ERK5 MAP Kinase Regulates Neurogenin1 during Cortical Neurogenesis

Paige Cundiff1, Lidong Liu2, Yupeng Wang2, Junhui Zou2, Yung-Wei Pan3, Glen Abel2, Xin Duan6, Guo-li Neurogenin 1 (Neurog1). Previous studies have focused on mechanisms that control the expression of these proteins while little is known about whether their pro-neural activities can be regulated by kinase signaling pathways. Using primary cultures and ex vivo slice cultures, here we report that both the transcriptional and pro-neural activities of Neurog1 are regulated by extracellular signal-regulated kinase (ERK) 5 signaling in cortical progenitors. Activation of ERK5 potentiated, while blocking ERK5 inhibited Neurog1-induced neurogenesis. Furthermore, endogenous ERK5 activity was required for Neurog1-initiated transcription. Interestingly, ERK5 activation was sufficient to induce Neurog1 phosphorylation and ERK5 directly phosphorylated Neurog1 in vitro. We identified S179/S208 as putative ERK5 phosphorylation sites in Neurog1. Mutations of S179/S208 to alamines inhibited the transcriptional and pro-neural activities of Neurog1. Our data identify ERK5 phosphorylation of Neurog1 as a novel mechanism regulating neuronal fate commitment of cortical progenitors.

Abstract
The commitment of multi-potent cortical progenitors to a neuronal fate depends on the transient induction of the basic-helix-loop-helix (bHLH) family of transcription factors including Neurogenin 1 (Neurog1). Previous studies have focused on the intrinsic induction of expression of Neurog1/Neurog2/Ascl1 proteins, extrinsic factors could activate protein kinase signaling pathways and modulate the pro-neural activity of Neurog1/Neurog2/Ascl1 via protein phosphorylation. However, most research so far has focused on understanding the transcriptional regulation of these bHLH transcription factors; there is currently limited evidence that their transcriptional activities or their ability to specify neuronal commitment are regulated post-translationally.

We recently reported that the ERK5 (Mapk7), a member of the mitogen-activated protein (MAP) kinase family, provides an instructive signal to specify cortical progenitors to a neuronal fate [18]. In this study, we tested the hypothesis that the pro-neural activity of Neurog1 may be regulated by ERK5 during cortical neurogenesis.

Results
Activation of ERK5 potentiates while inhibition of ERK5 attenuates Neurog1-stimulated neurogenesis

Our previous studies established that ERK5 is necessary and sufficient to promote neuron fate specification of cortical progenitors [18]. Because ERK5 is a MAP kinase that can phosphorylate and regulate the activity of several transcription factors, we set out to determine whether it could regulate the expression of the bHLH transcription factors Neurog1/Neurog2/Ascl1 in a manner that promotes neuronal commitment.

Introduction
During mammalian cortical neurogenesis, neuronal cell fate specification is dependent on the temporal and spatial expression of the bHLH family of transcription factors including Neurog1, Neurogenin 2 (Neurog2), and Ascl1 (Mash1) [1–10]. These transcription factors specify neuronal phenotype at the expense of glial fate and subsequent choice of sub-neuronal phenotypes during cortical development (glutamatergic vs. GABAergic). For example, although there may be a high degree of redundancy between Neurog1 and Neurog2 [11,12], both are expressed in the dorsal telencephalon and direct multi-potent cortical progenitors to a pyramidal, glutamatergic neuron fate. Ascl1 directs cortical progenitors to a GABAergic neuron fate [9], and its expression is high in the ventral telencephalon but low in the dorsal telencephalon [12–14]. The pro-neural Neurog1 and Neurog2 induce the expression of NeuroD1, NeuroD2, and Nex, members of the NeuroD family of bHLH transcription factors, which induce terminal differentiation of the committed precursors into mature neurons.

