GROUP A P21-ACTIVATED KINASES IN RADIAL NEURONAL MIGRATION

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The p21-activated kinases (PAKs) are downstream effectors of signal transducers including Rac and Cdc42. PAKs are involved in cytoskeletal rearrangements during cell migration, mitosis, dendritic spine formation, and neurite outgrowth. While the general expression pattern of PAKs in the brain was known, possible effects of PAK kinase activity on early brain development had not been described. I studied the expression pattern of activated PAKs 1-3 by immunoblotting and immunofluorescence in embryonic mouse brains and the effect of disruption of PAK activity during development on radial migration and early neuronal remodeling at the cortical plate. Active PAKs were observed in progenitor cells, in which they were coexpressed with PAK1 phosphorylated at T212, and in the developing cortical plate. Disruption of PAK activity did not affect radial cell distribution in the neocortex or neurite number in early neurons in the cortical plate.
To my nephew, Max Eitan Halberstadt-Turits, whose brain is beginning its wonderful journey.
ACKNOWLEDGMENTS

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<td>cortical plate</td>
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<td>IZ</td>
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INTRODUCTION

Disruptions of development of the mammalian brain can cause various disorders, such as mental retardation and epilepsy. Neurons in the cerebral cortex arise from glial progenitors in the developing brain and migrate by complex sequences of events to their final destinations. Neuronal migration involves many steps requiring integration of extracellular cues, adhesion to extracellular guides, establishment of polarity, and cytoskeletal remodeling events of process extension, retraction, and somal translocation. The p21-activated kinases (PAKs), several of which are highly expressed in brain, interact with many of the molecular components involved in migration, including components that transduce external stimuli and internal modulators of the cytoskeleton. While some of the roles of PAKs in cell migration had been studied in cultured cells, their effects in in vivo had not been explored.

Neurogenesis and migration

The mature mammalian cerebral cortex is composed of six primary layers of neurons that arise from progenitors lining the lateral ventricles of the telencephalon. Glial cells derived from early neuroepithelial progenitors located in the ventricular zone, adjacent to the ventricle, give rise to post-mitotic neurons and extend fibers that guide migrating neurons. Migrating neurons move out of the ventricular zone, forming new cortical layers. Early born neurons migrate into layers adjacent to the ventricular zone, while later born neurons migrate past these layers in an inside out layering order. Initially, the prospective cortex consists of a neuroepithelium lining the ventricles. Glia in the ventricular zone give rise to neurons that migrate out of the ventricular zone and form the preplate. Later divisions give rise to neurons that migrate in an inside out order to form the layers of the cortex.

Two major types of cortical neurons, pyramidal projection neurons and inhibitory interneurons, arise from progenitors in the dorsal telencephalon and ventral telencephalon, respectively.
Neurons arising in the dorsal telencephalon migrate radially, while those from the ventral telencephalon migrate tangentially followed by oblique or radial migration to reach their final laminar position. In mice, the period of proliferation in the ventral telencephalon involves 11 cell cycles from, approximately, embryonic days 10 to 17 (E10 to E17), out of a total gestation of 20 days.

Neurons undergo significant changes during radial migration in a process that depends on multiple factors. Disruption of some of these factors has been shown to lead to abnormal migration. Radial migration can be divided into four stages in developing rat. Progenitor radial glia, which extend a long process to the pial surface, undergo interkinetic nuclear movement within the ventricular zone. Following division of a glial progenitor at the ventricular surface, a postmitotic newborn neuroblast [1] moves along its progenitors’ radial process to the subventricular zone, where [2] it may arrest for approximately 24 hours and develop a multipolar morphology. Some neuroblasts may then [3] reverse polarity, move back into the ventricular zone, and extend a leading process that contacts the ventricular surface. In the final stage, [4] neurons migrate radially toward the cortical plate. Neurons may migrate by locomotion in apposition to a radial glial process, while others may extend a leading process to the pia and migrate by nuclear translocation. Though some elements that affect radial migration are known, the full complement of factors and interactions is not understood.

**Cytoskeleton and migration**

Cellular motility is a multi-step process requiring temporal coordination of various elements of the cytoskeleton in spatially distinct areas within cells. When a cell (from a multicellular organism) moves in a directed manner it: adheres to an extracellular surface or matrix, establishes polarity, extends leading processes, releases and contracts trailing processes, and translocates its soma and nucleus. These steps require remodeling of the cytoskeleton, including actin and microtubules, and the generation of cellular forces. Several of the p21-activated kinases are closely linked to these processes and are associated with cellular migration.
PAK family of proteins

The p21-activated kinases are a family of serine-threonine kinases originally identified in a screen for Rho GTPase binding partners in rat brain cytosol, and are divided into two groups: A, consisting of PAKs 1-3, and B consisting of PAKs 4-6. Homologs are present in various organisms, including yeast, Drosophila, mouse, and human. PAKs 1-3 share extensive homology, while other PAKs are less well understood and vary from the canonical structure. The most extensively studied of the family is PAK1, which has diverse functions, including a role in cytoskeletal regulation and neurite extension.

PAK structure

The structure of PAK1 will be used to illustrate group A PAK structure. Group A PAKs have a N-terminal regulatory domain and a C-terminus kinase domain (Figure 1). Several phosphorylation sites are involved in activation and regulation of PAK1 function. Phosphorylation by Akt/PKB at Ser21 regulates PAK1 localization to focal adhesions. Phosphorylation at Thr212 by Cdc2 is cell cycle dependent and is associated with regulation of the microtubule structure during mitosis, while neuron specific p35/Cdk5 also phosphorylates Thr212 and is involved in neurite organization. Phosphorylation at Thr423 is necessary for stabilization of an active kinase conformation. PAK1 also supports the binding of several adapter proteins, including Nck, Grb2, Pix/Cool, and Gβγ. These adapters are involved in recruitment of PAK to membrane structures and in activation complexes involving PAK.
PAK1 is involved in the transduction of external stimuli to effectors of cytoskeletal remodeling. PAKs interact with other molecules by phosphorylating a wide range of downstream targets and by formation of multi-protein complexes. The kinase activity of PAK1 is activated through GTPase dependent and independent means. The GTPase activators of PAK, Rac and Cdc42, are major components of various signaling pathways affecting cell motility. Stimulation of these signaling networks causes PAK1 activation and recruitment to cellular protrusions. Once activated, PAKs can phosphorylate other target proteins, many of which are involved in remodeling of the cytoskeleton.

Cell migration and PAKs

PAKs are involved in cytoskeletal rearrangements associated with cellular polarity, motility, and remodeling. Upon stimulation, such as by adhesion to extracellular matrix (ECM), PAK1 becomes activated and localized from the cytosol to sites of dynamic cytoskeletal remodeling including cortical actin at the leading edge of polarized cells, at focal adhesions, lamellae, and at sites of membrane ruffling. PAK1 affects rates of migration, though these effects, whether increasing or decreasing migration, depend on the experimental conditions, including the type of cell studied, the extracellular matrix, and the means of stimulating the cell and activating PAK1.

