Cortical Interneurons Require *Jnk1* to Enter and Navigate the Developing Cerebral Cortex

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Proper assembly of cortical circuitry relies on the correct migration of cortical interneurons from their place of birth in the ganglionic eminences to their place of terminal differentiation in the cerebral cortex. Although molecular mechanisms mediating cortical interneuron migration have been well studied, intracellular signals directing their migration are largely unknown. Here we illustrate a novel and essential role for c-Jun N-terminal kinase (JNK) signaling in guiding the pioneering population of cortical interneurons into the mouse cerebral cortex. Migrating cortical interneurons express *Jnk* proteins at the entrance to the cortical rudiment and have enriched expression of *Jnk1* relative to noninterneuronal cortical cells. Pharmacological blockade of JNK signaling in *ex vivo* slice cultures resulted in dose-dependent and highly specific disruption of interneuron migration into the nascent cortex. Time-lapse imaging revealed that JNK-inhibited cortical interneurons advanced slowly and assumed aberrant migratory trajectories while traversing the cortical entry zone. *In vivo* analyses of JNK-deficient embryos supported our *ex vivo* pharmacological data. Deficits in interneuron migration were observed in *Jnk1* but not *Jnk2* single nulls, and those migratory deficits were further exacerbated when homozygous loss of *Jnk1* was combined with heterozygous reduction of *Jnk2*. Finally, genetic ablation of *Jnk1* and *Jnk2* from cortical interneurons significantly perturbed migration *in vivo*, but not *in vitro*, suggesting JNK activity functions to direct their guidance rather than enhance their motility. These data suggest JNK signaling, predominantly mediated by interneuron expressed *Jnk1*, is required for guiding migration of cortical interneurons into and within the developing cerebral cortex.

Key words: development; forebrain; GABAergic interneuron; intracellular signaling; neuronal migration; psychiatric disorder

Introduction

Cortical interneurons play vital roles regulating neurotransmission in the cerebral cortex and their dysfunction is implicated in severe brain disorders including epilepsy and schizophrenia. Mechanisms contributing to the pathological vulnerability of cortical interneurons are unclear, but they may arise during embryonic development when interneurons embark on long distance tangential migration from the medial and caudal ganglionic eminences to the overlying cerebral cortex (Anderson et al., 1997; Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 2001; Nery

et al., 2002; Miyoshi et al., 2010). For this reason, molecular mechanisms guiding cortical interneuron migration have been extensively studied (for review, see Faux et al., 2012; Marín, 2013). Many ligands and receptors mediating cortical interneuron dispersion from the ventral forebrain (Powell et al., 2001), motility (Polleux et al., 2002; Pozas and Ibáñez, 2005), chemorepulsion from the striatum (Marín et al., 2001), chemoattraction to the cerebral cortex (Yau et al., 2003; Flames et al., 2004), and formation and maintenance of migratory streams have been identified (Tiveron et al., 2006; Li et al., 2008; López-Bendito et al., 2008; Sánchez-Alcañiz et al., 2011; Wang et al., 2011). However, intracellular signals controlling cortical interneuron migration, particularly those directing migration at critical positions along their trajectories, are largely unknown.

The c-Jun N-terminal protein kinase (JNK) pathway plays obligate roles in mammalian forebrain development (Kuan et al., 1999), and moreover, genetic disruption of JNK function leads to cognitive disorders in humans (Kunde et al., 2013). JNKs are members of the mitogen-activated protein kinase (MAPK) signaling pathway, and are encoded by three related genes in mammals: *Jnk1* (*Mapk8*), *Jnk2* (*Mapk9*), and *Jnk3* (*Mapk10*). All three *Jnk* genes are expressed in the developing mouse brain, but only combinatorial deletion of *Jnk1* and *Jnk2* results in embryonic lethality and profound alterations in neuronal survival and programmed cell death (Kuan et al., 1999; Sabapathy et al., 1999).

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Genetic deletions of *Jnk1* or upstream Jnk kinases results in aberrant radial migration of cortical projection neurons (Hirai et al., 2006; Wang et al., 2007; Westerlund et al., 2011; Yamasaki et al., 2011), strongly implicating JNK signaling as a major regulator of neuronal migration in the developing forebrain.

In the current study, we use a combination of *ex vivo* and *in vivo* approaches to demonstrate that cortical interneurons have a cell-intrinsic requirement for JNK signaling—largely mediated by the activity of *Jnk1*—to enter and successfully navigate the developing cerebral cortex. JNK-inhibited cortical interneurons dramatically slow their advancement and take inappropriate trajectories at the entrance to the cortical rudiment. Similarly, cortical interneurons in *Jnk1*- and *Jnk1/2*-deficient embryos exhibit significant delays in their cortical migration. Conditional ablation of *Jnk1* and *Jnk2* from cortical interneurons delays cortical entry, disrupts migratory streams, and perturbs interneuron morphology *in vivo*, but has no effect *in vitro*, suggesting JNK signaling only regulates migration of cortical interneurons in the presence of cortical guidance cues. These findings implicate the JNK pathway, and *Jnk1* in particular, as a key molecular node in the intracellular regulation of cortical interneuron migration *in vivo*.

Materials and Methods

Animals. Animals were housed and cared for by the Office of Laboratory Animal Resources at West Virginia University. Timed-pregnant dams (day of vaginal plug = embryonic day 0.5) were killed by rapid cervical dislocation and mouse embryos were immediately harvested for tissue culture, gene expression, or histological analyses. The following mouse strains were acquired and maintained on a C57BL/6J (The Jackson Laboratory) background: *Dlx5/6-Cre-IRES-EGFP* (*Dlx5/6-CIE*; Stenman et al., 2003), *Mapk8^{tm1Rjd}* floxed (*Jnk1^{fl/fl}*; Das et al., 2007; kindly provided by Dr. Roger Davis), *Mapk8^{tm1Flv}* knock-out (*Jnk1^{-/-}*; Dong et al., 1998; The Jackson Laboratory), and *Mapk9^{tm1Flv}* knock-out (*Jnk2^{-/-}*; Yang et al., 1998; The Jackson Laboratory). For slice culture experiments, CF-1 (Charles River) dams were crossed with hemizygous *Dlx5/6-CIE* males. Constitutive mutant embryos were generated by crossing *Jnk1^{+/-}*; *Jnk2^{+/-}* females to *Jnk1^{-/-}* or *Jnk2^{-/-}* males. Conditional mutant embryos were generated by crossing *Jnk1^{fl/fl}*; *Jnk2^{-/-}* females to *Dlx5/6-CIE*; *Jnk1^{fl/+}*; *Jnk2^{-/-}* males. All animal procedures were performed in accordance to protocols approved by the Institutional Animal Care and Use Committee at West Virginia University.

Fluorescently activated cell sorting and qRT-PCR. Methods for fluorescently activated cell sorting (FACS), extracting RNA, synthesizing cDNA, and performing qRT-PCR were reported previously (Meechan et al., 2012). Briefly, cortices from *Dlx5/6-CIE+* embryos were individually dissociated with papain (Worthington Biochemical), resuspended in FACS buffer, sorted based on endogenous GFP fluorescence, and *Dlx5/6-CIE(+)* and *Dlx5/6-CIE(-)* sorted samples were homogenized separately in TRIzol reagent (Ambion). Total RNA was isolated and cDNA synthesized using the Improm-II RT kit (Promega). qPCR was conducted using EvaGreen (Bio-Rad) reagent and a CFX384 thermal cycler (Bio-Rad). PCR for *Jnk1* (F: AGCAGAAGCAAACGTGACAAC/R: GCTGCACACACTATTCCTTGAG) and *Jnk2* (F: CCAAGTAGGATTGCCTGCTTA/R: TGGTCACATGCATACGAGTC) was performed. *Gapdh* was used as the endogenous reference gene control. Fold change was determined by the delta-delta CT method (Livak and Schmittgen, 2001).