It has been postulated that in addition to the intrinsic molecular properties of these bHLH transcription factors, extracellular factors present in the microenvironment may also influence the cell fate choice of progenitors [15–17]. Thus, it is conceivable that, in addition to the intrinsic induction of expression of Neurog1/Neurog2/Ascl1 proteins, extrinsic factors could activate protein kinase signaling pathways and modulate the pro-neural activity of Neurog1/Neurog2/Ascl1 via protein phosphorylation. However, most research so far has focused on understanding the transcriptional regulation of these bHLH transcription factors; there is currently limited evidence that their transcriptional activities or their ability to specify neuronal commitment are regulated post-translationally.

We recently reported that the ERK5 (Mapk7), a member of the mitogen-activated protein (MAP) kinase family, provides an instructive signal to specify cortical progenitors to a neuronal fate [18]. In this study, we tested the hypothesis that the pro-neural activity of Neurog1 may be regulated by ERK5 during cortical neurogenesis.

Acknowledgments
This work was supported by grant NIH AG19193 (Z.X.), a Rosetta-Merck 2006–2007 Molecular Profiling Pre-doctoral Fellowship (P.C.), and by an NIH Pre-doctoral Training grant T32GM07750 (P.C.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: zxia@u.washington.edu
ERK5 Regulation of Neurogenin1

ERK5 signaling regulates Neurogenin1-initiated gene expression

Since Neurog1 induces neuronal cell fate specification and differentiation primarily through the transcriptional activity of Neurog1-specific genes, we investigated if ERK5 regulates the transcriptional activity of Neurog1. Using a Nucleofector® method, we transiently transfected freshly isolated, rat E13 cortical progenitor cells with a Flag-Neurog1 expression vector and dnMEK5 as indicated (Fig. 2). Cells were co-transfected with a luciferase reporter construct driven by a Neurog1-responsive, 3-tandem boxes promoter (Box-Luc). Ectopic expression of Neurog1 increased transcription initiated from the Box-Luc promoter (Fig. 2 A). These data provide evidence that the ERK5 signaling pathway regulates the pro-neural activity of Neurog1.

Does ERK5 signaling modulate the activities of endogenous bHLH transcription factors? Although Neurog1, Neurog2, and Ascl1 are capable of binding and activating the E-box and native NeuroD2 promoter [6,19], Ascl1 expression is extremely low in dorsal telencephalon [12]. Therefore, Neurog1 and Neurog2 are most likely the main endogenous transcription factors capable of stimulating the E-box-Luc or NeuroD2 promoter-driven luciferase in cortical progenitor preparations isolated from E13 rat dorsal telencephalon. We transiently transfected E13 cortical progenitors with the 3xE-box-Luc or a NeuroD2 promoter-driven luciferase (NeuroD2-Luc) without introducing exogenous Neurog1 to monitor the activity of endogenous bHLH transcription factors. Cells were co-transfected with dnMEK5 to block ERK5 signaling or the cloning vector as a control. Inhibition of ERK5 signaling significantly reduced transcription of both reporters initiated by endogenous bHLH transcription factors present in rat E13 cortical cells (Fig. 2, B and C). Together, data in Figure 2 suggest that ERK5 regulates the transcriptional activity of Neurog1.

ERK5 regulation of Neurog1 transcriptional activity may be mediated through phosphorylation

Because ERK5 is a MAP kinase that can directly phosphorylate and regulate the activity of transcription factors [20], we postulated that ERK5 may regulate the transcriptional activity of Neurog1 through direct phosphorylation. A protein sequence analysis revealed two perfectly matched, putative proline-directed MAP kinase phosphorylation sites (PX(S/T)P, S179 and S208, and two imperfect sites (S/T)P, S201 and T237, within the C-terminus of Neurog1 (Fig. 3 A). Although mutations of S201 or T237 to non-phosphorylatable alanines had no effect on Neurog1’s transcriptional activity (Fig. 3 B), replacing S179 or S208 with alanines almost completely abolished Neurog1’s ability to initiate transcription in HEK293 cells (Fig. 3 C) and in cortical neurons (Fig. 3 F). The distinct effects of the four mutations on Neurog1’s transcriptional activity were not due to differential expression of the mutant proteins (Fig. 3, D and E). These results suggest that Neurog1’s transcriptional activity requires the function of S179 and S208. Furthermore, phosphorylation of S179 and S208 may regulate the transcriptional activity of Neurog1. Because the double mutant SA179/208 was as effective as, if not more potent than, the single mutants we focused our efforts on the double mutant for the remaining investigation.