Neuronal migration and PAKs

Mutations involving several proteins that interact with PAK1 cause neuronal migration defects. Defects in the neuron specific p35/Cdk5 kinase, which phosphorylates PAK1 at Thr212, leads to inverted cortical layering and abnormal neuronal migration. Rac1, acting via PAK1, participates in...
dendrite formation in cortical neurons. Both Rac1 and Cdc42 activate PAKs and are involved in normal rates of migration and neuronal positioning. PAK1 affects neuronal migration in cell culture, but has not been studied in live tissue.

**PAK expression and mutations**

PAKs 1 and 3 are enriched in brain tissue, suggesting a possible brain-specific role, while PAK2 is expressed in most tissues. Mice lacking PAK1 show no overt brain defects but have some immune defects, while PAK2 knockout mice are embryonic lethal. Mutations in PAK3 are associated with nonsyndromic X-linked mental retardation in humans, while PAK3 knockout mice have normal brain morphology but exhibit abnormal synaptic plasticity and cognition. Disruption of PAK3 expression in hippocampal slice culture causes elongated dendritic spines, an increase in filopodial protrusions, and decrease in mature synapses. A forebrain-specific dominant negative mutant PAK1 mouse strain was shown to have abnormalities in dendrite and axon development, indicating an in vivo role for PAK1 in neuronal remodeling, consistent with other assays of PAK1 showing effects on dendrite morphology. Paks 1 and 3 affect dendritic spines, synaptogenesis, and synaptic plasticity. Zhong JL et al. analyzed expression of PAK1 and its T212 phosphorylated form, which are both expressed in developing rat and mouse cortex. Expression of PAK1 increases during brain development and remains high through adulthood, while the T212 phosphorylated form increases around E14 and remains high until around P8 when it declines. Both PAK1 and its T212 phosphorylated form are expressed within the subventricular zone, cortical plate, and marginal zones, areas through which neurons migrate. The T212 site is phosphorylated by p35/cdk5, which affect neuronal morphology and migration.

**Proposed research**

Various constructs are available for studying group A PAKs, including dominant negative and constitutively active constructs. The effects of PAK on cellular morphology and migration depend, as mentioned earlier, on the specific experimental conditions. The environment within the developing brain is different from that in cell culture, yet nearly all studies published to date on PAKs’ effects have used cell cultures. An exception, which studied PAK1’s effects in whole animals, used a dominant negative PAK construct driven by a neuron specific promoter, but this
promoter was activated only after the period of neuronal migration. While existing studies have examined expression of PAKs by tissue type, expression in mature neurons, or in dissociated neurons, no studies have looked at expression in migrating embryonic neurons or at possible effects in early neurons.

PAKs may be involved in neuronal migration and morphological development in vivo. To determine if active PAKs were expressed in regions of neuronal migration and development, the expression pattern of the group A PAKs, and their kinase-active forms, in developing mouse cortex was studied by immunoblotting and immunohistochemistry. To test a possible contribution of active PAKs to brain development, PAK activity was disrupted by acute electroporation of plasmids expressing dominant negative forms of the PAKs and the effect on migration and morphology examined.
RESULTS

Expression

The expression of PAKs in the developing mouse brain during periods of neuronal migration and brain maturation was examined using immunobloting (Figure 2). Cortical lysates from E14.5, E15.5, E17.5, P12, and adult wild-type mouse brains were probed with antibodies to PAK1, active PAK, and PAK1T212(PO4). The antibody to PAK1 is partially cross reactive with PAKs 2 and 3. The antibody to active PAK recognizes PAKs 1-3 phosphorylated at T423, T402, or T421, respectively (indicating kinase active PAK). The antibody to PAK1T212(PO4) is specific to PAK1 and this phosphorylation site is not present in the other members of the family.

PAK1T423(PO4) was not detected at E14.5 or E17.5, but was detected at P12 and adult (Figure 2 A). At P12, no PAK2T402(PO4) is detected, but this changes in adult brain, where a prominent band is detected along with PAK1T423(PO4) and PAK3T421(PO4). Since PAK1T423(PO4), representing the kinase-active form of the protein, is expected to be present in brain at the earlier stages, E14.5-E17.5, the failure to detect it may be due to insufficient protein quantity or loss of phosphorylation at T423 during sample preparation or separation. Expression of total PAK remained high throughout all stages, with highest levels in adult.

PAK1T212(PO4) expression (Figure 2 B) increased during embryonic and postnatal development and then declined in adult. Expression was low at E14.5, slightly higher at E15.5, significantly stronger at E17.5, and reached high levels at P12, then declined to low levels in adult. Expression of total PAK remained high throughout all stages, but showed differences over time possibly due to varying phosphorylation of the protein or detection of other PAK proteins.
Figure 2 Immunoblot for PAK in cortical lysates.

Immunoblot of cortical lysates from E14.5, E17.5, P12, and adult wild-type brains for (A) active PAK and (B) PAK1T212(PO₄) (including E15.5). Stripped and reprobed for PAK1 and for actin as loading control. Active PAK 1-3 phosphorylated at T423, T402, or T421, respectively, whose expression pattern during these stages of development has not been previously described, were not detected at E14.5 or E17.5, but PAK1T423(PO₄) and PAK3T421(PO₄) were detected at P12, while all three were detected in adult. In contrast, PAK1T212(PO₄) was detected at all stages, increasing from very weak at E14.5 to a maximum at P12, and declining to low levels in adult. PAK was detected at all stages, though with a changing banding profile suggesting differential regulation, with strongest expression in adult. Blot in A, 20 µg/lane total protein separated for 50 minutes on mini-gel and nitrocellulose membrane treated following transfer with guanidine solution for 30 minutes prior to incubation with antibody. Blot in B, 60 µg/lane total protein separated for 6 hours without guanidine treatment.

The expression of PAK in the developing mouse brain during periods of neuronal migration and brain maturation was also examined using immunofluorescent staining (Figure 3). PAK was detected throughout the cortex. At E14.5 expression was lower in the ventricular and subventricular zones, highest in the intermediate zone and cortical plate, and declined in the marginal zone. At E17.5, a similar pattern was noted, though with expression also in the subplate. At P12, PAK was more strongly expressed in some projection neurons, for instance in layer V.
Medial coronal sections of E14.5 and E17.5 embryos and P12 pups were stained with antibody to PAK1, which also weakly recognizes PAKs 2 and 3. Expression was seen throughout the cortex. At E14.5 and E17.5 expression was highest in the intermediate zone, while at P12 it was highest in projection neurons and their apical process. LV lateral ventricle, VZ ventricular zone, SVZ subventricular zone, IZ intermediate zone, CP cortical plate, MZ marginal zone. Scale 100 µm, detail 20 µm.

The expression of two phosphorylated forms of PAK, PAK1T212(PO_4) and active PAK, in the developing mouse brain was examined by immunofluorescent staining (Figures 4-6). To examine relative expression patterns of PAK1T212(PO_4) and active PAKs, antibodies for both were used together (Figure 4).

At E14.5 (Figure 4), a period of significant progenitor cell division, PAK1T212(PO_4) is found in cells of the ventricular zone lining the lateral ventricle, in a few cells in the SVZ (not shown), and more diffusely in the upper intermediate zone, but not in the developing cortical plate and marginal zone. Active PAK is found in cells of the ventricular zone lining the lateral ventricle, often being coexpressed with PAK1T212(PO_4). Active PAKs are also found in the lower intermediate zone and in cells of the cortical plate. Other than the few cells lining the ventricle that
coexpressed both forms, they showed reduced expression in the remainder of the ventricular zone.

