomato was generated in the lab. Membrane-bound EGFP (CAAX-EGFP) and mCherry (CAAX-mCherry) were gifts from Dr Mahendra Sonawane (Tata Institute of Fundamental Research, India). p*Eomes*-Cre was obtained from Dr Tarik Haydar (University of Maryland, Baltimore, USA). p*Dcx*-Cre was previously described in (*60*). pSilcaggs-*Neurog2* shRNA-EGFP and pSilcaggs-scrambled-EGFP were gifts from François Guillemot (Francis Crick Institute, London, UK).

Tissue Preparation:

Electroporated mice were harvested on postnatal day 30. Mice were anaesthetised using Thiopentone and transcardially perfused with Saline, followed by 4%(wt/vol) paraformaldehyde in phosphate buffer. Brains were kept for overnight fixation and then cryoprotected by transferring to 30% sucrose-PBS until sectioning. The brains were sectioned at 40µm using a Vibratome (Leica VT2000S).

Immunohistochemistry:

Brains were sectioned at 40um and processed for free-floating immunohistochemistry. Sections were washed and permeabilised with phosphate buffer containing 0.3% (vol/vol) Triton X-100. Blocking was done with 5% (vol/vol) horse serum in phosphate buffer with 0.3% (vol/vol) Triton X-100 for one hour at room temperature. Primary antibody treatment was performed in phosphate buffer containing 0.3% (vol/vol) Triton X-100 with 2.5% (vol/vol) horse serum and incubated overnight at 4ºC. The sections were washed in phosphate buffer, followed by the appropriate secondary antibody for two hours at room temperature and DAPI. Sections were mounted on Superfrost plus glass microscope slides (Cat #71869-10) and protected with Fluoroshield (Sigma cat no F6057 or F6182).

Antibodies used: Biotinylated GFP (Abcam; ab6658, 1:200), Rabbit RFP (Abcam; ab65856, 1:200), Neurog2 (ThermoFisher Scientific; PA5-78556, 1:1000), β Catenin (BD Biosciences; A610151, 1:200).

Image acquisition and analysis:

Images of neurons and dendritic spines were imaged using Olympus FluoView 1200 and 3000 confocal microscopes with FluoView software. A step size of 0.8um was kept to maximise the z-axis resolution of the dendritic branches. All the image analysis was done on Fiji-ImageJ. A nonlinear operation such as gamma correction was not performed in the images. Brightness and contrast adjustments were performed identically for control and mutant conditions.

Dendritic morphology and spine analysis:

3D morphology reconstruction of CAAX-EGFP/CAAX-mCherry labelled neurons was done using the Neurolucida 2017 software. The apical and basal dendrites were identified based on the directionality of the dendrites. The primary dendrite directed towards the pial surface of the cortex was considered apical, and the rest were labelled as basal. Total length, mean length and Sholl analysis were performed using the Neurolucida Explorer 2017 software.

For dendritic spine analysis, high-resolution images of the distal branches of the apical and basal dendrites were acquired on the confocal system. The ImageJ plugin – Dendritic Spine Counter (https://imagej.net/plugins/dendritic-spine-counter) was used to classify spines into mushroom, stubby, thin or filopodium by calculating the neck length, neck width and head width of the spines. Approximately 15-20 dendrites with ~25 spines each, obtained from 4 biological replicates, were analysed. Kruskal Wallis Test was performed to compare the three conditions. All statistical analysis was performed using GraphPad Prism 10.0.0 software.

RNA Sequencing and analysis:

For RNA Sequencing, the E15.5 electroporated layer II/III (Control: EGFP in *Lhx2*$^{lox/lox}$, Progenitor mutant: Cre-EGFP in *Lhx2*$^{lox/lox}$; Postmitotic mutant EGFP in *NexCre/+ Lhx2*$^{lox/lox}$) was microdissected from the cortical tissue at P5 after sectioning on a tissue chopper. RNA extraction and sequencing were performed on four replicates of each condition. 1ug of RNA was used for

cDNA library preparation. Samples were sequenced using the Illumina HiSeqX platform to achieve 2 x 150 bp reads to generate 60 million paired-end reads per sample.

FastqQC was performed as described in (FastQC) and read with Phred scores higher than 30 were aligned using HISAT2 (*61*). Feature counts function of the R Subread package was used to quantify the number of reads per transcript. Differential expression analysis was performed using DESeq2 (*62*). A log2FoldChange cutoff of 0.58 and P-value < 0.05 was used to identify DEGs. GO analysis was performed using ShinyGO online software (*63*).