Organotypic slice cultures. Embryos were collected and dissected in ice-cold complete HBSS (cHBSS; Tucker et al., 2006). *Dlx5/6-CIE+* brains were embedded in 3% low melting point agarose (Fisher Scientific), sectioned at 300 μ m on a Leica VT1000 S vibratome, transferred to laminin/poly-D-lysine-coated transwell inserts (BD Falcon; Polleux and Ghosh, 2002), and grown at 37°C with 5% CO₂ in slice culture media (Tucker et al., 2006) for 24 h. Stock solutions of pharmacological inhibitors [20 mM SP600125 (Enzo Life Sciences), 20 mM AS601245 (Enzo Life Sciences), and 100 mM U0126 (EMD Chemicals)] were prepared in DMSO, stored at -20°C, and added to slice culture media immediately

Figure 1. Early arriving cortical interneurons express Jnk proteins. **A–F**, Localization of total Jnk (red) and EGFP (green) expression in *Dlx5/6-CIE+* embryonic brain sections. Jnk proteins are widely expressed in the developing forebrain at E12.5 and E13.5, and are particularly enriched in postmitotic zones. **A**, At E12.5, few *Dlx5/6-CIE+* cortical interneurons have entered the cortex (CTX) from subcortical locations. **B, C**, By E13.5, many *Dlx5/6-CIE+* cortical interneurons have invaded the cortical rudiment and formed streams of migratory cells in the MZ and SVZ/IZ of the nascent cortex. **D–F**, *Dlx5/6-CIE+* interneuronal cell bodies (asterisks) and processes (arrowheads) colocalize with total Jnk protein in the E13.5 cortex. **G**, Separation of *Dlx5/6-CIE(+)* and *Dlx5/6-CIE(-)* cells from a dissociated E14.5 cortex by FACS sorting. Three cortices were independently processed for gene expression analyses by qRT-PCR. **H**, qRT-PCR indicates *Jnk1* transcript is enriched twofold in *Dlx5/6-CIE(+)* cells compared with *Dlx5/6-CIE(-)* cells (Student's *t* test; *p* = 0.02), whereas *Jnk2* transcript is uniformly expressed in the two populations. CP, Cortical pool.

before use. DMSO was used as a vehicle control at matching solvent concentrations to inhibitors.

Live imaging experiments. Live vibratome slices were transferred to Millicell cell culture inserts (Millipore) in FluoroDishes (World Precision Instruments) filled with slice culture media containing a 1:1000 dilution of DMSO (for controls) or 20 μ M SP600125 JNK inhibitor. FluoroDish preparations were immediately transferred to a Zeiss LSM 510 Meta Confocal Microscope with stable environmental controls maintained at 37°C with 5% humidified CO₂. Time-lapse z-series were

Figure 2. Cortical interneurons require JNK activity to enter the developing cerebral cortex. **A**, Schematic representation of slice culture assay. E12.5 *Dlx5/6-CIE* brains are isolated, sectioned, and cultured 24 h in control or SP600125 (SP) conditions. **B–D**, In control cultures, cortical interneurons robustly migrate into the cortical rudiment during the 24 h culture period. Migratory front is marked by a flanking arrowhead (MZ region) and arrow (SVZ region) in each image. **E–H**, Entry of cortical interneurons into the cerebral cortex is disrupted by SP600125, a pan-JNK inhibitor, in a dose-dependent fashion. **I**, For quantification of interneuron migration in slice cultures, cortices were segmented into five equidistant bins and the percentages of cortical interneurons appearing in all cortical bins were determined for each section, and averaged across all sections per treatment group. **J**, Dose-dependent accumulation of cortical interneurons in lateral and decline of interneurons in medial cortical bins following SP600125 treatment. Two-way ANOVA reveals statistically significant interactions between treatment and bin location ($F_{(16,275)} = 57.5; p < 0.0001$). Differences within bins were determined *post hoc* by Fisher's LSD tests (**** $p < 0.0001$; *** $p \leq 0.0006$; ** $p < 0.008$).

acquired every 10 min for 24 h with an LD Plan-Neofluar 20×/0.4 Korr objective lens.

MGE explant culture and analysis. Brains from embryonic day 12.5 (E12.5) *Dlx5/6-CIE*; *Jnk1^{fl/+};Jnk2^{-/-}* (control) or *Dlx5/6-CIE*; *Jnk1^{fl/fl};Jnk2^{-/-}* (conditional double knock-out) embryos were removed in ice-cold cHBSS, embedded in 3% low melting point agarose, and sectioned at 300 μ m. Medial ganglionic eminence (MGE) tissue was isolated from appropriate sections and trimmed to generate subventricular zone-enriched explants. Each brain was processed individually and yielded approximately two MGE explants plus material for genotyping, which was performed retrospectively. A 1:1 collagen-Matrigel mixture was made by preparing a 2 mg/ml working stock of neutralized collagen (BD Life Sciences; Type 1 Rat Tail High Concentration) in phenol-red free Neurobasal medium (Invitrogen) and diluting it 1:1 with phenol-red free Matrigel (Corning Life Sciences). Ice-cold collagen-Matrigel mixture was added to each well of an eight-well Lab-Tek chambered coverglass, and MGE explants were pipetted into matrix-filled wells. MGE-embedded matrices were allowed to solidify for 45 min at 37°C with 5% CO₂, fed with phenol-red free Neurobasal medium supplemented with GlutaMAX (Invitrogen), penicillin-streptomycin (Thermo Scientific), and B-27 (Invitrogen), and cultured for 2 d. Cultured explants were imaged on a Zeiss LSM 510 confocal microscope at a uniform optical thickness. Area measurements were performed on projected montages in ImageJ. Outgrowth area was determined by subtracting the explant area from the total area occupied by both the explant and migrating cells (Fig. 8F), and normalized to explant size. Explants were measured blind to genotype and evaluated statistically by a two-tailed unpaired Student's *t* test using Prism 6 (GraphPad) software.

Immunostaining of slice cultures. Slices were fixed overnight in 4% paraformaldehyde in 1× PBS at 4°C. Slices were rinsed from fixative with 1× PBS, and blocked overnight at 4°C in permeability solution (Polleux and Ghosh, 2002) with 5% normal goat serum. Rabbit anti-GFP (Invitrogen; 1:1500) and mouse anti-nestin (BD Transduction Laboratories; 1:1000) primary antibodies were diluted in block, applied to slices, and incubated overnight at 4°C. Slices were thoroughly rinsed in 1× PBS, and incubated overnight at 4°C with secondary antibody solution containing goat anti-rabbit Alexa488 (1:4000; Invitrogen), goat anti-mouse Alexa546 (1:2000; Invitrogen), and the nucleic acid counterstain Draq5 (1:4000; Axxora) diluted in block. Slices were rinsed in 1× PBS and slide mounted in an aqueous mounting medium containing anti-fade reagent.

Cryosectioning and staining. E13.5 heads were removed and fixed with 4% paraformaldehyde overnight, rinsed in 1× PBS, and progressed through a sucrose series (10, 20, and 30%). Cryoprotected heads were embedded with TFM Tissue Freezing Medium (Triangle Biomedical) and flash frozen in liquid

nitrogen-cooled 2-methyl butane. Heads were serially sectioned (12 μm) in the coronal plane and slides stored at -20°C before use. Slides were rehydrated with $1\times$ PBS for 20 min, blocked (as above) for 2 h at room temperature, and incubated in primary antibody solution (block with primary antibodies) overnight at 4°C . Primary antibodies included rabbit anti-calbindin (Swant; 1:2000), mouse anti-nestin (BD; 1:1000), and chicken anti-GFP (Abcam; 1:1500). Slides were rinsed with $1\times$ PBS, incubated with secondary antibody and nucleic acid counterstain solution (as above) for 2 h at room temperature, rinsed in $1\times$ PBS, and coverslipped with an aqueous mounting medium containing anti-fade reagent.

Imaging and analysis. Immunofluorescently labeled slice cultures and cryosections were imaged on a Zeiss LSM 510 Meta Confocal Microscope with a $10\times$ Fluar or $20\times$ Plan-Apo objective lens. Confocal micrographs were uniformly adjusted for levels, brightness, and contrast in Adobe Photoshop. For quantification of migration, cortical length was measured from the corticostriatal boundary to the cortical arch of each image, and cortices were segmented into five equidistant bins (Figs. 2, 5). Similarly, for quantification of radial distribution (Fig. 8), a defined region of the lateral cortical wall was cropped from montaged images and equidistantly segmented into six bins from pial to ventricular surfaces. For both studies, the numbers of cells present in each bin were counted and their percentile distributions across all bins were determined for each tissue section. Bin distributions were averaged across sections of the same treatment group or genotype, and statistical significances were determined by two-way ANOVA followed by Fisher's LSD *post hoc* analyses (GraphPad Prism 6).

4D live imaging movies were analyzed using Imaris (Bitplane). Movies (12 control and 12 inhibitor) were evaluated in the first 12 h and last 12 h of each recording. Ten interneurons were tracked in each time segment for all movies. Interneurons were selected for tracking if they could be followed for at least 4 h. Tracks were discontinued if a cell remained stationary for 40 contiguous minutes, or if the tracked cell could no longer be unambiguously identified. Average velocity, displacement, and straightness values were obtained for each movie (20 cells). Each treatment group contained 120 cells analyzed from 12 movies ($n = 12$). Two-tailed unpaired Student's *t* tests (GraphPad Prism 6) were used to determine statistical differences between groups.

Results

Interneurons express Jnk proteins as they first enter the cortical rudiment

We used transgenic mice expressing EGFP under the control of a Dlx5/6 Cre-IRES-EGFP transgene (hereafter referred to as Dlx5/6-CIE) to follow the migration of newly born interneurons into the developing mouse cortex. Dlx5/6-CIE+ interneurons first arrive at the entrance to the cortical rudiment on E12.5 (Fig. 1A). By E13.5, Dlx5/6-CIE+ interneurons have crossed the corticos-

Figure 3. Disruption of cortical interneuron migration by pharmacological inhibition of JNK signaling is specific and reversible. **A–D**, An independent, pan-JNK inhibitor, AS601245 (**B**), diminishes interneuron entry into the cortex (CTX) compared with control (**A**) cultures, while blockade of MAPK signaling with the Mek1/2 inhibitor U0126 (**C**) does not. **D**, Quantification reveals significant reductions in migration for slices treated with 5 μM AS601245, but no change in slices treated with 10 μM U0126 (two-way ANOVA: $F_{(8,165)} = 15.98$; $p < 0.0001$; Fisher's LSD: **** $p < 0.0001$; *** $p = 0.0002$; ** $p = 0.009$; * $p = 0.049$). **E–H**, Cortical interneuron migration recovers after removal of 20 μM SP600125. **E**, Quantification reveals that interneuron migration improves when 20 μM SP600125 is washed out and replaced by control medium half way through the 24 h culture period (two-way ANOVA: $F_{(8,165)} = 37.56$; $p < 0.0001$; Fisher's LSD: **** $p < 0.0001$). **F**, Slice cultured in control medium, rinsed at 12 h, and cultured for an additional 12 h in control medium. **G**, Slice cultured in 20 μM SP600125, rinsed at 12 h, and cultured for an additional 12 h in fresh 20 μM SP600125. **H**, Slice cultured in 20 μM SP600125, rinsed at 12 h, and cultured for an additional 12 h in control medium.

triatl boundary and have migrated approximately half the length of the lateral cortical wall (Fig. 1B,C). As they enter the neocortex, Dlx5/6-CIE+ interneurons form two medially oriented migratory streams flanking the emerging cortical plate; an upper marginal zone (MZ) stream and lower subventricular zone/intermediate zone (SVZ/IZ) stream (Fig. 1C). Thus, the first cohort of Dlx5/6-CIE+ cortical interneurons forms streams of migratory cells that rapidly enter the cortical rudiment over a single developmental day.

JNK signaling regulates cell survival (Kuan et al., 1999), radial migration (Westerlund et al., 2011), and axon tract formation

Figure 4. Migratory properties of cortical interneurons are perturbed following SP600125 treatment at E12.5. **A–D**, In control slices, *Dlx5/6-CIE*⁺ interneurons robustly migrate through the cortical entry zone over a 24 h imaging period. Cells travel in MZ region (arrowhead in **D**) and SVZ region (dashed lines in **D**) of the section. **E–H**, In slices treated with 20 μ M SP600125, *Dlx5/6-CIE*⁺ cells remain motile, but advance slowly and frequently take aberrant trajectories. Cells pile up in MZ (arrowhead in **H**), failing to advance further. **I, J**, Tracks from 10 individual cells in the first 12 (**I**) and last 12 (**J**) h of a control movie. Individual cell tracks are long, straight, and medially directed. **K, L**, Tracks from 10 individual cells in the first 12 (**K**) and last 12 (**L**) h of an inhibitor movie. Cell tracks are short, crooked, and less medially oriented in SP600125-treated slices, particularly in the first 12 h of imaging. Tracks are pseudocolored by time (bars in **I–L**). **M–O**, Comparison of interneuron migration in SP600125-treated and control slices during the first 12 h of recordings. SP600125 treatment leads to statistically significant reductions in migratory velocity (**M**), track length (**N**), and track straightness (**O**) corresponding to directionality. **P–R**, Comparison of interneuron migration in SP600125-treated and control slices in the last 12 h of recordings. Interneuron migratory velocity (**P**) increases in SP600125-treated slices during the second 12 h of recording due to waning inhibitor efficacy, but remains significantly reduced from controls. Track length (**Q**) of JNK-inhibited interneurons also increase during the last 12 h, but displacement values remain significantly altered. Directionality (**R**) of interneurons from SP600125-treated slices remains significantly altered in the last 12 h. Significance levels after performing two-tailed unpaired Student's *t* tests are denoted as follows: *****p* < 0.0001, ****p* < 0.001, ***p* < 0.01, **p* < 0.05.

(Hirai et al., 2006; Yamasaki et al., 2011) in the developing forebrain. However, the extent to which JNK signaling influences the migration of cortical interneurons is unknown. We examined the expression pattern of total Jnk protein between E12.5 and E13.5 to determine whether JNK signaling could regulate the initial migration of cortical interneurons into the cerebral cortex. Jnk

protein is widely expressed in the developing forebrain (Fig. 1*A,B*), but appears to be enriched in the nascent cortical plate and mantle region of the ventral forebrain, where newly generated postmitotic neurons reside. Moreover, Jnk proteins are expressed by tangentially migrating *Dlx5/6-CIE*⁺ cortical interneurons as they enter the cerebral cortex (Fig. 1*D–F*). qRT-

Movie 1. Live imaging of cortical interneuron migration through the cortical entry zone at E12.5 in a control slice (Fig. 4). **Video clip 1**, Ventrolateral, bottom left; dorsomedial, upper right. Dlx5/6-CIE+ cells organize into migratory streams and rapidly invade the nascent cortical rudiment over the 24 h imaging period. **Video clip 2**, Cell track 1 (purple spot and tail) represents a Dlx5/6-CIE+ cell tracked during the first 12 h imaging period. Cell 1 maintains a medially directed trajectory, but shifts from SVZ to VZ regions of the slice during the tracking period. **Video clip 3**, Cell track 2 (red spot and tail) represents a Dlx5/6-CIE+ cell tracked during the last 12 h imaging period. Cell 2 maintains a long, medially directed trajectory, but shifts positions within the SVZ/IZ stream during the tracking period. Spots mark tracked cells, and tails represent where the cell traveled over the last hour of time.

Movie 2. Live imaging of cortical interneuron migration through the cortical entry zone at E12.5 in an SP600125-treated slice (Fig. 4). **Video clip 1**, Ventrolateral, bottom left; dorsomedial, upper right. Dlx5/6-CIE+ cells tentatively migrate into the cortical entry zone during the first 12 h of recording, often migrating slowly into the cortex only to reverse directions and leave. Migration improves during the last 12 h of recording, but many cells pile up in the MZ stream or spread out in the SVZ/IZ region without advancing past the cortical entry zone. **Video clip 2**, Cell track 1 (purple spot and tail) represents a Dlx5/6-CIE+ cell tracked during the first 12 h imaging period. Cell 1 migrates a short distance medially, traveling from the SVZ/IZ to the MZ regions of the slice, only to stall, turn, and migrate back toward the corticostriatal border. **Video clip 3**, Cell track 2 (red spot and tail) represents a Dlx5/6-CIE+ cell tracked during the last 12 h imaging period. Cell 2 migrates in a radial trajectory, traveling from the VZ to the MZ region of the slice, only to turn and migrate laterally, like Cell 1. Spots mark tracked cells, and tails represent where the cell traveled over the last hour of time.

PCR analysis of FACS sorted Dlx5/6-CIE+ and Dlx5/6-CIE(−) cells from dissociated E14.5 cortices indicated that *Jnk1* mRNA is enriched at nearly twofold (average fold change $\pm$ SEM = 1.99 ± 0.22) in Dlx5/6-CIE+ cortical interneurons, while *Jnk2* is uniformly expressed in both populations (Fig. 1H). Thus, Dlx5/6-CIE+ cortical interneurons express Jnk proteins as they enter the cortical rudiment and, compared with Dlx5/6-CIE(−) cortical cells, selectively express *Jnk1* at higher levels.

Interneuron entry into the cortical rudiment requires intact JNK signaling

To evaluate the initial entry of interneurons into the cerebral cortex under controlled conditions, we prepared live-vibratome slices from E12.5 Dlx5/6-CIE+ brains and cultured them for 1 d *in vitro* (Fig. 2A). Initially, very few cortical interneurons were present in the cortical rudiment (Fig. 2B). Over the next day *in vitro* (DIV), Dlx5/6-CIE+ cortical interneurons rapidly invaded the lateral aspect of the cerebral cortex (Fig. 2C,D), recapitulating their cortical entry *in vivo* (Fig. 1A–C).

To determine whether interneuron entry into the cerebral cortex depends on intact JNK signaling, we cultured E12.5 Dlx5/6-CIE+ slices for 1 DIV in the presence of different concentrations of SP600125, a selective, reversible, pan inhibitor of JNK signaling (Bennett et al., 2001). At 1 μ M, SP600125 had little effect on interneuron entry into the cortex (Fig. 2E). As the concentration of SP600125 was increased from 5 to 40 μ M, however, a dose-dependent inhibition of interneuron migration into the cerebral cortex was observed (Fig. 2F–H). To quantify interneuron entry into the cortex, we determined the frequency in which Dlx5/6-CIE+ interneurons were found in five equidistant cortical bins (Fig. 2I). When compared with control sections, interneurons from SP600125-treated sections showed a dose-dependent accumulation in the most lateral cortical bin, and dose-dependent reductions in medial bin locations (Fig. 2J). Thus, pharmacological blockade of JNK signaling prevents the initial entry of Dlx5/6-CIE+ cortical interneurons into the cerebral cortex in a dose-dependent fashion.

Blockade of interneuron entry into the cerebral cortex by JNK inhibition is specific and reversible

To determine whether disruption of cortical interneuron migration was specific to JNK inhibition, we evaluated whether pharmacological blockade of the MAPK signal transduction cascade had a similar effect on the initial entry of cortical interneurons into the cortical rudiment. Unlike JNK inhibition, U0126, a potent and selective inhibitor of Mek1/2 (Favata et al., 1998), failed to prevent the entry of Dlx5/6-CIE+ interneurons into the cerebral cortex (Fig. 3C). Indeed, no differences were found between the advancement of Dlx5/6-CIE+ interneurons in slices treated with 10 μ M U0126 and control slices (Fig. 3D), confirming previous assessments of U0126 on cortical interneuron migration (Polleux et al., 2002).

To further validate the specificity of JNK inhibition on cortical interneuron migration, we cultured E12.5 Dlx5/6-CIE+ slices for 24 h in the presence of AS601245, an independent pan-inhibitor of JNK signaling (Carboni et al., 2004). Similar to SP600125 treatment, application of AS601245 significantly impaired entry of Dlx5/6-CIE+ interneurons into the cortical rudiment (Fig. 3B,D). Indeed, a 5 μ M concentration of AS601245 impaired interneuron entry to an extent approximating a 20 μ M concentration of SP600125 (Figs. 2G, 3B). Thus, application of two independent inhibitors of JNK signaling prevented interneuronal migration into the nascent cortical rudiment, while pharmacological inhibition of MAPK signaling had no effect, suggesting JNK signaling, but not MAPK signaling, is required for the initial migration of cortical interneurons into the cortical rudiment.

We next asked whether blockade of interneuron migration into the cerebral cortex by SP600125 treatment was reversible. E12.5 Dlx5/6-CIE+ slices were exposed to 20 μ M SP600125 for 12 h, rinsed, and grown for an additional 12 h in either control medium (“washout” condition; Fig. 3H) or medium containing freshly prepared 20 μ M SP600125 (“SP600125” condition; Fig.

Figure 5. Genetic ablation of *Jnk1* impairs migration of cortical interneurons *in vivo*. **A**, Sampling strategy for mutant embryo analyses (Figs. 5–7). Five E13.5 brains were serially sectioned and bilaterally sampled at four rostrocaudal locations per genotype (Cx, cortex; L, LGE; M, MGE; C, CGE; S, septum; T, thalamus). Cortices were divided into five equidistant bins, and the percentages of calbindin-positive cells appearing in cortical bins were determined. Data from rostral (slices 1 and 2) and caudal (slices 3 and 4) positions were analyzed separately. **B–E**, Distribution of calbindin-positive cells in *Jnk1*^{+/+} (**B**), *Jnk1*^{-/-} (**C**), and *Jnk1*^{-/-}; *Jnk2*^{+/-} (**D**) cortices at rostral positions. Furthest extent of interneuron progression is marked by closed (MZ) and open (SVZ/IZ) arrowheads in each image. **E**, Statistically significant interactions were found between genotype and bin location (two-way ANOVA: $F_{(8,60)} = 2.742$; $p = 0.0120$). Compared with *Jnk1*^{+/+} controls, migration of calbindin-positive cortical interneurons was significantly impaired in *Jnk1*^{-/-}; *Jnk2*^{+/-} embryos (Fisher's LSD: *** $p = 0.0005$; * $p = 0.019$ in Bin 1, 0.042 in Bin 3). **F–I**, Distribution of calbindin-positive cells in the caudal cortex of *Jnk1*^{+/+} (**F**), *Jnk1*^{-/-} (**G**), and *Jnk1*^{-/-}; *Jnk2*^{+/-} embryonic brains (**H**). **I**, Statistically significant interactions were observed between genotype and bin location (two-way ANOVA: $F_{(8,60)} = 2.463$; $p = 0.0223$). Migration of calbindin-positive cortical interneurons was significantly impaired when *Jnk1*^{-/-} (Fisher's LSD: ** $p = 0.005$; * $p < 0.049$) and *Jnk1*^{-/-}; *Jnk2*^{+/-} cortices (Fisher's LSD: ** $p = 0.003$; * $p = 0.017$) were compared with *Jnk1*^{+/+} controls. LGE, lateral ganglionic eminence.

3G). When compared with control slices (Fig. 3F), slices in both washout and SP600125 conditions displayed impaired migration (Fig. 3G,H). Dlx5/6-CIE+ interneurons showed robust recovery in washout conditions, however, and migrated much further into the cortical rudiment than Dlx5/6-CIE+ interneurons in slices continuously exposed to SP600125 (Fig. 3E–H). Thus, SP600125 treatment impairs interneuron entry into the cortex, but this effect is largely reversible upon removal of JNK inhibition.

JNK inhibition impairs directed migration of cortical interneurons into the cortical rudiment

To further examine consequences of JNK inhibition on the migration of cortical interneurons, time-lapse recordings were made from E12.5 Dlx5/6-CIE+ slices exposed to control medium or medium containing 20 μ M SP600125. Live images of the cortical entry zone and lateral aspect of the cortical rudiment were acquired every 10

min for 24 h in control and JNK-inhibited conditions, and migratory properties of Dlx5/6-CIE+ cortical interneurons were examined as they entered and navigated the cerebral cortex.

In control slices, Dlx5/6-CIE+ interneurons robustly entered the cortical rudiment and traveled tangentially to fill the lateral cortical wall (Fig. 4A–D; Movie 1, video clip 1). Many Dlx5/6-CIE+ interneurons traversed the cortical entry zone by 12 h of recording (Fig. 4C), and by 24 h, Dlx5/6-CIE+ interneurons had formed MZ and SVZ/IZ streams on their medial progression through the cortex (Fig. 4D), often migrating beyond the field of view.

In stark contrast, migration of Dlx5/6-CIE+ interneurons through the cortical entry zone was severely compromised when slices were treated with 20 μ M SP600125 (Fig. 4E,H; Movie 2, video clip 1). Dlx5/6-CIE+ interneurons remained motile, yet failed to advance in medially oriented trajectories. After 12 h, far fewer Dlx5/6-CIE+ interneurons had entered the cortex (Fig.

4G) compared with control conditions (Fig. 4C). By 24 h, progression of *Dlx5/6*-CIE+ interneurons into the cortex had improved (Fig. 4H), but failed to match the extent of interneuron migration seen in controls (Fig. 4D), as previously quantified (Fig. 2J). *Dlx5/6*-CIE+ cells often stacked up in the MZ stream (Fig. 4H), and remained dispersed in the SVZ/IZ region without forming an organized migratory stream.

To quantify changes in migratory behavior, individual *Dlx5/6*-CIE+ interneurons were analyzed in control and SP600125-treated slices. Representative cell tracks of cortical interneurons in control (Fig. 4I,J; Movie 1, video clips 2–3) and JNK-inhibited (Fig. 4K,L) conditions are shown for movies depicted in Figure 4. Navigational errors were apparent during the first 12 h of imaging in SP600125-treated slices, when *Dlx5/6*-CIE+ interneurons stall their advancement at the entrance to the cortex, and take short, aberrant paths toward the pial or ventricular surfaces (Fig. 4K; Movie 2, video clip 2). Lengths of individual cell tracks (track length) increased during the last 12 h of imaging in SP600125-treated slices, but *Dlx5/6*-CIE+ interneurons typically remained misguided (Fig. 4L; Movie 2, video clip 3), and the entire migratory front of cortical interneurons remained less advanced compared with controls.

For statistical comparisons, 240 migratory interneurons from 12 control and 12 SP600125-treated slices were tracked and their trajectories were analyzed. Twenty *Dlx5/6*-CIE+ interneurons were tracked from each slice: 10 during the first 12 h, and 10 during the last 12 h of imaging. Speed of interneuron advancement significantly declined after SP600125 treatment, with greatest deficits occurring during the first 12 h period (Fig. 4M). Maximum, mean, minimum, and SD velocities of *Dlx5/6*-CIE+ interneurons diminished significantly in SP600125-treated slices (values = avg ± SEM; Control: max = 139.6 ± 4.4 , mean = 59.6 ± 2.0 , min = 7.6 ± 0.5 , SD = 35.1 ± 1.0 $\mu\text{m/h}$; SP600125: max = 90.3 ± 3.3 , mean = 38.5 ± 1.8 , min = 4.9 ± 0.4 , SD = 21.8 ± 0.9 $\mu\text{m/h}$). Accordingly, average track lengths from the beginning to ending point of their trajectories (displacement) as well as average cumulative distances traveled (total) were significantly impaired during the first 12 h period (Control: displacement = 204.6 ± 8.1 , total = 297.2 ± 12 μm ; SP600125: displacement = 98.9 ± 8.3 , total = 208.4 ± 10.7 μm ; Fig. 4N). Migratory velocities of interneurons in SP600125-treated slices increased during the last 12 h of imaging (Fig. 4P), but remained significantly lower than controls (Control: max = 129.6 ± 2.9 , mean = 54.3 ± 1.7 , min = 7.0 ± 0.6 , SD = 32.8 ± 0.8 $\mu\text{m/h}$; SP600125: max = 107.8 ± 2.7 , mean = 44.5 ± 1.6 , min = 4.9 ± 0.5 , SD = 26.8 ± 0.8 $\mu\text{m/h}$). As velocity increased during the last 12 h of imaging, so did distances traveled by individual cortical interneurons. Although both displacement and total track lengths increased, displacement length remained reduced to statistically significant levels in the last 12 h of imaging (Control: displacement = 197.6 ± 6.4 , total = 270.5 ± 8.4 μm ; SP600125: displacement = 170.6 ± 11.3 , total = 261.7 ± 12.6 μm ; Fig. 4Q).

Figure 6. Unlike *Jnk1*, genetic ablation of *Jnk2* does not disrupt migration of cortical interneurons *in vivo*. At both rostral (A–D) and caudal (E–H) levels, calbindin-positive interneurons are equally distributed in *Jnk2*^{+/+} (A, E), *Jnk2*^{−/−} (B, F), and *Jnk2*^{−/−}; *Jnk1*^{+/−} (C, G) cortices. No statistically significant interactions were found between genotype and bin location at either rostral (two-way ANOVA: $F_{(8,60)} = 0.131$; $p = 0.998$; D) or caudal positions (two-way ANOVA: $F_{(8,60)} = 0.135$; $p = 0.997$; H).

Directionality of migration as reflected in overall track straightness values, however, was significantly altered in interneurons from SP600125-treated slices during both first (Control: $r = 0.70 \pm 0.01$; SP600125: $r = 0.47 \pm 0.02$) and last 12 h intervals (Control: $r = 0.74 \pm 0.02$; SP600125: $r = 0.64 \pm 0.02$; Fig. 4O,R).

Improvements observed in migratory speed and track length during the last 12 h of imaging in SP600125-treated slices likely reflect declining efficacy of the pharmacological inhibitor over the 24 h imaging period. When interneurons were allowed to grow into the cerebral cortex for 12 h in control conditions before treatment with SP600125, deficits in interneuron migration were comparable to those observed in acutely treated slices. For example, mean interneuron velocity was reduced to 37.2 ± 1.2 $\mu\text{m/h}$, which was slightly less than the mean velocity observed during the first 12 h of SP600125 treatment. Furthermore, to evaluate whether the JNK requirement persisted beyond cortical entry at E12.5, we analyzed migratory properties of control and JNK-inhibited cortical interneurons at the leading front of migration in E14.5 cortices. Similar to SP600125 treatment at E12.5, pharmacological inhibition of JNK at E14.5 led to statistically significant reductions in total track length (Control: 267.3 ± 17.4 ; SP600125: 188.1 ± 8.5 μm ; $*p = 0.01$), mean velocity (Control: 51.7 ± 0.8 ; SP600125: 32.1 ± 1.6 $\mu\text{m/h}$; $***p = 0.0004$), and directionality (Control: 0.75 ± 0.05 ; SP600125: 0.56 ± 0.05 ; $*p = 0.04$). Thus, inhibition of JNK signaling significantly slows the advancement and alters the trajectories of migratory cortical interneurons in the cortical entry zone at E12.5 and at the leading front of migration in E14.5 cortices.

Jnk1 regulates cortical interneuron migration at the cortical entry zone *in vivo*

To determine whether loss of *Jnk* genes compromised migration of cortical interneurons *in vivo*, we evaluated the distribution of

Figure 7. Cortical interneurons have a cell-intrinsic requirement for JNK signaling to migrate through the cortical entry zone *in vivo*. *Jnk1* was conditionally ablated in *Dlx5/6-CIE*+ cells of *Jnk2*^{-/-} embryos. **A–D**, Distribution of *Dlx5/6-CIE*+ (green) and calbindin-positive (red/yellow) cortical interneurons in the rostral cortex of *Dlx5/6-CIE; Jnk1^{fl/+}; Jnk2^{-/-}* (**A**) and *Dlx5/6-CIE; Jnk1^{fl/fl}; Jnk2^{-/-}* embryonic brains (**B**). **C, D**, Statistically significant interactions were found between genotype and bin location for *Dlx5/6-CIE*+ (two-way ANOVA $F_{(4,40)} = 11.55$; $p < 0.0001$; **C**) and calbindin-positive cortical interneurons (two-way ANOVA $F_{(4,40)} = 12.42$; $p < 0.0001$; **D**). Migratory advancement of interneurons was significantly impaired in *Dlx5/6-CIE; Jnk1^{fl/fl}; Jnk2^{-/-}* cortices (Fisher's LSD: *Dlx5/6-CIE*: **** $p < 0.0001$, *** $p = 0.0003$; calbindin: **** $p < 0.0001$, ** $p = 0.002$). **E–H**, Distribution of *Dlx5/6-CIE*+ and calbindin-positive cortical interneurons in the caudal cortex of *Dlx5/6-CIE; Jnk1^{fl/+}; Jnk2^{-/-}* (**E**) and *Dlx5/6-CIE; Jnk1^{fl/fl}; Jnk2^{-/-}* embryonic brains (**F**). **G, H**, Statistically significant interactions were found between genotype and bin location for both *Dlx5/6-CIE*+ (two-way ANOVA: $F_{(4,40)} = 8.336$; $p < 0.0001$; **G**) and calbindin-positive (**H**) cortical interneurons (two-way ANOVA: $F_{(4,40)} = 11.98$; $p < 0.0001$). Similar to rostral levels, migratory advancement of interneurons was significantly impaired at caudal levels in *Dlx5/6-CIE; Jnk1^{fl/fl}; Jnk2^{-/-}* cortices (Fisher's LSD: *Dlx5/6-CIE*: *** $p = 0.0002$ in bin1, 0.0007 in bin3; calbindin: **** $p < 0.0001$, *** $p = 0.0007$, * $p = 0.029$).

calbindin-expressing cortical interneurons in an allelic series of *Jnk1* and *Jnk2* single and combinatorial mutant embryos at E13.5 (Figs. 5, 6), after the initial cohort of interneurons have entered the cerebral cortex. At this age, calbindin expression labels most tangentially migrating interneurons in the cortical rudiment (Anderson et al., 1997). Five embryos of each genotype were coronally sectioned, and the lateral cortical wall was bilaterally sampled at four rostrocaudal locations spanning sections containing the rostral MGE to mid caudal ganglionic eminence (CGE; Fig. 5A). The cortical rudiment was subdivided into five equidistant bins spanning the corticostriatal boundary laterally (Bin1) to cortical arch medially (Bin5; Fig. 5A), and the percentage of calbindin-positive cortical interneurons found in each cortical bin was determined for each section and averaged across all embryos of the same genotype. Since placement of equidistant bins was done with respect to the length of each cortical hemisphere, our sampling strategy accounts for variation in cortical length that might occur between sections.

In *Jnk1*^{+/-} embryos, calbindin-positive interneurons displayed normal tangential migration into the cortical rudiment at both rostral and caudal probe locations (Fig. 5B,F). However, calbindin-positive interneurons in both MZ and SVZ/IZ streams of *Jnk1*^{-/-} embryos (Fig. 5C,G) were less advanced than calbindin-positive interneurons in *Jnk1*^{+/-} embryos (Fig. 5B,F). Accordingly, the proportion of calbindin-positive interneurons increased laterally (Bin1) and declined medially (Bin3–5; Fig. 5E,I). In this and subsequently presented data, statistically significant increases in interneuron abundance are seen in Bin1 and statistically significant decreases in interneuron abundance are seen in Bin3 when migratory deficits are present. Bin2 likely remains unchanged due to transitional normalization occurring between Bins 1 and 3, and while reductions of interneuron abundance occur in Bins 4–5, they often do not reach statistical significance due to the relatively small percentages of cells found in those bins at E13.5. Although reductions of interneuron migration were apparent at both rostral and caudal probe locations in

Figure 8. *In vitro* migration of cortical interneurons from explants of MGE tissue does not rely on JNK signaling. **A**, Illustration of experimental design (see Materials and Methods). **B**, **C**, Explant of MGE tissue isolated from an E12.5 *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* embryonic brain section and cultured for 2 d in a collagen:Matrigel matrix. Interneurons have migrated from explant borders in a radial pattern. **D**, **E**, MGE explant from a E12.5 *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* brain grown under identical conditions, and exhibiting a similar pattern of interneuron outgrowth. **F**, Areas measured from explant shown in **B** and **C**, illustrating that outgrowth area (dark blue) was determined by subtracting the explant area (light blue) from the total area occupied by both explant and migrating cells. **G**, Plot representing mean values of normalized outgrowth area (outgrowth area/explant area) for 10 explants from each genotype. DIC images (**B**, **D**) were used to identify explant boundaries, while perimeters of migrating cells were traced from images containing the EGFP channel alone (**C**, **E**).

Jnk1^{-/-} embryos, reductions in medial progression only reached statistical significance at caudal probe locations (Fig. 5*E,I*).

To determine whether reduction of *Jnk2* in combination with loss of *Jnk1* exacerbated migratory phenotypes of cortical interneurons, we analyzed the distribution of calbindin-positive cortical interneurons in embryos lacking *Jnk1* and a single genomic copy of *Jnk2* (*Jnk1*^{-/-};*Jnk2*^{+/-}), since *Jnk1/2* double nulls die before interneuron migration into the cerebral cortex (Kuan et al., 1999). In *Jnk1*^{-/-};*Jnk2*^{+/-} embryos, advancement of calbindin-positive interneurons in both MZ and SVZ/IZ streams was significantly diminished relative to *Jnk1*^{+/-} controls at both rostral and caudal probe locations (Fig. 5*D,H*). Statistically significant interactions were found between genotype and

bin position at rostral and caudal probe locations (Fig. 5*E,I*), suggesting loss of *Jnk1* alone and loss of *Jnk1* combined with reduction of *Jnk2* inhibits tangential progression of cortical interneurons *in vivo*. At caudal locations, calbindin-positive interneurons in *Jnk1*^{-/-};*Jnk2*^{+/-} and *Jnk1*^{-/-} embryos were comparably regressed (Fig. 5*G–I*), but at rostral locations, calbindin-positive interneurons in *Jnk1*^{-/-};*Jnk2*^{+/-} embryos were significantly less advanced than those in *Jnk1*^{-/-} embryos (Fig. 5*C–E*). To ensure that potential differences in brain size did not influence interpretation of our results, we measured absolute cortical lengths from every section we analyzed and found no statistically significant differences in cortical length between genotypes by one-way ANOVA (*Jnk1*^{+/-} = 937.8 ± 17.4, *Jnk1*^{-/-} = 1005.0 ± 21.0, *Jnk1*^{-/-};*Jnk2*^{+/-} = 935.4 ± 27.6 μm). Thus, at rostral positions, reducing *Jnk2* in combination with the loss of *Jnk1* has a greater impact on cortical interneuron migration than loss of *Jnk1* alone.

To determine whether *Jnk2* plays a comparable role to *Jnk1* in the initial migration of cortical interneurons *in vivo*, we generated embryos with the reciprocal *Jnk2*^{+/-}, *Jnk2*^{-/-}, and *Jnk2*^{-/-};*Jnk1*^{+/-} genotypes, and evaluated tangential progression of calbindin-positive cortical interneurons at E13.5. Normal migration of cortical interneurons was observed in *Jnk2*^{+/-} embryos at both rostral (Fig. 6*A,D*) and caudal (Fig. 6*E,H*) probe locations, and their cortical bin distribution closely matched *Jnk1*^{+/-} embryos (Fig. 5*E,I*). Unlike in *Jnk1*^{-/-} embryos, however, tangential progression of calbindin-positive interneurons was completely unperturbed in *Jnk2*^{-/-} embryos at rostral (Fig. 6*B,D*) and caudal probe locations (Fig. 6*F,H*). Moreover, removing a genomic copy of *Jnk1* in the context of *Jnk2* deletion did not alter the advancement of calbindin-positive cortical interneurons (Fig. 6*C,D,G,H*). Neither MZ nor SVZ streams were regressed in *Jnk2*^{-/-} or *Jnk2*^{-/-};*Jnk1*^{+/-} embryos relative to migratory streams in *Jnk2*^{+/-} controls (Fig. 6*A–G*), and the distribution of calbindin-positive cortical interneurons in cortical bins were nearly identical across all three genotypes at rostral and caudal locations (Fig. 6*D,H*). As before, cortical rudiment lengths were consistent between genotypes (*Jnk2*^{+/-} = 1032.6 ± 22.0, *Jnk2*^{-/-} = 1033.2 ± 27.6, *Jnk2*^{-/-};*Jnk1*^{+/-} = 1014.5 ± 9.3 μm). Apparently, retaining a single genomic copy of *Jnk1* is sufficient to leave cortical interneuron migration intact and unperturbed in the *Jnk2*^{-/-} background at E13.5.

Cortical interneurons have a cell-intrinsic requirement for *Jnk1* *in vivo*

Genetic ablation of *Jnk1* disrupts the initial migration of interneurons into the cortical rudiment, but since *Jnk1* is expressed in both migratory cortical interneurons and noninterneuronal cells of the developing cortex (Fig. 1), the requirement for JNK signaling in regulating cortical interneuron migration may not be cell autonomous. Indeed, radial migration of cortical projection neurons is accelerated in *Jnk1*^{-/-} embryos (Westerlund et al., 2011), which could lead to nonautonomous disruptions in the tangential migration of cortical interneurons. To determine whether the initial cohort of cortical interneurons has a cell-intrinsic requirement for JNK signaling to migrate into the cortical rudiment, we used mice expressing the *Dlx5/6-CIE* transgene to conditionally ablate *Jnk1* within cortical interneurons *in vivo*. Since migration of cortical interneurons was completely unperturbed after constitutive loss of *Jnk2*^{-/-} (Fig. 6), but reduction of *Jnk2* in *Jnk1* nulls exacerbated interneuron migratory phenotypes (Fig. 5), we conditionally ablated *Jnk1* in interneurons of *Jnk2*^{-/-} embryos. This allowed evaluation of

Figure 9. Cortical interneurons lacking *Jnk1* and *Jnk2* disperse from migratory streams and adopt branched morphologies *in vivo*. **A–C**, Radial distribution of Dlx5/6-CIE+ cells (green) in the lateral cortex of E13.5 Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* (**A**) and Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* embryos (**B**). Equivalently sized cortical probes were equidistantly binned along the radial axis (Bin 1 = MZ – Bin 6 = VZ) and the percentage of Dlx5/6-CIE+ cells appearing in each bin was determined ($n = 5$ embryos/genotype). **C**, Radial distribution of Dlx5/6-CIE+ cells is altered in Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* cortices (two-way ANOVA: $F_{(5,48)} = 13.26$; $p < 0.0001$). Proportionately fewer Dlx5/6-CIE+ cells are located in bin 4 (SVZ region; Fisher's LSD = $***p < 0.0001$), while more are located in bin 3 (IZ region; Fisher's LSD = $***p < 0.0001$). **D–G**, Dlx5/6-CIE+ (green) and calbindin-positive cells (red/yellow) from E13.5 Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* cortices. **D**, Cortical interneurons within the MZ travel in a coherent stream with few gaps (open arrowhead). **E**, Interneurons within the cortical SVZ/IZ stream bare medially directed (image right) leading processes. **F–G**, Examples of isolated Dlx5/6-CIE+ cells with simple, unbranched leading processes. **H–K**, Dlx5/6-CIE+ (green) and calbindin-positive cells (red/yellow) from E13.5 Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* cortices. **H**, Mutant MZ streams are patchy, containing many gaps (open arrowheads). **I**, Cortical interneurons are loosely organized within the mutant SVZ. Many interneuronal cell bodies (arrows) have no apparent leading processes, and many interneuronal processes (arrowheads) have no apparent cell bodies, suggesting many interneurons are orthogonally positioned to the plane of section. Interneurons with attached processes (asterisks) are often nonmedially directed. **J, K**, Dlx5/6-CIE+ cells from mutant cortices often have branched morphologies. Also note aberrant branching of interneurons in **B** and **I** compared with **A** and **E**.

interneurons that were completely deficient in both *Jnk1* and *Jnk2*, which would otherwise be impossible due to the mid-gestation embryonic lethality of constitutive *Jnk1/2* double mutation.

We predicted Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* embryos would not have deficiencies in interneuron migration, since interneuron migration was unperturbed in *Jnk2^{-/-};**Jnk1^{+/+}* embryos. Indeed, normal tangential migration of cortical interneurons was observed at E13.5 in Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* embryos (Fig. 7A,E). We analyzed Dlx5/6-CIE+ cortical interneurons, as well as the calbindin-expressing cohort of cortical interneurons,

which constitute most of the Dlx5/6-CIE+ cells in the cortical rudiment at E13.5. Both populations of labeled cells appeared normally distributed within the cortex of Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* embryos at rostral (Fig. 7C,D) and caudal probe locations (Fig. 7G,H). In Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* embryos, however, both Dlx5/6-CIE+ and calbindin-positive cortical interneurons were significantly regressed in their cortical advancement at rostral (Fig. 7B–D) and caudal (Fig. 7F–H) probe locations compared with interneurons in control brains. The distribution of interneurons within Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* cortices was significantly altered from control Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* cortices at rostral (Fig. 7C,D) and caudal probe locations (Fig. 7G,H). Similar to *Jnk1^{-/-}* and *Jnk1^{-/-};**Jnk2^{+/-}* embryos, cortical interneurons accumulated laterally and diminished medially within Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* cortices, with the largest statistical differences between genotypes occurring in Bin1 and Bin3 (Fig. 7C,D,G,H). Distributions of cortical interneurons within Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* embryos were comparable to *Jnk1^{-/-};**Jnk2^{+/-}* embryos, since both rostral and caudal portions of their trajectories were significantly regressed relative to their respective controls (Fig. 5E,I). This finding is consistent with *Jnk2* loss of function enhancing a *Jnk1^{-/-}* migratory phenotype at rostral probe locations. Cortical lengths were not significantly different between control and conditional *Jnk1/2* double knock-out embryos (Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* = 949.1 ± 50.1 , Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* = 922.7 ± 56.0 μ m). Collectively, these data suggest the initial cohort of cortical interneurons has a cell-intrinsic requirement for JNK signaling—largely mediated through the activity of *Jnk1*, but partially compensated for by *Jnk2* when *Jnk1* is lost—in migrating through the cortical entry zone *in vivo*.

Although our results indicate cortical interneurons have a cell-intrinsic requirement for JNK signaling to appropriately enter the cerebral cortex, there are two

possible explanations for this requirement: (1) JNK signaling could intrinsically enhance the migratory capacity of cortical interneurons or (2) JNK signaling could be required by cortical interneurons to sense or respond to environmental cues located in the cortical rudiment. To distinguish between these possibilities, we cultured explants of MGE tissue from E12.5 control or conditional double knock-out embryos in a reduced, serum-free *in vitro* environment devoid of cortical cues (Fig. 8A). MGE explants isolated from either Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* (Fig. 8B,C) or Dlx5/6-CIE;*Jnk1^{fl/fl};**Jnk2^{-/-}* (Fig. 8D,E) forebrain slices produced Dlx5/6-CIE+ migratory interneurons that dis-

persed in a radial pattern from the explant margins. When migratory outgrowth was measured blind to genotype (Fig. 8F), no statistical differences in interneuron migration were observed between *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* and *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* explants of MGE tissue (Fig. 8G). These data strongly suggest that JNK signaling does not act in cortical interneurons to autonomously promote or enhance their motility.

Integrity of cortical migratory streams depends on JNK signaling *in vivo*

Since we observed statistically significant deficits in the entry of cortical interneurons into the cortical rudiment after conditionally ablating *Jnk1* from interneurons of *Jnk2*-null mutants *in vivo* (Fig. 7), but failed to see migratory anomalies in the absence of cortical cues *in vitro* (Fig. 8), we hypothesized that cortical interneurons have a cell-intrinsic requirement for JNK signaling to navigate the early cortical rudiment *in vivo*. To determine whether JNK-deficient cortical interneurons are misrouted when first entering the cerebral cortex, we quantified the radial distribution of cortical interneurons within the lateral cortical wall of *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* control and *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* mutant embryos at E13.5 (Fig. 9A–C). Six equidistant bins were made along the radial axis of the lateral cortex (adjacent to the corticostriatal boundary), and the percentage of *Dlx5/6-CIE*+ cortical interneurons appearing in Bins 1 (MZ) through 6 (ventricular Zone; VZ) were determined for each genotype. In *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* embryos, *Dlx5/6-CIE*+ interneurons were predominantly distributed in the MZ (Bin 1) and SVZ/IZ (Bins 3–4) streams (Fig. 9A,C). In *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* embryos, however, the proportions of *Dlx5/6-CIE*+ interneurons located in the MZ stream (Bin 1) and SVZ portion of the SVZ/IZ stream (Bin 4) were diminished compared with control cortices (Fig. 9B,C). In addition, the percentage of *Dlx5/6-CIE*+ interneurons in the IZ (Bins 2–3) was elevated in conditional double mutants compared with controls (Fig. 9B,C). When compared statistically, distributions of *Dlx5/6-CIE*+ migratory interneurons within Bins 1–6 were significantly altered between *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* and *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* genotypes with the largest shifts occurring in Bins 3 and 4 (Fig. 9C), suggesting JNK-deficient interneurons were dispersing from the SVZ.

Moreover, repositioning of migratory interneurons within the radial axis of the *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* mutant cortex was accompanied by diminished integrity to both MZ and SVZ/IZ migratory streams. The MZ and SVZ/IZ streams of *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* control embryos (Fig. 9D,E) were consistently more cohesive than MZ and SVZ/IZ streams of *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* mutant embryos (Fig. 9H,I). *Dlx5/6-CIE*+ and calbindin-positive cortical interneurons were more loosely organized in *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* cortices, leading to frequent gaps within MZ and SVZ/IZ streams (Fig. 9H,I). Leading processes of cortical interneurons within the SVZ/IZ stream of *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* cortices were typically straight and medially oriented (Fig. 9E), while disordered and

Figure 10. Migration of cortical interneurons through the cortical entry zone *in vivo* requires intact JNK signaling, largely mediated by interneuron expressed *Jnk1*. Schematic drawings of the cortical rudiment at E13.5; ventrolateral is left, dorsomedial is right. Red shading represents JNK-dependent cortical “entry zone”. LOF, loss of function. Control *Jnk1^{+/+};Jnk2^{-/-};Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* genotypes, as well as *Jnk2^{-/-}* and *Jnk2^{-/-};Jnk1^{+/+}* genotypes, display normal tangential migration at rostral (top left) and caudal (top right) cortical locations at E13.5. Cortical interneuron migration within *Jnk1^{-/-}* embryos is diminished to statistically significant levels (red interneurons) at caudal (middle right), but not rostral locations (middle left). *Jnk1^{-/-};Jnk2^{+/+}* constitutive mutants and *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* conditional mutants have significantly diminished migration (red interneurons) at both rostral (bottom left) and caudal locations (bottom right). Additionally, interneurons completely devoid of both *Jnk1* and *Jnk2* are abnormal positioned in the radial axis of the cortex, disrupting the integrity of MZ and SVZ/IZ streams, and exhibit branching anomalies.

nontangentially oriented in the SVZ/IZ stream of *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* mutant cortices (Fig. 9I). In addition, interneuronal processes were often noncontiguous with their cell bodies (Fig. 9I), suggesting migratory orientations of *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* interneurons were frequently orthogonal to the coronal plane of section. Finally, processes of cortical interneurons in *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* embryos were often more highly branched (Fig. 9J,K) than those of control interneurons (Fig. 9F,G). Thus, conditional ablation of *Jnk1* from interneurons of *Jnk2^{-/-}* embryos disrupts the radial distribution of cortical interneurons at the entrance to the cortical rudiment, diminishes the integrity of MZ and SVZ/IZ migratory streams, and leads to morphological alterations in migratory cortical interneurons. Collectively, these data suggest cortical interneurons have an intrinsic requirement for JNK signaling in navigating the cortical entry zone *in vivo*.

Discussion

In the current study, we identified novel and essential roles for JNK signaling in cortical interneuron migration. We established that cortical interneurons express *Jnks* as they enter the cerebral cortex and, compared with noninterneuronal cortical cells, have enriched expression of *Jnk1*. Using *ex vivo* slice cultures, we demonstrated pharmacological blockade of JNK results in dose-dependent disruption of interneuron migration into the cerebral cortex, and that JNK-inhibited cortical interneurons slow their advancement and take aberrant trajectories as they navigate the cortical entry zone at E12.5. JNK inhibition at E14.5 similarly perturbed migratory properties of cortical interneurons, indicating their requirement for JNK persists at later embryonic stages. *In vivo* analyses of single and multi-allelic mutations of two *Jnk* genes revealed that loss of *Jnk1* impairs cortical interneuron migration, while loss of *Jnk2* does not, unless *Jnk2* is reduced in *Jnk1* nulls. Finally, we showed conditional deletion of *Jnk1* in interneurons of *Jnk2* nulls recapitulates migratory deficits observed in constitutive *Jnk1^{-/-};Jnk2^{+/+}* embryos, and in addition, disrupts migratory streams and cortical interneuron morphology.

Despite this clear intrinsic requirement for JNK activity *in vivo*, JNK-deficient interneurons migrate normally *in vitro*, suggesting JNK regulates cortical interneuron migration in response to cortical guidance cues. Together, our results suggest that cortical interneurons have an intrinsic requirement for *Jnk1* to enter and navigate the developing cerebral cortex, making the JNK signaling pathway a key intracellular mediator of cortical interneuron migration *in vivo*.

JNKs and neuronal migration

JNK signaling orchestrates diverse cellular processes in the developing nervous system including cell survival (Kuan et al., 1999; Sabapathy et al., 1999), axon elongation and stability (Hirai et al., 2006; Wang et al., 2007; Yamasaki et al., 2011), axon guidance (Qu et al., 2013), and radial migration (Hirai et al., 2006; Wang et al., 2007; Westerlund et al., 2011; Yamasaki et al., 2011). The extent to which individual *Jnk* genes have distinct functions remains to be elucidated (Haeusgen et al., 2009; Yamasaki et al., 2012). Here, we report *Jnk1* plays a greater role than *Jnk2* in cortical interneuron migration. This may either reflect functional differences between *Jnk1* and *Jnk2*, or unequal expression of *Jnk1* and *Jnk2* in migratory cortical interneurons. The contribution of *Jnk3* to cortical interneuron migration is currently unknown.

Radial migration in the developing cortex relies on JNK signaling, yet it is unclear whether *Jnk1*, *Jnk2*, and *Jnk3* play equivalent roles. Deletion of upstream JNK activators including *Map3k12* (Hirai et al., 2006), *Map2k4* (Wang et al., 2007), or *Map2k7* (Yamasaki et al., 2011) inhibits radial migration. Surprisingly, deletion of *Jnk1* has the opposite effect: radial migration into the cortical plate is accelerated in *Jnk1* nulls (Westerlund et al., 2011). These conflicting results could be explained if *Jnk2* and/or *Jnk3* play opposite roles to *Jnk1* in radial migration, since deletion of upstream JNK activators would diminish activity of all available Jnk isoforms, whereas single deletion of *Jnk1* would not. We find pharmacological inhibition of all JNK activity, as well as genetic deletion of *Jnk1* or *Jnk1* and *Jnk2* together, inhibits tangential progression of cortical interneurons. These results indicate that at least *Jnk1* and *Jnk2* act in parallel to promote cortical interneuron migration *in vivo*. Thus, both radial and tangential migration requires *Jnk1*-mediated signaling, but *Jnk1* function exerts opposite effects on radially versus tangentially migrating neuroblasts. Differential expression of downstream JNK targets may underlie the molecular bases of these distinctions. For example, the microtubule regulatory protein SCG10/stathmin-2 is a major downstream effector of *Jnk1* in radially migrating neuroblasts (Westerlund et al., 2011); cortical interneurons may rely on other JNK substrates, however.

JNK signaling at the “cortical entry zone”

Our data suggest the first cohort of cortical interneurons requires JNK activity to enter and navigate the lateral cortical rudiment. We call this segment of their migratory pathway the cortical entry zone, which bridges the subcortical telencephalon and neocortex. Extracellular guidance cues located at the cortical entry zone likely direct and concentrate cortical interneurons into newly emerging migratory streams. Indeed, *Cxcl12* is highly expressed within the SVZ/IZ at the cortical entry zone (Stumm et al., 2003; 2007; Tiveron et al., 2006; López-Bendito et al., 2008), and is required for interneuron migration into the cortex via the SVZ/IZ stream (Tiveron et al., 2006; López-Bendito et al., 2008). Thus, cortical interneurons respond to extracellular guidance cues located at the cortical entry zone to correctly navigate the nascent cerebral cortex, and our data suggest JNK signaling crit-

ically regulates the spatial and temporal precision for which this occurs.

We provide substantial genetic evidence indicating that JNK signaling regulates interneuron migration at the cortical entry zone *in vivo* (Fig. 10). Constitutive ablation of *Jnk1* diminishes cortical interneuron migration at E13.5, with significant reductions in migration occurring at caudal cortical locations. Loss of *Jnk2* alone or loss of *Jnk2* with heterozygous reduction of *Jnk1* has no discernable effect, however, suggesting a single genomic copy of *Jnk1* is sufficient to maintain normal migratory properties of cortical interneurons—even in the absence of *Jnk2*. In contrast, heterozygous reduction of *Jnk2* in *Jnk1* nulls impairs cortical interneuron migration more than loss of *Jnk1* alone, since tangential migration into both rostral and caudal portions of their cortical trajectory are compromised. Thus, compared with *Jnk1*, *Jnk2* plays a relatively minor role in cortical interneuron migration, and its influence is only apparent rostrally when *Jnk1* function is lost. Conditional deletion of *Jnk1* in interneurons of *Jnk2* nulls, which completely eliminates *Jnk1* and *Jnk2* function in cortical interneurons, significantly impairs cortical interneuron migration throughout the rostrocaudal axis of the developing cortex. Not only is cortical entry compromised when *Jnk1* and *Jnk2* function is eliminated from cortical interneurons, but MZ and SVZ/IZ streams are less cohesive, radial positioning of migratory interneurons is altered, and migratory cortical interneurons are more highly branched. In a reduced *in vitro* environment, however, JNK-deficient interneurons migrate normally, suggesting cortical interneurons require JNK to sense or respond to cortical guidance cues rather than promote motility. Together, our *in vivo* genetic, *ex vivo* pharmacologic, and *in vitro* migration data suggest cortical interneurons have a cell-intrinsic requirement for JNK signaling to guide their migration into and within the developing cerebral cortex.

Two cell biological processes control tangential migration of cortical interneurons: (1) dynamic remodeling of the leading process through branching and elongation and (2) translocation of the nucleus into the leading process, or nucleokinesis. Both events require precise molecular control of actin and microtubule cytoskeleton (Bellion et al., 2005; Godin et al., 2012), and must be coordinated in a stepwise fashion to orient migration toward chemoattractant guidance cues (Martini et al., 2009). Although cellular and molecular mechanisms underlying the requirement for JNK in migrating cortical interneurons are currently unknown, we hypothesize JNK signaling acts as a molecular bridge between cortically encountered guidance cues and cytoskeletal machinery driving migration. One potential mediator is doublecortin, a microtubule-associated protein regulated by JNK phosphorylation (Gdalyahu et al., 2004; Jin et al., 2010), and required for proper branching, nucleokinesis, and directed migration of cortical interneurons (Kappeler et al., 2006; Fricourt et al., 2007). The degree to which extracellular guidance cues activate JNK signaling to modulate doublecortin, or other regulators of cytoskeletal dynamics in migrating cortical interneurons, such as *Lis1* (Gopal et al., 2010), *p27^{Kip1}* (Godin et al., 2012), or *Rac-GTPases* (Tahirovic et al., 2010; Tivodar et al., 2014), remains to be determined.

Relevance to neuropsychiatric disorders

Cortical interneurons are cellular targets for the pathogenesis of several developmental disorders of cortical connectivity, including schizophrenia and autism (Marín, 2012). Recent work in a mouse model of 22q11.2 deletion syndrome (22q11.2DS), which confers significant genetic risk for schizophrenia and autism in

humans (Murphy et al., 1999; Fine et al., 2005; Niklasson et al., 2009), has revealed that embryonic migratory deficits in cortical interneurons lead to laminar repositioning of parvalbumin-positive interneurons in adult cortices (Meechan et al., 2009, 2012). Similar to JNK-deficient interneurons, cortical interneurons in 22q11.2DS brains display delayed cortical entry at E13.5 and irregularities in their migratory streams (Meechan et al., 2009, 2012). Interneuron migratory deficiencies in 22q11.2DS mice are cell autonomous and largely result from reductions in the chemokine receptor *Cxcr4* (Meechan et al., 2012). Independent analysis of a similar mouse model confirmed that functional defects in *Cxcl12/Cxcr4* signaling underlie interneuron deficiencies in 22q11.2DS mice (Toritsuka et al., 2013), and implicated *Dgcr8* as the mediator. It is currently unknown whether genes within the 22q11.2 locus, including *Dgcr8*, interact with the JNK pathway, but similarities in interneuron migratory deficits are highly suggestive of a connection. One plausible explanation is that *Cxcl12-Cxcr4* signaling activates JNK to guide interneuron migration and promote migratory stream integrity at the cortical entry zone, but this hypothesis remains to be tested.

Uncovering cellular and molecular mechanisms underlying the pathogenesis of cortical connectivity disorders is essential for developing effective strategies for their treatment. Our current work identifies the JNK signaling pathway, and *Jnk1* in particular, as a major regulator of interneuron migration *in vivo*—making the JNK pathway a novel candidate to consider in the etiology of cortical circuit disorders and a target for potential therapeutic interventions.

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