At E17.5, active PAKs were detected at high levels in the subplate, cortical plate, and marginal zone. There was continued expression of both phosphorylated forms in cells at or adjacent to the apical surface of the ventricular zone with reduced expression in the remainder of the ventricular and subventricular zones. Active PAKs were also expressed in what are most likely axonal tracts in the intermediate zone. PAK1T212(PO$_4$) was found diffusely throughout the intermediate zone through the marginal zone, with higher expression in the upper intermediate zone and subplate. The high expression of active PAKs in the subplate, cortical plate, and marginal zone may reflect involvement of PAKs in early neuronal remodeling and synaptogenesis.

At P12 and adult, PAK1T212(PO$_4$) and active PAKs are expressed throughout the cortex. PAK1T212(PO$_4$) was located predominantly in the soma, while active PAKs were found in the apical dendrites of some cells. In the adult, active PAKs were more diffusely distributed than at P12, possibly reflecting changes in synaptogenesis.
Figure 4 Phosphorylated PAK expression in developing cortex.

Medial coronal sections of E14.5 and E17.5 embryos, P12 pups, and adult were stained with antibodies to PAK1T212(PO₄) (red) and active PAK (green). PAK1T423(PO₄) is expressed in some cells lining the ventricle and in the cortical plate at E14.5 and E17.5. At P12 and adult it was expressed throughout the cortex and in the apical dendrites of some cells (arrows), but was more diffuse in adult. PAK1T212(PO₄) and PAK1T423(PO₄) are coexpressed in cells lining the ventricle at E14.5 and E17. At P12 and adult PAK1T212(PO₄) was expressed in many cells throughout the cortex. WM white matter, LV lateral ventricle, VZ ventricular zone, SVZ subventricular zone, IZ intermediate zone, CP cortical plate, MZ marginal zone. Scale 100 µm, detail 10 µm.

To determine if active PAK was expressed in progenitor cells, staining for PAK1T212(PO₄) and active PAK was repeated with the glial progenitor cell marker RC2 at E14.5 and E17.5 (Figure 5).

Both phosphorylated forms were detected in cells coexpressing RC2 at the apical surface of the
ventricular zone. At E14.5, cells coexpressing all three markers were also found in the SVZ in intermediate progenitor cells. The staining at E17.5 showed low levels of active PAKs in the VZ/SVZ and high levels in the subplate and cortical plate. The presence of both PAK1T212(PO₄) and active PAKs in progenitor cells suggests that regulation of PAK activity may be necessary for normal progenitor cell division.
Figure 5 Expression of PAK1T212(PO₄) and active PAK with the glial marker RC2.

Medial coronal sections of E14.5 and E17.5 embryos were stained for PAK1T212(PO₄) (red), active PAK (green) and RC2 (blue). In cells at the ventricular surface at E14.5 and E17.5, all three markers are coexpressed, indicating that PAK is phosphorylated at these sites in progenitor glial cells. Cells in the SVZ at E14.5 also coexpressed the three markers. LV lateral ventricle, VZ ventricular zone, SVZ subventricular zone, IZ intermediate zone, CP cortical plate, MZ marginal zone. Scale A and B 100 µm, detail 10 µm.

To determine if active PAKs were expressed in neuronal cells in the developing cortical plate, staining for active PAKs was repeated at E14.5 and E17.5 with the early neuronal marker Tuj1, which recognizes the early neuron specific βIII-tubulin protein (Figure 6). At both E14.5 and E17.5, βIII-tubulin is found in the upper layers from the subventricular zone to the marginal zone. At E14.5, little active PAK was detected at the cortical plate, while at E17.5 it was detected in the...
intermediate zone and high levels were detected throughout the cortical plate. Though active PAK is highly expressed in the same regions in which early neurons are found, active PAK immunoreactivity does not strictly correspond to recognizable cellular features, so that while some active PAK may appear to colocalize with βIII-tubulin, it is not possible to say with confidence that active PAK is coexpressed with βIII-tubulin, and hence with neuronal cells, at E17.5.

Electroporation and radial migration

To study a possible role of PAKs in radial migration, PAK function was acutely knocked down by electroporation of dominant negative constructs into cells lining the lateral ventricles of E14.5 embryos.
mice. Following electroporation, brains were sectioned, cultured in organotypic slice culture for 2-4 days, and the radial distribution of cells expressing the electroporated constructs was scored.

PAKs display a range of effects, including kinase dependent and independent effects, on cell morphology and motility. Kinase dependent effects can be inhibited by expression of the auto-inhibitory domain (AID) of PAKs 1-3, comprising amino acids 83-149, which acts as a dominant negative construct to inhibit the kinase function of PAKs 1-3. In transgenic mice, when expressed under control of a α-CamKII promoter, the AID reduced PAK activation in the cortex of older mice by approximately 30-50%. Effects that may be due to additional interactions of PAKs can be examined by expression of another dominant negative construct comprising amino acids 1-265 of PAK1. This construct inhibits the kinase function of the PAKs, but since it comprises the full regulatory domain of PAK1, it also incorporates the binding sites for other interacting partners of PAK and may therefore interfere with normal interactions of the PAKs with other proteins.

To test these effects, these two regions of PAK1 were used in two different plasmids (Figure 7). PAK AID cDNA was inserted into the plasmids pIRES2-EGFP under control of a CMV promoter (Clontech, Mountain View, CA) and into pCIG2 under control of a chicken β-actin promoter, yielding pCMVIG PAK AID and pCIG PAK AID, respectively. cDNA for the N-terminal regulatory domain of PAK1, comprising amino acids 1-265, was inserted into pIRES2-EGFP, yielding pCMVIG PAK DN. The corresponding parent plasmids, pCIG2 or pIRES2-EGFP, lacking the PAK inserts but expressing EGFP, were used as controls. All plasmids express GFP via an internal ribosomal entry site (IRES).
Figure 7 PAK constructs.

At top is a schematic of the full PAK1 protein, showing the p21-binding domain (PBD), the auto inhibitory domain (AI), the N-terminal regulatory domain (yellow), the catalytic kinase domain (green), as well as sites of phosphorylation at T212 and T423. The pCMVIG PAK DN plasmid consists of amino acids 1-265 of PAK1, comprising the N-terminal regulatory domain, under a CMV promoter. The pCMVIG PAK AID plasmid contains just the auto inhibitory domain under a CMV promoter. Finally, the pCIG PAK AID plasmid contains the auto inhibitory domain under a chicken β-actin promoter with a CMV enhancer. All three plasmid inserts are followed by an IRES-EGFP sequence for coexpression of EGFP.

Cells migrating from the ventricular zone undergo a complex radial migratory process, starting with division at the apical surface of the ventricular zone, migration into the subventricular zone where the cells may arrest or migrate back down towards the ventricular zone, followed by migration into the cortical plate. These stages can take 24 hours or longer, depending on the types of cells, age of the embryo, and the thickness of the developing cortex. In addition, plasmid expression takes approximately 24 hours from the time of electroporation. Therefore, any effects of the plasmids were examined at least 24 hours after electroporation. To examine whether inhibition of PAK function affected early or later stages of migration, the effect of acutely expressing the PAK constructs on the radial distribution of cells was examined for 2 and 3 day incubation periods (days in vitro or div). A 1-day experiment was also conducted, but insufficient sections were collected for comparison. The plasmids pCMVIG PAK AID and pCMVIG PAK DN were used for these experiments, with pIRES2-EGFP as control.