We investigated if activation of ERK5 induces Neurog1 phosphorylation. When Flag-Neurog1 was expressed alone in HEK293 cells or when co-transfected with dnERK5 as a control, it appeared as multiple bands on a 12% SDS gel, running at approximately 37 kDa (Fig. 4 A). However, when co-transfected with caMEK5+wtERK5 to activate ERK5 signaling in transfected cells, the majority of the Flag-Neurog1 exhibited reduced electrophoretic mobility suggesting that Neurog1 is phosphorylated in cells when ERK5 is activated. Indeed, the reduced electrophoretic mobility of Neurog1 was abolished when whole cell lysates were treated with calf intestine alkaline phosphatase (CIP) (Fig. 4 B), confirming that the gel shift is due to phosphorylation of Neurog1. In contrast, ERK5 activation did not reduce the electrophoretic mobility of the mutant SA179/208 Neurog1 (Fig. 4 C). Thus, activation of ERK5 signaling leads to wt, but not the mutant SA179/208 Neurog1 phosphorylation in HEK293 cells.

To determine if ERK5 directly phosphorylates Neurog1, active ERK5 was immunoprecipitated using an anti-Flag antibody from HEK293 cells transfected with HA-tagged caMEK5 and Flag-tagged wtERK5. The immunoprecipitated ERK5 was incubated with 32P-ATP and purified recombinant GST-Neurog1 (151-244)
Figure 1. Activation of ERK5 potentiates while inhibition of ERK5 attenuates Neurog1-stimulated neurogenesis. For panels A–D, neurosphere assays. Freshly dissociated E13 cortical progenitors were co-infected with lentiviruses encoding Neurog1, constitutive active (ca) or dominant negative (dn) MEK5, or wild-type ERK5 as indicated. Cells infected with GFP-virus were used as a control. Neurospheres were allowed to form in culture for 5 d, and then transferred to PDL/laminin coated plates in bFGF-free medium to promote spontaneous differentiation for 3 d. Neurospheres infected with lentiviruses were identified by GFP expression. Neurons were identified by the pan-neuronal marker, β-III tubulin. A, Representative images of neurospheres infected with either GFP control virus (control) or wild-type Neurog1, and immunostained for β-III tubulin.
fusión protein (Fig. 4 D) as substrates in an in vitro kinase assay. HEK293 cells were also co-transfected with HA-tagged dnMEK5 and Flag-tagged wtERK5 as a control for the active ERK5. The kinase activity of ERK5 was monitored by its autophosphorylation (32P-ERK5) (Fig. 4 E). The wild-type GST-Neurog1 (151–244) was robustly phosphorylated by active ERK5 but not by the control inactive ERK5 (Fig. 4, E and F). Importantly, active ERK5 did not significantly phosphorylate the GST-Neurog1 SA179/208 mutant protein (Fig. 4, E and F). These data suggest that ERK5 directly phosphorylates Neurog1 on S179, S208, or both.

To investigate if Neurog1 phosphorylation occurs in rat E13 cortical progenitors, freshly dissociated E13 rat cortical cells were infected with lentiviral stocks encoding GFP control or wt Neurog1. Cell lysates were collected 3 d later and treated with CIP (Fig. 4 G). Treatment with CIP reduced the electrophoretic mobility of Neurog1, indicating that Neurog1 expressed in E13 cortical progenitor cells exists as a phosphorylated protein. Similarly, Neurog1 was phosphorylated when expressed ex vivo in rat E15 cortex slices (Fig. 4 H).