In vitro primary neuronal cultures:

Neuronal cultures were performed from E15.5 embryos of *Lhx2*$^{lox/lox}$ and *NexCre/+; Lhx2*$^{lox/lox}$ mice. Embryos were dissected in ice-cold L-15 medium (Thermo Fischer Scientific SKU #41300039). pCAGGS-IRES-Cre-TdTomato + CAAX-EGFP (Progenitor mutant) and pCAGGS-IRES-TdTomato + CAAX-EGFP (Control) were electroporated in the dissected brains of *Lhx2*$^{lox/lox}$ mice. For the postmitotic loss condition, CAAX-EGFP was electroporated in *NexCre/+, Lhx2*$^{lox/lox}$ (postmitotic mutant) and littermate *Lhx2*$^{lox/lox}$ controls. The electroporated cortical explants were maintained on a Millicell Insert (Millipore, catalogue no. 217 PICM03050) with Neurobasal medium (ThermoFisher Scientific SKU #21103049) containing B-27 supplement (ThermoFisher Scientific SKU #17504044) and 1% GlutaMAX (ThermoFisher Scientific SKU #35050061) for 4 hours at 37°C with 5% CO_2. The explants were dissociated into a single-cell suspension using 0.25%Trypsin (ThermoFisher Scientific SKU #15400054) and then dissociated by trituration after washes. The dissociated cells were plated on Poly-D-Lysine (Sigma catalogue no. P7280) coated coverslips in Neurobasal medium containing B-27 supplement, Penicillin/Streptomycin and 1% GlutaMAX for 15 days at a 5% CO_2 atmosphere.

Dendritic morphology was analysed using the NeuronJ plugin in ImageJ. Apical and basal dendrites were identified based on their pyramidal morphology. Only neurons with just one apical dendrite were considered for analysis.

In vitro patch-clamp electrophysiology:

Slice preparation for in vitro patch-clamp recording:

Mice were anaesthetised by intraperitoneal injection of a ketamine-xylazine mixture. After the onset of deep anaesthesia, assessed by cessation of the toe-pinch reflex, transcardial perfusion of the

ice-cold cutting solution was performed. The cutting solution contained (in mM) 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 7 dextrose, 3 sodium pyruvate, and 200 sucrose (pH 7.3, ~300 mosM) saturated with carbogen (95% O_2, 5% CO_2). Thereafter, the brain was removed quickly, and 350-µm thick near-horizontal slices were prepared with a vibrating blade microtome (Leica Vibratome) while submerged in an ice-cold cutting solution saturated with carbogen. The slices were then incubated for 10–15 min at 34° C in a holding chamber containing a holding solution (pH 7.3, ~300 mosM) with the composition of (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 2 $CaCl_2$, 2 $MgCl_2$, 10 dextrose, and 3 sodium pyruvate saturated with carbogen. Thereafter, the slices were kept in the holding chamber at room temperature for at least 30 minutes before recordings started.

Whole-cell current-clamp recordings:

For electrophysiological recordings, slices were transferred to the recording chamber and were continuously perfused with carbogenated artificial cerebrospinal fluid (ACSF-extracellular recording solution) at a flow rate of 2–3 mL/min. All neuronal recordings were performed under current-clamp configuration at physiological temperatures (32–35° C) achieved through an inline heater that was part of a closed-loop temperature control system (Harvard Apparatus). The ACSF contained (in mM) 125 NaCl, 3 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, and 10 dextrose (pH 7.3; ~300 mosM). Slices were first visualised under a 10× objective lens to visually locate layer II/III of the somatosensory cortex (S1). A 63× water-immersion objective lens was employed to perform visually guided patch-clamp recordings from mCherry-labelled pyramidal neurons in superficial layers of S1 through a Dodt contrast microscope (Carl Zeiss Axioexaminer). Whole-cell current-clamp recordings were performed from layer II/III S1 pyramidal neurons with a Dagan BVC-700A amplifier.

Borosilicate glass electrodes with electrode tip resistance between 3 and 7 MΩ were pulled (P-97 Flaming/Brown micropipette puller; Sutter) from thick glass capillaries (1.5-mm outer diameter and 0.86-mm inner diameter; Sutter) and used for patch-clamp recordings. The pipette solution contained (in mM) 120 K-gluconate, 20 KCl, 10 HEPES, 4 NaCl, 4 Mg-ATP, 0.3 Na-GTP, and 7 K_2-phosphocreatine (pH 7.3 adjusted with KOH; osmolarity ~300 mosM). Series resistance was monitored and compensated online with the bridge-balance circuit of the amplifier. Experiments were discarded only if the initial resting membrane potential was more depolarised than –60 mV if

series resistance rose above 50 MΩ, or if there were fluctuations in temperature and ACSF flow rate during the experiment. Voltages have not been corrected for the liquid junction potential, which was experimentally measured to be ~8 mV. Neuronal response to a 50-pA hyperpolarising current pulse was continuously monitored to observe and correct series resistance changes using the bridge balance circuit throughout the course of the experiment.

Pharmacological blockers:

All recordings were performed in the presence of synaptic receptor blockers in the ACSF. Drugs and their concentrations used in the experiments were 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an AMPA receptor blocker; 10 µM (+) bicuculline and 10 µM picrotoxin, both $GABA_A$ receptor blockers, and 2 µM CGP55845, a $GABA_B$ receptor blocker (all synaptic blockers from Abcam) in the ACSF.

Subthreshold measurements:

We characterised S1 pyramidal neurons with several electrophysiological measurements using standard protocols (*57–59*, *64*, *65*), detailed below. Resting membrane potential (V_{RMP}) was measured as the voltage at which the cell rested when no current was injected (Supplementary Figure S2A, B). Input resistance (R_{in}) was measured as the slope of a linear fit to the steady-state voltage-current (*VI*) plot obtained by injecting subthreshold current pulses of amplitudes spanning –250 to 0 pA in steps of 50 pA (Figure 2A). The sag ratio was measured from the voltage response of the cell to a hyperpolarising current pulse of 250 pA (Supplementary Figure S2A, B). Sag ratio was defined as ($V_{initial}$ /V_{SS}), where V_{SS} and $V_{initial}$ depict the steady-state and peak (during the initial 50-ms period after current injection) voltage deflections (from V_{RMP}), respectively. To assess temporal summation, five alpha excitatory postsynaptic potentials (α-EPSPs) with 50-ms intervals were injected as currents of the form $I_\alpha = I_{max}\, t\, exp(-\alpha t)$, with α = 0.1 ms^{-1}. The temporal summation ratio (S_α) in this train of five EPSPs (Supplementary Figure S2C, D) was computed as E_{last}/E_{first}, where E_{last} and E_{first} were the amplitudes of the last and first EPSPs in the train, respectively.

The chirp stimulus, a sinusoidal current with its frequency linearly spanning 0–15 Hz in 15 s and of constant amplitude that was adjusted to be below the firing threshold, was used for characterising the impedance profiles (Supplementary Figure S2E). The voltage response of the neuron was

recorded for chirp current stimulus injection at V_{RMP}. The ratio of the Fourier transform of the voltage response to the Fourier transform of the chirp stimulus formed the impedance profile. The frequency at which the impedance amplitude reached its maximum was the resonance frequency (f_R). Resonance strength (Q) was measured as the ratio of the maximum impedance amplitude to the impedance amplitude at 0.5 Hz. The total inductive phase (ϕ_L) was defined as the area under the inductive part of the impedance phase profile as a function of frequency.

Suprathreshold measurements:

Action potential (AP) firing frequency was computed by extrapolating the number of spikes obtained during a 700-ms current injection to 1 s. The amplitude of these pulse current injections was varied from 0 pA to 1250 pA in steps of 250 pA to construct the firing frequency vs injected current (*f–I*) plot (Figure 2C, D). Various AP-related measurements (*10*) were derived from the first action potential in the voltage response of the cell, resting at V_{RMP} , to a 500-pA pulse current injection (Figure 2E–J; Supplementary Figure S3B, C). AP amplitude (V_{AP}) was computed as the difference between the peak voltage of the spike (V_{peak}) and V_{RMP} (Supplementary Figure S3B). The temporal distance between the timing of the first spike and the time of the current injection was defined as latency to the first spike T_{1AP} (Figure 2E, F). The duration between the first and the second spikes was defined as the first inter-spike interval T_{1ISI} (Figure 2G, H). AP half-width (A_{APHW}) was the temporal width measured at the half-maximal points of the AP peak concerning V_{RMP}. The maximum and minimum rate of change of voltage (dV/dt) of the AP temporal derivatives was calculated from the temporal first derivative of the voltage trace (Figure 2I, J; Supplementary Figure S3C). The voltage in the AP trace corresponds to the timepoint at which the dV/dt crossed 20 V/s defined as the AP threshold V_{th} (Supplementary Figure S3C).

Analyses of electrophysiological data and statistics:

All data acquisition and analyses were performed with custom-written software in IGOR Pro (WaveMetrics), and statistical analyses were performed using the Wilcoxon rank sum test in the R computing package (http://www.r-project.org/).

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