After 2 div (Figure 8), most cells were in the lower half of the cortex, with the majority within the ventricular, subventricular, and lower intermediate zones. The maximum number of cells was in the upper ventricular zone. Quantification of the distance from the ventricle indicates a similar
overall distribution of cells for the different plasmids. Slight variations between the values were not found to be statistically significant (see below). Only one embryo was available for the pCMVIG PAK DN plasmid results and therefore no error bars are shown. The 3 div incubations (Figure 9) showed a similar pattern, though the greatest number of cells had shifted slightly higher to the SVZ, consistent with an increased time for cells to migrate away from the ventricular surface. When the distance from the ventricle was quantified, a similar distribution of cells between the different plasmids was found (Table 1). No statistically significant difference between the experimental and control conditions for either 2 div or 3 div incubations was found, indicating that expression of these domains of PAK did not affect the radial distribution of the cells.
Figure 8 PAK AID and PAK DN electroporation, 2 days in vitro.

Plasmids expressing auto inhibitory and dominant negative domains of PAK1 were electroporated into embryos at E14.5 and sections incubated for 2 days. The distribution of GFP expressing cells in the cortex across 10 radial bins was counted. Approximate radial region boundaries are indicated with dashed lines. Cell distributions did not differ statistically from control plasmid by t-test or by one-way repeated measures ANOVA (p<0.05). LV lateral ventricle, VZ ventricular zone, SVZ subventricular zone, IZ intermediate zone, CP cortical plate, MZ marginal zone. Scale 100 µm.
Figure 9 PAK AID and PAK DN electroporation, 3 days in vitro.

Plasmids expressing auto inhibitory and dominant negative domains of PAK1 were electroporated into embryos at E14.5 and coronal sections incubated for 3 days. The distribution of expressing cells in the cortex across 10 radial bins was counted. Approximate radial region boundaries are indicated with dashed lines. Cell distributions did not differ statistically from control plasmid by t-test or by one-way repeated measures ANOVA (p<0.05). LV lateral ventricle, VZ ventricular zone, SVZ subventricular zone, IZ intermediate zone, CP cortical plate, MZ marginal zone. Scale 100 µm.
Table 1 Probability values for 2 and 3 div electroporations.

(A) Two-sided pairwise Student’s t-test relative to control plasmid electroporation and corresponding bin and days incubation. No test is available for PAK DN 2 div due to insufficient data. No statistically significant differences were found at a significance level of p < 0.05. (B) Probability values of ANOVA, testing variance across bins for each condition. No statistically significant differences were found at a significance level of p < 0.05.

The pCIG2 plasmid, containing a chicken β-actin promoter, provided much stronger expression of the GFP expressed by the plasmid and allowed detection in regions further from the ventricular zone, including into the cortical plate and the marginal zone. This plasmid also yielded cleaner signals with lower background noise and did not require additional staining for GFP for detection. While processes extending from cells labeled with the plasmid derived from pIRES2-EGFP could not be clearly visualized, the pCIG2-derived plasmids showed fine processes, including growth cones and neurites, extending from labeled cells. It has been reported that plasmids using a CMV promotor show rapid reduction in expression in committed neuronal progenitors. The pCIG2-derived plasmid was therefore used to examine cortical morphology in the cortical plate, as well as a second method to verify the effects the AID on radial cell distribution.

The plasmid pCIG PAK AID was injected into the lateral ventricles of E14.5 mice, electroporated, and coronal sections incubated for 3 and 4 days, then the distribution of cells scored as before. After 3 div (Figure 10), comparison to sections expressing control plasmid pCIG2 did not show a significant difference. Under both conditions most cells are in the intermediate zone. There was insufficient data for the AID plasmid to calculate standard errors or to apply Student’s t-test, but visual inspection indicated that there was not a significant difference between the cell distributions. After 4 div (Figure 11), the distribution of cells had shifted upward.
into the cortical plate, consistent with a longer duration for cell migration, but again the distribution of cells was very similar between AID and control electroporations. Student's t-test did not show any statistically significant difference between bins at p < 0.05. Therefore, these results also did not indicate a role for PAKs in radial migration.

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**Figure 10** Electroporation of pCIG PAK AID and incubation for 3 div.

Plasmid expressing the auto inhibitory domain of PAK1 was electroporated into embryos at E14.5 and coronal sections incubated for 3 days. The distribution of expressing cells in the cortex across 10 radial bins was counted. Cell distributions did not differ from control. VZ ventricular zone, SVZ subventricular zone, IZ intermediate zone, CP cortical plate, MZ marginal zone. Scalebars 100 µm.
Figure 11 Electroporation of pCIG PAK AID and incubation for 4 div.

Plasmid expressing the auto inhibitory domain of PAK1 was electroporated into embryos at E14.5 and coronal sections incubated for 4 days. The distribution of expressing cells in the cortex across 10 radial bins was counted. Cell distributions did not differ statistically from control plasmid by t-test. VZ ventricular zone, SVZ subventricular zone, IZ intermediate zone, CP cortical plate, MZ marginal zone. Scale 100 μm.

Morphology

To study the effect of inhibition of PAK kinase activity during early neuronal differentiation and remodeling in the cortical plate, I examined the processes extended by young cells expressing the PAK AID. Plasmids pCIG2 PAK AID (experiment) or pCIG2 (control) were electroporated into wild-type E14.5 brains and coronal sections incubated for 4 days to allow the cells to reach the cortical plate. The morphology of cells in the cortical plate and upper intermediate zone in randomly selected fields of single z-plane confocal images were analyzed by Sholl analysis. The number of processes at a radius of 12.5 μm and 25 μm from the center of the cells' soma were
counted and results for each radius compared by two-sided Student's t-test (Figure 12). At 12.5 μm, control cells had 1.40 ± 0.11 (n=156) processes, while PAK AID electroporated cells had 1.64 ± 0.22 (n=58) processes (mean ± sem). At 25 μm, control cells had 0.95 ± 0.08 (n=155) processes, while PAK AID electroporated cells had 0.95 ± 0.13 (n=57) processes (mean ± sem). No statistically significant differences were found at either radius by Student's t-test at p < 0.05. These results do not support a role for active PAK in early neurite outgrowth at the cortical plate.

Figure 12 Morphology of neurons.
Plasmids pCIG2 PAK AID or pCIG2 as control were electroporated into wild-type E14.5 brains and sections incubated for 4 days. The number of processes at a radius of 12.5 μm and 25 μm were counted for a set of randomly selected cells in the upper IZ and lower CP and results for each radius compared by two-sided Student's t-test. IZ intermediate zone, CP cortical plate, MZ marginal zone. Scale 50 μm (control), 100 μm (AID).
**DISCUSSION**

PAKs are expressed in brain, are involved in cell shape changes and migration, and have effects on synapse maintenance and cognition in post-embryonic mice. To determine if they are involved in earlier processes of brain development, including radial neuronal migration and early neurite formation in vivo, the embryonic expression pattern of active PAK and the effects of disrupting PAK activity and interactions were studied.

**Active PAK is expressed in cortical plate and progenitor cells**

PAKs are differentially regulated and expressed during development. PAKs 1-3 are maintained in their kinase-active conformation through phosphorylation at T423, T402, or T421, respectively. An antibody recognizing these phosphorylated forms was used to detect active PAK.

Immunoblotting for active PAKs in cortical lysates detected active PAK 1 and 3 at P12 and active PAK 1-3 in adult, but did not detect active PAKs at E14.5 or E17.5. PAK1, and possibly PAKs 2 and 3, were expressed throughout these stages with a varying expression pattern, indicating possible changes in regulation or phosphorylation at multiple sites. At E14.5 through adult, PAKs were detected by immunofluorescence within the cortex, with higher expression in the axonal tracts in the intermediate zone at embryonic stages.

Active PAKs were highly expressed within the cortical plate at E17.5, a region containing many young neurons. Expression of active PAKs was present, but lower, in the axonal tracts of the intermediate zone, and was lowest in the subventricular zone. At E14.5, some active PAK was detected at the cortical plate, but this region occupies a small volume at this stage relative to the entire cortex. In contrast, at E14.5, PAK1T212(PO₄) was restricted more to the intermediate zone, with lower levels in the cortical plate, while at E17.5 it was present more in upper levels of the intermediate zone.
Active PAK has been previously found in the cortices of 3 and 8 week old mice, but information about its embryonic expression had not been examined. The most striking result in the immunofluorescent staining in this study was the high expression of active PAKs in the subplate, cortical plate, and marginal zone at E17.5. This high expression may reflect the involvement of PAKs in the cytoskeletal changes involved in neuronal remodeling and synaptogenesis that occur as neurons mature in the cortical plate. Conversely, the low-level of active PAK in the ventricular and subventricular zones may reflect lack of PAK activity in early migratory stages of neurons or in interkinetic movement of progenitor cells. Phosphorylation at T212 reduces the activity of PAK1, suggesting that suppression of PAK1 activity may be necessary for radial migration through the intermediate zone. These expression patterns imply that migratory and morphological changes occurring during the early movements of neurons within the ventricular and subventricular zones and their radial migration through the intermediate zone do not require active PAK, and may indeed require suppression of PAK activity. An additional role for PAK activity and regulation in dividing progenitor cells is suggested by the presence of active PAKs and PAK1T212(PO4) in RC2-positive cells adjacent to the apical ventricular surface and in the subventricular zone.

My results for PAK expression are consistent with previously published results. PAKs 1 and 3 are enriched in brain. In P0 mice, PAK3 is expressed in the intermediate zone and cortical plate, but not in the subventricular zone, while in adult rat it is expressed in the primary dendrites of pyramidal neurons. At E14.5, E16.5, and P0 PAKs 1-3 are consistently expressed in the cortex of mice, with a slight reduction at P5, while PAK1 is enriched in the cortical plate, subplate, and intermediate zone of E17 mice and is expressed at E18.5 in the intermediate zone and more highly in the cortical plate. PAK1T212(PO4) expression in the cortex increases from E12 to E16, remains high through P7, then rapidly declines by P10. PAK1T212(PO4) is also expressed in mitotic cells in the ventricular zone. While PAK1T212(PO4) was detected in this study at P12 in C57BL/6 mice, this difference may be due to the use of the CD-1 strain of mice by Zhong et al. The Increase in Phosphorylation of PAK1 at T212 occurs over the same period during which
p35/Cdk5 expression increases. Cdk5 phosphorylate PAK1 at T212 and negatively regulates PAK1 activity, so that Cdk5 is the most likely cause of the increase in PAK1T212(PO4).

The antibody to phosphorylated active PAK did not detect active PAK in E14.5 or E17.5 cortical lysates (Figure 2), yet immunofluorescent staining indicated presence of active PAK at these stages (Figures 4-6). The quantity of active protein present at these stages may not be sufficient for detection, or difficulty in sample preparation may have led to loss of phosphorylation. Immunoprecipitation of PAK may facilitate detection at these stages. Further, while active PAK was detected in the cortical plate in regions also expressing the neuronal protein βIII-tubulin, specific cell types could not be identified due to the diffuse nature of the active PAK staining. Active PAK was detected in progenitor cells lining the ventricles, suggesting that it is present in mitotic cells, which could have been confirmed by staining with a mitotic marker such as an antibody to phosphorylated histone H3. The antibodies used to detect the active PAKs as well as PAK1 also react with PAKs 2 and 3. Staining of PAK1 and/or PAK3 knockout mice would help to differentiate the contribution of each of these family members.

**PAK activity is not necessary for radial neuronal migration**

Disruption of PAK activity did not affect radial migration. PAK activity was acutely disrupted in vivo by electroporation of plasmids expressing dominant negative forms of the PAKs into the proliferating cells lining the lateral ventricles in E14.5 mice. Two dominant negative sequences were used for disruption of activity: the auto inhibitory domain (AID), which inhibits the kinase activity of PAKs 1-3, and the regulatory domain of PAK1, which both inhibits the kinase activity and may disrupt kinase independent interactions. The radial distribution of cells expressing the construct was examined following incubation for periods of 2-4 days. No effect was detected on cell distribution with either construct for the timeframes examined.

In light of studies in cell culture, it was expected that PAKs might affect radial cell migration. PAKs are involved in cytoskeletal events necessary for cell movement and are downstream of proteins known to affect formation of the cortex. PAKs affect the rate and persistence of motion of cultured cells. Suppression of PAK kinase activity typically decreases cell movement, but may
also increase it, depending on cell type and culture conditions. The brain specific kinase Cdk5, which acts in a complex with p35 to phosphorylate PAK1 at T212, causes layering defects when its expression is disrupted. Dominant negative forms of Rac, which activates PAK1, cause migration defects causing cells to fail to leave the subventricular zone. The lack of an effect when PAK function was suppressed in this study suggests that neurons may migrate in vivo using processes that do not require PAK activity.

PAKs are expressed in the intermediate zone, through which neurons migrate, of developing brain during periods of neuronal migration. Active PAK was highest in the cortical plate at E17.5, following neuronal migration, while PAK1T212(PO₄) was expressed in the intermediate zone. Phosphorylation of PAK1 at T212 by Cdk5, which can inhibit PAK1 kinase activity, may enhance retention of a single leading neurite process, possibly facilitating polarized radial cell migration to the cortical plate. PAK activity is either not necessary for radial migration, or the contribution of PAKs to migration is partial so that loss of activity is not pronounced, or the method used in this study to suppress PAK and observe the changes had insufficient power to detect any effect. If suppression of PAK activity in the intermediate zone is necessary for radial migration, then ectopic expression of a constitutively active PAK in migrating neurons might disrupt migration and lead to premature neurite outgrowth. Constitutively active PAK1, in which threonine 423 is replaced with glutamic acid, could be placed in a plasmid under the control of an early neuronal promoter such as Tα1 and introduced by electroporation into embryonic mouse brain.

**PAK activity does not affect early neuronal processes in vivo**

Active PAKs do not appear to affect neurite branching in cells within the cortical plate. Acutely disrupting PAK activity in vivo by electroporation of a plasmid expressing the PAK AID did not affect neurite number. This result was unexpected, given the high expression of active PAKs in the cortical plate during the equivalent period in vivo and the known promotion of neurite outgrowth by PAK in cultured cells.

Previous work has shown that regulation of PAK affects neuronal morphology. Suppression of PAK activity in cultures of cortical neurons transfected with the PAK AID resulted in a significant
decrease in branching, similar to the effect of electroporation with a dominant negative Rac1 construct, while constitutively active PAK1 lead to an increase in neurites. Expression of a PAK1 construct modified to prevent phosphorylation at T212 in primary cortical neurons led to an increase in the number, and a disordering, of neurites, whereas expression of a PAK1 construct mimicking phosphorylation at T212 prevented this change in morphology. Expression of the auto inhibitory domain in forebrain neurons of postnatal mice under control of the α-CamKII promoter resulted in a reduction of dendritic spines and a shift towards larger spines in cortical neurons.

When neurons reach the cortical plate, they differentiate and, in the following days, form the elaborate morphologies characteristic of differentiated neurons. During radial migration, cells may exhibit transient branching, but generally maintain a bipolar appearance during locomotion. Neurons continue to elaborate processes during the first postnatal days and weeks, but the electroporated cells were examined after only 4 days in vitro, corresponding to an age of E18.5. Examining neuronal morphology at later postnatal time points may have revealed morphological changes that were not yet evident. In utero electroporation, in which a plasmid is electroporated into embryos that are allowed to develop within the mother and are born normally, would have permitted examination of postnatal brains in which PAK activity had been suppressed.

PAKs may also be involved in apical dendrite orientation via cGMP-dependent protein kinase I (cGKI). Mice lacking cGKI have misoriented apical dendrites. PAK1 is phosphorylated by cGKI at Ser21, inhibiting PAK1’s association with the adapter Nck and affecting cell polarization. Thus, PAK1’s association with other proteins is regulated by cGKI and may be necessary for cGKI’s effect on apical dendrite orientation.

**PAK inhibition may not have been effective**

Electroporation of dominant negative constructs may not have reduced PAK activity sufficiently to cause an observable effect on radial migration or neurite outgrowth. Staining with the active PAK antibody of electroporated sections was not successful and could not be used to determine if expression of active PAK was indeed reduced by coexpression of the AID domain. The plasmid used for most of the migration studies, pIRES2EGFP, contains a CMV promoter that drives...
expression of the inserted construct. The CMV promoter has reduced expression in neurons compared to plasmids containing a chicken β-actin promotor. Reduced expression of the construct by the plasmid may have both failed to suppress PAK activity as well as not expressed it in cells in which PAKs are active. More limited testing of the pCIG2 PAK AID plasmid, which uses a chicken β-actin promotor, showed high expression in the cortical plate, but did not show effects on migration or morphology. If a subtle migration defect was present, use of the more highly expressing plasmid may have yielded clearer results.

Electroporation of the plasmids into PAK1 or PAK3 knockout mice, which already have reduced expression of PAK, may have sufficiently decreased expression for an effect to be detectable. Alternatively, electroporation of shRNA contained within a plasmid may have reduced PAK expression using a method complementary to the dominant negative constructs. Since PAKs 1-3 may have redundant functions, knocking down all three by RNAi might be necessary. This could be done by attempting to find a region suitable for shRNA knockdown in a region of homology among the PAKs. Alternatively, vectors that express multiple shRNAs are available, or RNAi could be used in one of the knockout mice for PAK1 or PAK3 to reduce expression of the remaining PAKs.

**Expression of PAKs in progenitor cells suggests role in neuronal proliferation**

Expression of active PAK and PAK1T212(PO₄) in progenitor cells indicates a possible role for PAK activity and regulation in neuronal proliferation. Division orientation and timing affect normal neuronal proliferation and stem cell renewal, processes that are crucial to the size and structure of brain regions and the proper differentiation of cell types. During brain development in mice, progenitor cells undergo symmetric divisions in which the cleavage plane is perpendicular to the apical surface of the ventricular zone, leading to self renewal of progenitor cells and an increase in their number. Later, asymmetric divisions in which the axis is parallel to the apical surface leads to generation of a post-mitotic neuron and a self renewing progenitor cell. Later still, terminal symmetric divisions dominate, in which a progenitor cell divides into two post-mitotic neurons, leading to depletion of the pool of progenitor cells. Symmetric self-renewing stem cell
divisions are more prevalent prior to E14.5, after which symmetric neurogenic divisions become more common. In addition, the duration of the cell cycle increases during development. PAKs may affect division axis orientation and division timing in progenitor cells.

PAK1 affects cell cycle progression and division axis orientation. A peptide that mimics PAK1T212(PO_4) was found at centrosomes and mitotic spindles and caused increased astral microtubule length during metaphase. Transfection of cells in culture with the PAK AID led to cell cycle arrest. PAK1 is phosphorylated at T212 by Cdc2, which is expressed in proliferating neuronal precursor cells and is probably the kinase responsible for phosphorylation of PAK1 at T212 in neuronal progenitor cells, while the p35/Cdk5 complex, which also phosphorylates PAK1 at T212, is not expressed in mitotically active cells.

PAK1 is activated by targeting to the centrosome, where it dissociates from a PIX/GIT complex and phosphorylates Aurora-A, whereas inhibition of PAK causes a delay in centrosome maturation. Aurora-A is a conserved protein, found (among others) in *Drosophila* and mammals, involved in cell division, mitotic spindle formation, chromosome segregation, and cytokinesis. *Drosophila* larval neuroblasts divide asymmetrically to form a ganglion mother cell (GMC) and a neuroblast. GMCs divide to give rise to neurons or glia, while neuroblasts continue to undergo self-renewing asymmetric divisions. In wildtype larvae, the spindle axis during asymmetric neuroblast division is oriented with the apical/basal cortical polarity axis. Aurora-A mutants fail to align the spindle axis and instead undergo symmetric division in which neuroblasts give rise to two neuroblasts, rather than a neuroblast and a GMC, and also show an increase in cell cycle length. The interaction between PAK1 and Aurora-A may provide a link between PAK1 activation and symmetric versus asymmetric divisions and may affect the timing of cell divisions, possibly altering neuronal subtypes or cortical structure. The lack of observable deficits in PAK1 and PAK3 mutant mice may be due to compensatory factors.

**Model for possible role of PAKs in early brain development**

Based on these results, and the known activities and expression of the PAKs, a model for the possible role of PAKs in early brain development is proposed (Figure 13). During neuronal
proliferation, both PAK1T212(PO₄) and active PAKs 1-3 are found in mitotic progenitor cells, possibly affecting cell cycle progression and cell orientation for symmetric versus asymmetric divisions, leading to neuronal differentiation or neuroblast renewal. During migration, PAK1 activity may be suppressed in the intermediate zone to maintain a leading process and facilitate radial migration. During neuronal maturation, active PAK, found in the cortical plate, may contribute to neuronal maturation. Finally, during synaptogenesis and into adulthood PAK activity at synapses affects synapse formation and maintenance. If PAK activity is necessary for proper spindle orientation and mitotic progression during proliferation of neuronal precursors, then disrupting PAK activity in the cells may change the number of neurons or neuronal precursors and may affect cell fate and neuronal subtype specification.
Figure 13 Model of possible role of PAKs in brain development.

During proliferative stages, E11-E17, PAK is expressed and is phosphorylated at T212 (red) and T423 (green) in mitotic cells in the ventricular zone. PAK is phosphorylated at T423 when it is recruited to centrosomes and is phosphorylated at T212 by Cdc2 during cell division. PAK then phosphorylates and activates Aurora-A, possibly contributing to spindle orientation and neuroblast renewal, or asymmetric division leading to neuronal fate. During migration, E11-E17, PAK1 may be phosphorylated by Cdk5 at T212 in the intermediate zone, reducing PAK1 activity, limiting the branching of neurites and contributing to directed radial migration to the cortical plate. During early neuronal maturation, approximately E15-P0, PAK is phosphorylated at T423 and active in the cortical plate, where it may contribute to neuronal maturation. In later stages, PAKs may contribute dendrite branching, synaptogenesis and synapse maintenance, and may contribute to apical dendrite orientation.

Conclusions

Neuronal migration, proliferation, and differentiation are complex processes requiring cytoskeletal rearrangements. PAKs interact with and affect many of these processes. The presence of active PAKs in regions of early neuronal remodeling in the cortical plate may affect early neuronal development in addition to PAKs previously known role in dendritic spines and synapses. PAK activity, however, does not appear to be necessary for normal radial migration or for initial neurite outgrowth in the cortical plate.
MATERIALS AND METHODS

Animals

Mice, strain C57BL/6, were maintained in a 12 hour light/dark cycle. For timed pregnancies, two females were set up for overnight breeding with one male. Noon of the next day was considered E0.5.

Constructs

Amino acids 1-265 of hPAK1 cDNA were inserted into pIRES2-EGFP (Clontech, Mountain View, CA) at XhoI and BamHI sites and expressed under control of a CMV promoter, to make the pCMVIG PAK DN plasmid (Mona Buhusi and Amanda Gates, UNC Chapel Hill). Amino acids 83-149 of hPAK1 cDNA, comprising the auto inhibitory domain of PAKs 1-3 were amplified by PCR from plasmid pCMV6-myc-PAK1(83-149) (gift of Tina Leisner, UNC Chapel Hill), with suitable linker sites attached, and inserted into pIRES2-EGFP at EcoRI and BamHI sites to make pCMVIG PAK AID which express the AID domain under control of the CMV promoter, or pCIG2 (gift of Dante Bortone, UNC Chapel Hill) at XhoI and Xmal to make pCIG2 PAK AID plasmids which expresses the AID domain under the chicken β-actin promotor with a CMV early-immediate enhancer. Primers used in PCR for insertion into pIRES2-EGFP were 5’-CGCCGCGGATCCACACAATTCATGTCGGTTTTGATGC -3’ and 5’-CGCCGCCGAATTCTGACTTATCTGTAAAGCTCATGTATTTCTGGC-3’, while for insertion into pCIG2 they were 5’-CGCCGCGCTCGAGATGCACACAATTCATGTCGGTTTTGATGC-3’ and 5’-CGCCCGCCCAGGTATGACCTATCTGTAAAGCTCATGTCGGTTTTGATGC-3’. Inserts were verified by sequencing.

Brain lysates

Brain tissue was collected from embryos of timed pregnant mice, from postnatal pups, and from adult mice. Mice were anesthetized by isoflurane inhalation (Halocarbon Products Co., River
Edge, NJ), sacrificed by decapitation, and heads placed on ice. Timed pregnant female mice were deeply anesthetized by IP injection of Avertin 2.5% 0.02 ml, embryos were removed and placed in ice cold complete HBSS and the mother sacrificed by cervical dislocation. For cortical lysates, the cortex was dissected under a microscope with brains maintained in cold complete HBSS. Brains were dounced in cold RIPA lysis buffer (Tris pH 7.0 20 mM, NaCl 150 mM, EDTA 5 mM, EGTA 1 mM, SDS 0.1%, Nonidet P40 1%, sodium deoxycholate 1%) supplemented with inhibitors (NaF 10 mM, Na3VO4 1mM, protease inhibitors (P2714, Sigma, Saint Louis, MO), PMSF 1 mM, Calyculin A 20 mM (C5552, Sigma, Saint Louis, MO)), centrifuged at 14K RPM for 15 minutes in a benchtop microcentrifuge at 4C, then DNA in the supernatant was sheared by passage through a 22 5/8 gauge needle, and lysates frozen in an ethanol/dry ice bath and stored at –80C. Protein concentration was quantified by BCA assay (Pierce 23227) and kept at ≤ 2 mg/mL to minimize dephosphorylation.

**Immunoblot detection**

Samples were mixed with Laemli buffer, boiled 3 minutes, and separated in a 4% stacking / 7.5% resolving SDS-PAGE gel in a Hoeffer chamber at 25 mA for 6 hours or in a BioRad mini-gel chamber at 20-30 mA for 50 minutes. The gel was fixed 0.5 hours in methanol buffer and proteins transferred to nitrocellulose membrane at 1 mA/cm² for 1.5 hours using semi-dry graphite transfer electrodes. Membranes were optionally incubated with guanidine solution (guanidine HCl 6 M, Tris pH 7.5 20 mM, DTT 5 mM, PMSF 1 mM), rinsed and washed in TBS (Tris HCl pH 7.4, NaCl 150 mM), blocked for 1 hour at RT in block solution (TBS, 0.1% Tween 20, 5% w/v non-fat milk). Antibodies to PAKs 1-3 phosphorylated at T423, T402, or T421, respectively, 1:500 (2601, Cell Signaling Technology Inc., Danvers, MA) were incubated overnight at 4C and to PAK1T212(PO4) were incubated 1:1000 at 2 µg/µL (clone PK-18 P3237, Sigma-Aldrich Co., Saint Louis, MO) for 1 hour at RT in block solution. The membrane was washed 3x with TTBS (TBS with 0.1% Tween-20) for 5 minutes at RT, incubated with anti-rabbit or anti-mouse HRP-conjugated secondary antibodies (111-035-003 or 115-035-003, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in block solution, washed again 3x with TTBS for 5 minutes at RT, incubated 1 minute with HRP assay (32106, Pierce Biotechnology Inc., Rockford, IL), and films exposed.
Membranes were then stripped 5 minutes (acetic acid 0.5M, NaCl 0.5M), washed, and reprobed with anti-PAK antibody 1:500 (C19, SCBT sc-881) for 1 hr at RT, which was detected as above. Finally, membranes were reprobed with antibody to actin 1:5000 (MAB1501, Chemicon, Temecula, CA) for 1 hr at RT as a loading control.

**Immunofluorescent staining**

Embryonic mice were removed from timed pregnant female mice that had been deeply anesthetized by IP injection of Avertin 2.5% 0.02 mL/g. The embryonic brains were then removed in cold complete HBSS and transferred to 4% PFA in PBS pH 7.3-7.4. Postnatal and adult mice were anesthetized by isoflurane inhalation, an incision made to expose the heart, then transcardially perfused with warm PBS followed by warm 4% PFA in PBS. Brains were removed and transferred to 4% PFA in PBS, then 10%, 20%, and finally 30% sucrose in PBS at 4C, embedded in freezing medium (Triangle Biomedical Sciences, Durham, NC), sectioned at 16 µm on a cryostat (Triangle Biomedical Sciences, Durham, NC), mounted on microscope slides, dried, and frozen at –80C. Sections were thawed, washed in PBS 2x 5 minutes, blocked for 1 hour in PBS supplemented with heat-inactivated goat serum 20% (Gibco, Invitrogen Corporation, Carlsbad, CA), BSA 2% (A2153, Sigma, Saint Louis, MO), and Triton X-100 0.2%. Sections were then incubated with primary antibody, washed 10x 1 minute with PBS, incubated with secondary antibody for 1 hr at RT, washed 5x 1 minute with PBS, and mounted in Vectashield with DAPI (H-1200, Vector Laboratories, Burlingame, CA). Primary antibodies, used at 1:250 dilution, were PAK1 1:250 (Clone C-19 sc-881, Santa Cruz Biotechnology Inc., Santa Cruz, CA), PAKs 1-3 phosphorylated at T423, T402, or T421, respectively (2601, Cell Signaling Technology Inc., Danvers, MA), PAK1T212(PO_4) (clone PK-18, P3237, Sigma-Aldrich Co., Saint Louis, MO), RC2 (Developmental Studies Hybridoma Bank, University of Iowa), Tuj1 (MMS-435p, Covance, Berkeley, CA). Secondary antibodies were AF488 or AF546 conjugated Molecular Probes A11008 or A11003, respectively, or Cy5 conjugated donkey anti-mouse IgM (Jackson Immunoresearch Laboratories Inc., West Grove, PA). Images were collected with a Zeiss LSM5 Pascal Confocal Laser Scanning Microscope (Microscopy Services Laboratory, Dr. Robert Bagnell, M.D. director, UNC Chapel Hill).
Electroporation, slice culture, and immunofluorescent staining

Ex-vivo electroporation was performed. Embryonic mice at E14.5 were removed from timed pregnant female mice that had been deeply anesthetized by IP injection of Avertin 2.5% 0.02 ml and embryos placed in cold complete HBSS. A solution containing plasmid DNA, either pCIG2 or pCIG2 PAK AID at 2 µg/µL, or pCMVIG PAK DN or pCMVIG PAK AID at 3 µg/µL in PBS pH 7.3 with Fast green 0.01% as tracer (Sigma, Saint Louis, MO) was injected into the lateral ventricles of E14.5 embryos using micropipettes (TW150-4, World Precision Instruments Inc., Sarasota FL) pulled in a pipette puller with a Picospritzer II (Parker Hannifin, Cleveland, OH) for 10 pulses at 3-4 msec/pulse. The embryos were decapitated and 5 electrical pulses at 55V of 100 msec duration with a 500 msec interval between each pulse were applied to each hemisphere using a BTX ECM 830 electroporator and 7 mm stainless steel tweezer electrodes (Harvard Apparatus, Holliston, MA). The positive electrode was situated over the dorsal lateral ventricle while the negative electrode was situated along the neck at the plane of decapitation on the contralateral side, causing selective uptake of the plasmid DNA into the cells lining the lateral ventricles. Brains were dissected out and mounted in 3% low-melting point agarose in complete HBSS, then sectioned at 300 µm on a Vibratome 1500 (Vibratome, Saint Louis, MO), mounted on organotypic slice culture membranes (Millicell PICMORG50, Millipore, Billerica, MA) previously coated with poly-L-lysine 0.1 mg/mL (P5899, Sigma, Saint Louis, MO) and laminin 0.01 mg/mL (L2020, Sigma, Saint Louis, MO), then incubated in 35 mm dishes containing 1.2 mL slice culture medium in 5% CO2 at 4C. One half of the medium was changed after 24 hours incubation. Following incubation sections were fixed in warm PFA 4% in PBS pH 7.3-7.4. Fixed sections were washed 5x 5 min in PBS, blocked in PBS supplemented with heat inactivated goat serum (Gibco, Invitrogen Corporation, Carlsbad, CA), BSA 3%, and Triton X-100 0.3% for one hour at RT. Sections were then incubated with primary antibodies in block solution at 4C O/N, washed 5x 5 min in PBS, and incubated with secondary antibodies and either Draq 5 1:10,000 (Axxora Ltd., Bingham, UK) or bis-benzimide 0.01 mg/mL (Hoechst 33342, Invitrogen Corporation, Carlsbad, CA) in block solution for 1 hr at RT. Primary antibodies were rabbit anti-GFP 1:1000 (A11122, Molecular Probes, Invitrogen Corporation, Carlsbad, CA), secondaries were AF488-conjugated
goat anti-rabbit 1:400 (A11008, Molecular Probes, Invitrogen Corporation, Carlsbad, CA) or FITC-conjugated goat anti-rabbit (111-095-003, Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Sections were then mounted in Vectashield (H-1000 or H-1200, Vector Laboratories, Burlingame, CA), Mowiol (4-88, Calbiochem, La Jolla CA), or Aqua Polymount (Polysciences Inc., Washington PA).

Quantification of radial cell distribution

Quantification was done based on a previously published method. Confocal Z-stacks were captured of the neocortex of electroporated slices using a 10x objective NA 0.3 on a Zeiss LSM5 Pascal Confocal Laser Scanning Microscope (Microscopy Services Laboratory, Dr. Robert Bagnell, M.D. director, UNC Chapel Hill) or with a Zeiss LSM 510 (Confocal/Multiphoton Microscopy Core Facility, Dr. Robert Petersen, PhD, director, UNC Chapel Hill). Maximum-brightness projections were made of each stack using ImageJ (NIH). Multiple projections from a single section were montaged in Photoshop (Adobe, San Jose, CA) and levels adjusted. The boundaries of the ventricular and pial surfaces and the centers of the soma of GFP-expressing cells were marked. Cells were marked in ImageJ either manually for sections electroporated with pIRES2EGFP, pCMVIG PAK DN or pCMVIG PAK AID, or automatically for sections electroporated with pCIG2 or pCIG PAK AID. Automatic marking was done by applying a Band Pass filter, thresholding, applying a watershed filter, and using the Analyze Particles feature in ImageJ. The ventricular and pial surfaces were segmented into 100 radial divisions and the position of cells were projected onto the closest radial line to determine the distance from the ventricle, which was then converted into a value relative to the total length of the radial line. Cells in the neocortex extending lateraly 500 µm from the neocortex/archicortex boundary were counted. Corresponding bins from experimental and control plasmid electroporations were compared by Student's t-test in Excel (Microsoft Inc., Redmond, WA) and by two-factor repeated measures ANOVA in SAS (SAS Institute Inc., Cary, NC). ANOVA analysis was performed by Dr. Robert Hamer, PhD, UNC Chapel Hill.
**Morphology analysis**

Sholl analysis was applied to cells within the cortical plate in randomly selected fields of single z-plane projections of confocal images captured using a 20x objective on a Zeiss LSM5 Pascal Confocal Laser Scanning Microscope or an Olympus FV500 Confocal Laser Scanning Microscope (Microscopy Services Laboratory, Dr. Robert Bagnell, M.D. director, UNC Chapel Hill). The number of processes intersecting the perimeter of circles of radius 12.5 μm and 25 μm centered at the soma were counted and compared by Student's t-test in Excel (Microsoft Inc., Redmond, WA).
REFERENCES