Phosphorylation of Neurog1 at S179 and S208 regulates the pro-neural activity of Neurog1

To examine if phosphorylation on S179 and S208 modulates the pro-neural activity of Neurog1, we infected LeX-stained cortical progenitors with lentiviruses encoding Flag-tagged, wt Neurog1, Flag-Neurog1 SA179/208, dnMEK5, caMEK5 together with wtERK5, or a combination of these constructs as indicated (Fig. 5). Lentiviral GFP was used as a control. All of the viral expression vectors were coupled to GFP through IRES and virus-infected cells were identified by anti-GFP immunostaining (green) (Fig. 5 A). Cortical progenitors were identified by nestin immunostaining (red). Virus-infected cells that express nestin stained orange in merged images. Quantification of the data demonstrated that ectopic expression of Neurog1 decreased the number of cells co-labeled with nestin (Fig. 5 B), suggesting that Neurog1 decreases the pool of cortical progenitors in the infected cell population.

Neuronal differentiation was assessed by immunostaining with β-III tubulin and the mature neuron marker, MAP-2 (Fig. 6, A and B). In contrast to the nestin staining, ectopic expression of Neurog1 increased the number of GFP+ cells co-labeled with β-III tubulin (Fig. 6 C) or MAP-2 (Fig. 6 D). The concomitant decrease in nestin expression and increase in β-III tubulin and MAP-2 expression suggest a pro-neural effect of Neurog1. Importantly, the pro-neural effect of Neurog1 was greatly attenuated by co-expression of dnMEK5 (Fig. 5 B and Fig. 6, C and D), consistent with the data in Figure 1. Significantly, mutations of S179 and S208 to alanines greatly reduced the neurogenic activity of Neurog1. Furthermore, expression of the SA179/208 mutant Neurog1 attenuated the neurogenic activity afforded by ERK5 activation (caMEK5+wtERK5). These data suggest that the pro-neural effect of Neurog1 is regulated by ERK5 phosphorylation and that Neurog1 is a downstream mediator of ERK5’s effect on neuronal fate specification.

We utilized ex vivo electroporation coupled to organotypic slice culture to examine the effect of SA179/208 mutations on Neurog1’s pro-neural activity. The organotypic slice cultures maintain some of the anatomy and cell-cell interactions of the intact cortex [21]. Plasmid DNA encoding vector control, wt Neurog1 and the Neurog1 SA179/208 mutant were injected into the lateral ventricles of dissected E15 rat brains. A GFP plasmid was co-injected as a marker to identify transfected cells. The electrodes were placed in a way to consistently favor plasmid DNA electroporation into the dorsolateral cortex. The cortices were sliced into 300 μm sections and cultured ex vivo. The cellular

**Figure 2.** ERK5 signaling is required for transcription initiated by ectopically expressed Neurog1 and endogenous bHLH transcription factors in E13 cortical progenitors. Rat E13 cortical progenitor cells were transiently transfected with a control vector (vector), Neurog1, and dnMEK5 as indicated. The transcriptional activity of Neurog1 was monitored using a co-transfected 3xE-Box-Luc or a NeuroD2-Luc reporter. Luciferase activity was normalized to a co-transfected LacZ reporter. A, Expression of wt Neurog1 stimulates 3xE-box luciferase activity, which was inhibited by dnMEK5. B, Expression of dnMEK5 inhibits 3xE-box luciferase activity afforded by endogenous bHLH transcription factors. C, NeuroD2-luciferase activity induced by endogenous bHLH transcription factors is inhibited by dnMEK5.

doi:10.1371/journal.pone.0005204.g002
Figure 3. S179 and S208 are required for Neurog1’s transcriptional activity. A, Schematic representation of the various functional domains of Neurog1. Four putative proline-directed MAP kinase phosphorylation sites (PX_5P) at S179, S201, S208, and T237 are present within the presumed transactivation domain in the C-terminus. B, Replacing S201 or T237 with alanine had no effect on Neurog1’s ability to stimulate NeuroD2-luciferase in HEK293 cells. V: vector control. C, