ASPM regulates Wnt signaling pathway activity in the developing brain

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Autosomal recessive primary microcephaly (MCPH) is a neural developmental disorder in which patients display significantly reduced brain size. Mutations in *Abnormal Spindle Microcephaly* (*ASPM*) are the most common cause of MCPH. Here, we investigate the underlying functions of Aspm in brain development and find that *Aspm* expression is critical for proper neurogenesis and neuronal migration. The Wnt signaling pathway is known for its roles in embryogenesis, and genome-wide siRNA screens indicate that ASPM is a positive regulator of Wnt signaling. We demonstrate that knockdown of Aspm results in decreased Wnt-mediated transcription, and that expression of stabilized β-catenin can rescue this deficit. Finally, coexpression of stabilized β-catenin can rescue defects observed upon in vivo knockdown of Aspm. Our findings provide an impetus to further explore Aspm's role in facilitating Wnt-mediated neurogenesis programs, which may contribute to psychiatric illness etiology when perturbed.

Supplemental material is available for this article.

Received April 19, 2011; revised version accepted August 15, 2011.

Development of the mammalian cortex requires several subprocesses, including progenitor cell proliferation, neurogenesis, neuronal migration, and establishment and refinement of synaptic connectivity. The Wnt pathway plays a prominent role in establishing the forebrain anterior–posterior axis (Fukuchi-Shimogori and Grove 2001) and promoting neural progenitor proliferation (Chenn and Walsh 2002; Zhou et al. 2004; Woodhead et al. 2006; Kim et al. 2009), dendritogenesis (Yu and Malenka 2003; Rosso et al. 2005; Wayman et al. 2006), axon establishment and guidance (Wang et al. 2002; Keeble et al. 2006), and synaptogenesis (Freese et al. 2010).

Autosomal recessive primary microcephaly (MCPH) is a disease characterized by an abnormally small head circumference that manifests during prenatal development (Aicardi 1992; Tunca et al. 2006). The underlying micrencephaly has a pronounced effect upon forebrain development and is accompanied by mental retardation (Bond et al. 2002, 2003; Roberts et al. 2002). Mutations in one of seven genes that localize to the centrosome are known to underlie development of MCPH (Guernsey et al. 2010; Kaindl et al. 2010; Nicholas et al. 2010; Yu et al. 2010). *Abnormal Spindle Microcephaly* (*ASPM*) harbors the most numerous cohort of causative MCPH mutations (Passemard et al. 2009). ASPM and its orthologs have been implicated in spindle organization, spindle orientation, mitotic progression, and cytokinesis (Fish et al. 2006; Paramasivam et al. 2007; van der Voet et al. 2009; Higgins et al. 2010). *Aspm* mutant mice display mild microcephaly without obvious increases in apoptosis, supporting the notion that MCPH is caused by defects in embryonic neural progenitor proliferation (Pulvers et al. 2010).

Recent work has identified ASPM as a positive regulator of the Wnt signaling pathway, suggesting a potential biological pathway through which ASPM may regulate neurogenesis (Major et al. 2008). FoxO activity negatively regulates *Aspm* expression while promoting expression of Wnt pathway antagonists in neural progenitor cells, suggesting a mechanism to link *Aspm* expression and Wnt activity (Paik et al. 2009). Additionally, *ASPM* overexpression, like many Wnt-activating components, is associated with increased cell proliferation and tumor development, supporting a common effect on proliferation (Kouprina et al. 2005; Hagemann et al. 2008; Klaus and Birchmeier 2008; Lin et al. 2008; Bikeye et al. 2010; Vulcani-Freitas et al. 2011). On the other hand, decreased expression of the schizophrenia risk gene *Disc1* or its binding partner, Dixdc1, results in diminished Wnt signaling activity with accompanying deficits in embryonic and adult cortical neurogenesis (Mao et al. 2009; Singh et al. 2010).

In this study, we explore the role of Aspm in cortical development and examine the functional interaction of Aspm with the Wnt signaling pathway. We report that in vivo knockdown of Aspm in the developing mouse brain results in defects in neurogenesis, neuronal migration, and cortical layer formation. We also demonstrate that Aspm promotes Wnt signaling activity, and that reduction of Aspm can be rescued by overexpression of the Wnt signal transducer β-catenin. Finally, we demonstrate that the in vivo overexpression of β-catenin can rescue defects in neurogenesis but not neuronal migration defects caused by Aspm reduction.

Results and Discussion

Aspm expression is necessary to maintain proliferation of neural progenitors at early stages of corticogenesis

In order to examine the role of Aspm in brain development, we screened small hairpins for Aspm knockdown capacity. We found two different small hairpins (shA1 and sha2) capable of knocking down levels of Aspm as assayed by quantitative PCR (qPCR) (Control and shA1, $n = 5$; shA2, $n = 3$) (Supplemental Fig. 1). We used these small hairpins to examine the effect of Aspm knockdown on neural progenitor cells at early stages of corticogenesis. We performed in utero electroporation at embryonic day 12 (E12) using a combination of either nontargeting small hairpin (control) or shASPM expression constructs and

[*Keywords*: microcephaly; neurogenesis; schizophrenia; Wnt; centrosome; neocortex]
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Article is online at http://www.genesdev.org/cgi/doi/10.1101/gad.16830211.

a GFP expression construct to mark electroporated cells. Brains were harvested at E15 and examined for distribution across cortical zones (Fig. 1A). We found that knockdown resulted in significantly fewer cells remaining in the proliferative regions of the ventricular/subventricular zones (VZ/SVZ) and significantly more cells residing in the intermediate zone (IZ) compared with controls (Control, $n = 3$; shA1, $n = 5$; shA2, $n = 3$) (Fig. 1B). Additionally, there was a strong trend toward fewer cells entering the cortical plate (CP) following Aspm knockdown ($P = .0627$) (Fig. 1B).

The fact that fewer cells remained in the VZ/SVZ following Aspm knockdown suggested defects in the maintenance of cell proliferation and premature neuronal differentiation. We examined these possibilities by looking at the overlap of the GFP-positive (GFP^+) cell population with Tuj1, a marker of differentiated neurons, at E15 (Fig. 1A). We found a significant increase in the fraction of GFP^+ cells that overlapped with the $Tuj1^+$ region of the cortex following Aspm knockdown (all groups, $n = 3$) (Fig. 1C). We also observed a significant decrease in mitotic activity at E15 based on phosphohistone H3 (PHH3) staining (all groups, $n = 4$) (Fig. 1 D,E). To examine the overall proliferative capacity of the control and Aspm knockdown populations, we performed in utero surgery at E12, followed by pulse labeling with 5-bromo-2-deoxyuridine (BrdU) at E14. Brains were harvested 24 h later (Fig. 1F). Quantification of BrdU labeling in the GFP^+ population showed a significant decrease within the shA1/shA2-expressing samples compared with controls (Control and shA1, $n = 5$; shA2, $n = 6$) (Fig. 1G), indicating an overall decrease in cell proliferation. Conversely, there was a significant increase within the GFP^+, $BrdU^+$ population of cells that no longer expressed Ki67 at E15 following Aspm knockdown compared with controls (Control and shA1, $n = 5$; shA2, $n = 6$) (Fig. 1H). Aside from shA1 and shA2, we found an additional small hairpin (shA3) that phenocopied shA1 and shA2 by all measured criteria following E12 electroporation (Supplemental Fig. 1B–G).

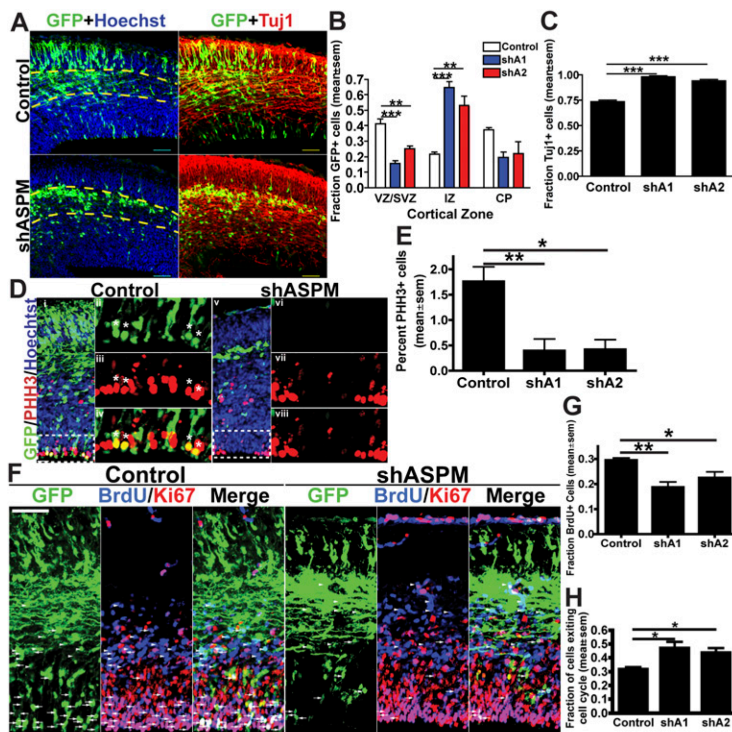

Figure 1. Aspm knockdown decreases neural progenitor proliferation in the developing cortex. (*A*) Images of E15 mouse cortices electroporated at E12 with nontargeting (*top* panels, Control) or ASPM-directed small hairpin (*bottom* panels, shASPM) and GFP expression plasmids. Images were stained for GFP and either Hoechst (*left*) or Tuj1 (*right*). Dashed lines in the *left* panels represent the CP/IZ (*top*) and IZ/ SVZ (*bottom*) borders. Bar, 50 μm. (*B*) Distribution of cells across cortical zones 72 h post-electroporation at E15. (*C*) Fraction of GFP^+ cells that overlap with Tuj1 staining in slices. (*D*) Images of E15 cortices electroporated with control small hairpin (*left*) or shASPM (*right*) plasmid. The *left* images in each set (see panels *i*,*v*) show the full span of the cortex. The *right* images in each set show GFP (panels *ii*,*vi*), PHH3 (panels *iii*,*vii*), or a merge of GFP and PHH3 (panels *iv*,*viii*) from the boxed area on the *left*. Asterisks indicate GFP, PHH3 double-positive cells. Bar, 25 μm. (*E*) Mitotic index of the GFP-positive cell population 72 h post-electroporation as measured by PHH3 staining. (*F*) Images of E15 cortices electroporated with control (*left*) or shASPM (*right*) plasmid. (*Left* panels) GFP staining. (*Middle* panels) Merge of BrdU (blue) and Ki67 (red). (*Right* panel) Merge of GFP with BrdU and Ki67. Arrowheads mark GFP, BrdU double-positive cells. Arrows mark GFP, BrdU, Ki67 triple-positive cells. Bar, 50 μm. (*G*) A 24-h BrdU labeling index as measured at E15. (*H*) Cell cycle exit index measured at E15.

Aspm expression is necessary for proper neuronal migration and cell fate acquisition during later stages of corticogenesis

In order to gain a broader understanding of the role of *Aspm* in corticogenesis, we performed in utero knockdown of Aspm at E15 and analyzed distribution of cells across cortical zones at E19 (Fig. 2A). We found a significant increase in the fraction of GFP^+ cells in the IZ and a significant decrease in the fraction of GFP^+ cells in the CP compared with controls (all groups, $n = 3$) (Fig. 2B). This suggested a severe defect in neuronal migration in the presence of reduced Aspm levels, where cells became arrested in the IZ but failed to complete migration into the CP. Additionally, we noted a significant increase in the fraction of cells arrested in the SVZ, also suggesting a migration defect (Fig. 2B). Interestingly, while we noted an overall increase in the fraction of cells found in the VZ/SVZ compartments overall, in knockdown versus control samples, this shift could be attributed mainly to the increase in GFP^+ cells found in the SVZ at this time point. We examined the GFP^+ population exclusive to the VZ and SVZ and found that there was a significant decrease in the fraction of cells found in the VZ and a significant increase in the fraction of cells in the SVZ compared with controls (all groups, $n = 3$) (Fig. 2C). This shift from the VZ to the SVZ among cells with reduced Aspm levels also suggested an enduring deficit in neurogenesis at this stage of development, since the VZ is mainly populated by self-renewing radial glia, while the SVZ contains a larger number of newly differentiated neurons and basal progenitors with limited self-renewal capacity (Englund et al. 2005).

We also asked whether Aspm knockdown affected layer-specific differentiation of cells in the CP. Electroporation of cells with our control small hairpin at E15 resulted in scattered cells throughout the deep layers of the cortex labeled by FoxP2 and a dense band of cells near the top of the cortex, representing the immature layers II/III and IV (Fig.

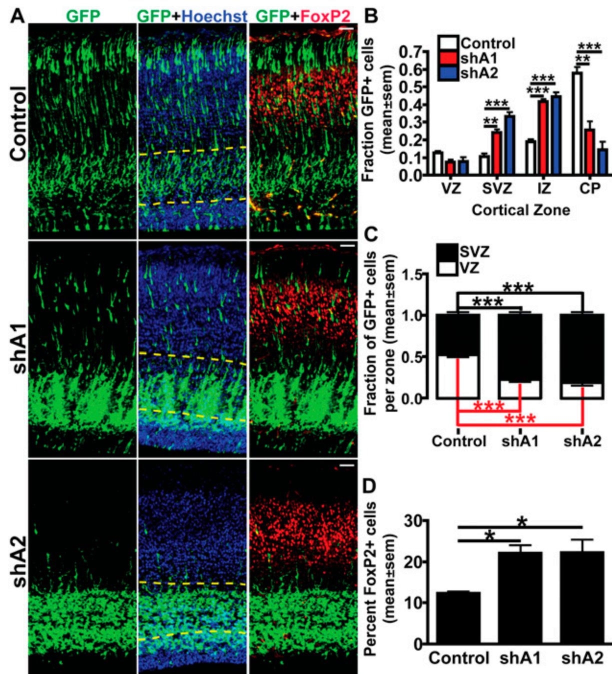

Figure 2. Aspm knockdown perturbs neuronal migration at late stages of corticogenesis. (*A*) Images of E19 cortices electroporated at E15 with control (*top* panels) or shASPM (*middle* and *bottom* panels) and GFP expression plasmids. Images were stained for GFP (*left*) and either Hoechst (*middle*) or FoxP2 (*right*). Dashed lines (*middle*) represent the CP/IZ (*top*) and IZ/ SVZ (*bottom*) borders. Bar, 100 μm. (*B*) Distribution of electroporated cells across cortical zones at E19. (*C*) Distribution of electroporated cells within the VZ and SVZ at E19. Upward-facing error bars and black stars denote significant differences in fraction of SVZ cells. Downward-facing error bars and red stars denote significant differences in fraction of VZ cells. (*D*) Percent of electroporated cells that overlap with FoxP2 staining in slices at E19.

2A). In the shASPM samples, significantly fewer cells were found in the CP overall (Fig. 2B) and the majority resides within the FoxP2$^+$ region (Fig. 2A), suggesting an alteration in production of layer-specific neurons following Aspm knockdown. We quantified the fraction of GFP$^+$ cells within the CP that were FoxP2$^+$ and found that there was a significant increase in this population following knockdown of Aspm compared with control samples (Control and shA2, n = 5; shA1, n = 4) (Fig. 2D). Together, these data suggest that few cells enter the CP due to migration defects and that more of these come from the earlier cohort of cells that give rise to deep layers of the cortex.

Expression of stabilized β-catenin rescues defects in Wnt signaling and in vivo cortical cell distribution caused by Aspm reduction

Aspm has recently been revealed as a positive regulator of Wnt signaling activity (Major et al. 2008; Paik et al. 2009). To better discern the role of Aspm in regulating the Wnt pathway, we measured Wnt-activated transcription in the presence of reduced Aspm levels. We transfected P19 cells with our control or shA1 knockdown construct and a luciferase reporter construct containing eight copies of the TCF/LEF-binding site (8XSuperTOPFLASH), which can be bound and activated by a core component of the Wnt signaling pathway, β-catenin (Molenaar et al. 1996; van de Wetering et al. 1997). We confirmed that shASPM expression resulted in a significant decrease in Wnt-mediated luciferase reporter activation compared with controls (n = 6) (Fig. 3A). We then asked whether overexpression of β-catenin could rescue dampened luciferase activation by expressing a β-catenin construct containing a stabilizing mutation (S37A). Expression of stabilized β-catenin resulted in a doubling of luciferase reporter activity compared with cells transfected with only vector and our control small hairpin. In cells expressing decreased levels of Aspm, addition of β-catenin brought luciferase reporter activity back to the level of control small hairpin samples not expressing additional β-catenin (Fig. 3A). Thus, β-catenin expression was able to rescue luciferase reporter activity in Aspm-reduced cells

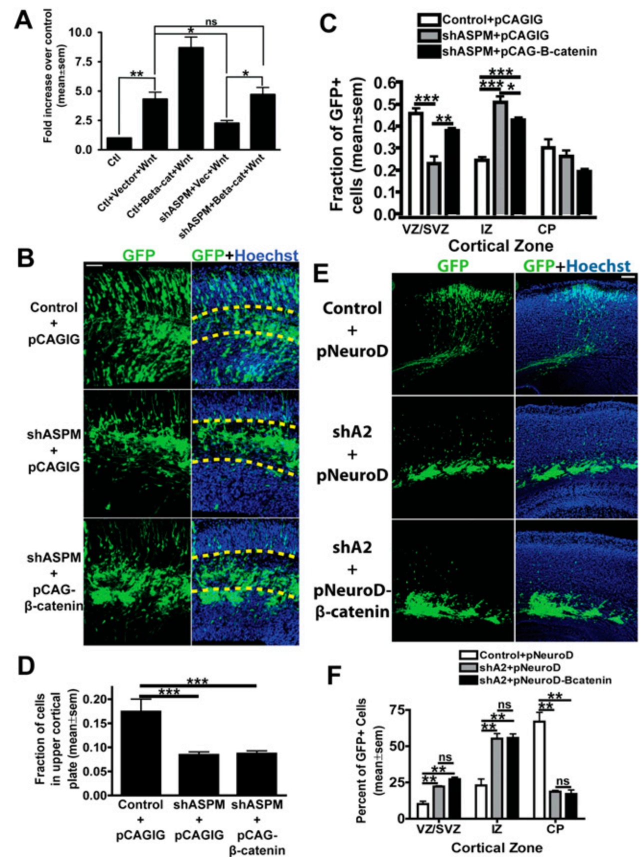

Figure 3. Expression of stabilized β-catenin rescues defects caused by Aspm knockdown. (*A*) Fold Wnt-mediated luciferase expression over control (Ctl, non-Wnt-containing medium) in P19 cells transfected with a combination of control (Ctl) or ASPM (shA) small hairpin expression constructs and vector (vector) or stabilized β-catenin (S37A mutant, βcat) expression constructs following exposure to Wnt-containing medium (Wnt). (*B*) Images of E16 cortices after electroporation at E13 with either control or shASPM, pCAGIG vector or pCAG-β-catenin, and GFP expression plasmids. (*Left* panels) GFP alone. (*Right* panels) GFP and Hoechst. Dashed lines in the *right* panels indicate the CP/IZ border (*top*) and IZ/SVZ border (*bottom*). Bar, 50 μm. (*C*) Distribution of electroporated cells across cortical zones at E16. (*D*) Fraction of GFP$^+$ cells found in the upper half of the CP. (*E*) Images of E19 cortices showing the distribution of GFP$^+$ cells following electroporation of control or shASPM and either pNeuroD vector or pNeuroD-β-catenin. (*Left* panels) GFP alone. (*Right* panels) GFP and Hoechst stain. Bar, 100 μm. (*F*) Distribution of electroporated cells across cortical zones at E19.

to levels seen in the presence of Wnt ligand-containing medium alone.

Given that Aspm can act as a positive regulator of Wnt-mediated transcription activity and that modulation of a Wnt component can compensate for deficits in Aspm-mediated Wnt activity in vitro, we asked whether increased Wnt pathway activity can rescue defects in corticogenesis caused by in vivo knockdown of Aspm. We performed in utero electroporation at E13 and harvested brains at E16, electroporating our control or shA2 small hairpin construct and either an empty GFP expression vector (pCAGIG) or the same vector encoding a stabilized β-catenin cDNA upstream of an IRES and the GFP cDNA (pCAG-β-catenin). We observed a significant shift of GFP^+ cells from the VZ/SVZ to the IZ when we compared samples electroporated with control or shA2 and pCAGIG (Control + pCAGIG, n = 3; shASPM + pCAGIG, n = 6; shASPM + pCAG-β-catenin, n = 4) (Fig. 3B,C). When we expressed stabilized β-catenin in samples concomitant with ASPM knockdown (shASPM + pCAG-β-catenin), the significant shift in cells from the VZ/SVZ was abolished compared with controls. However, we still observed a significant increase in the fraction of cells localized to the IZ in the presence of β-catenin overexpression. Compared with Aspm knockdown samples, there was a slight but significant decrease in the fraction of cells localized to the IZ in the presence of β-catenin overexpression (Fig. 3C).

At E16, we did not observe any obvious deficit in neuronal migration into the CP, although the large increase in the IZ population following Aspm knockdown in the absence or presence of β-catenin overexpression hinted at such a possibility (Fig. 3C). To address whether there was a more subtle defect in neuronal migration caused by Aspm knockdown at this stage, we divided the CP in half and separately counted the fraction of cells in the upper and lower regions. We found that Aspm knockdown caused a significant decrease in the fraction of cells that reached the upper CP, regardless of β-catenin expression (Control + pCAGIG, n = 3; shASPM + pCAGIG, n = 6; shASPM + pCAG-β-catenin, n = 4) (Fig. 3D). These data illustrate a subtle neuronal migration defect following early reduction of Aspm. It also demonstrates that while Wnt signaling may contribute to maintenance of proliferative activity in the absence of Aspm, it does not appear to exert a significant role in facilitating neuronal migration within the early CP. As further proof of this, restricting β-catenin overexpression to post-mitotic neurons between E15 and E19 also did not rescue the migration defect (all groups, n = 2) (Fig. 3E,F).

Our study is the first to examine the effect of Aspm knockdown on multiple stages of brain development. Previous work has implicated Aspm function in neurogenesis within the context of mouse brain corticogenesis (Fish et al. 2006; Pulvers et al. 2010). We expand on this role by demonstrating that acute knockdown of Aspm results in depletion of neural progenitors, decreased mitotic activity, and premature neuronal differentiation (Figs. 1, 2). Moreover, at relatively late stages of neurogenesis (E19), knockdown also results in redistribution of cells between the VZ and SVZ (Fig. 2). This result further suggests an enduring role for Aspm in the maintenance of the apical progenitor pool during all stages of neurogenesis. While we observed a modest reduction of *Aspm* in P19 cells by qPCR (Supplemental Fig. 1A), we were unable to assess knockdown in vivo due to a lack of reagents. We suspect that in vivo knockdown was stronger than our in vitro observation and note that acute knockdown can result in more pronounced effects than are observed in animal models that lack individual gene expression altogether (Bai et al. 2003).

Our study also uncovers a previously unappreciated role for Aspm in neuronal migration (Figs. 1, 2). This result is most pronounced at later stages of cortical development, when neurons must travel longer distances to reach their final destination in the CP. However, our study shows a significant effect of Aspm knockdown upon neuronal migration as early as E16 (Fig. 3D). It is interesting to note that while stabilized β-catenin expression can compensate for the effect of Aspm knockdown on deficits in neurogenesis at this stage, no such compensation is observed in terms of neuronal migration. This is consistent with previous observations that knockdown of positive regulators of Wnt signaling impinges on both neurogenesis and neuronal migration, but that only deficits in neurogenesis can be rescued via increased Wnt-mediated activity (Brandon et al. 2009; Mao et al. 2009).

Recent work has demonstrated that the schizophrenia risk gene *Disc1* is a positive regulator of Wnt signaling, and that decreased *Disc1* expression results in impaired adult neurogenesis and behavioral defects (Mao et al. 2009). This study demonstrates that overexpression of stabilized β-catenin can rescue deficits in neurogenesis caused by Aspm reduction, hinting at the possibility that ASPM function could play a role in regulating pathways and processes that contribute to manifestation of schizophrenia. While our study uncovers a functional interaction between Aspm and β-catenin, further studies will be necessary to elucidate the molecular mechanisms that allow Aspm to influence neurogenesis and the Wnt pathway. Importantly, both decreased brain size and deficits in adult neurogenesis are associated with schizophrenia (Ward et al. 1996; Reif et al. 2007; Crespi and Badcock 2008). While a plethora of *ASPM* mutations are associated with MCPH, it will be interesting to determine whether subtle alterations in *ASPM* expression can contribute to deficits in adult neurogenesis and neuropsychiatric disorders.

Materials and methods

In utero electroporation

In utero electroporation was performed as described elsewhere (Xie et al. 2007). Electroporations were performed at E12, E13, or E15, and brains were harvested at E15, E16, or E19, respectively.

DNA constructs and sequences

Aspm small hairpin sequences and cDNA expression constructs used in this study are described in the Supplemental Material.

Antibodies

All antibodies used in this study are listed in the Supplemental Material.

Luciferase assays

Luciferase assays were performed as described in Mao et al. (2009).

qPCR

qPCR was performed using material collected from P19 carcinoma cells transfected with small hairpin expression plasmids. Methods are described in detail in the Supplemental Material.

Statistical analysis

In all bar graphs, analysis was carried out using one-way analysis of variance followed by Newman-Keuls multiple comparison test; (ns) $P > .05$; (*) $P < .05$; (**) $P < .01$; (***) $P < .001$.

Acknowledgments

We thank Karuna Singh, Yingwei Mao, and Froylan Calderon de Anda for helpful discussion and editing. We thank Ester Kwon, Matthew Dobbin, Ling Pan, and Wen-Yuan Wang for technical assistance. Li-Huei Tsai is an investigator of the Howard Hughes Medical Institute and the director of the neurobiology program at the Stanley Center for Psychiatric Research. This work is partially supported by the Stanley Foundation.

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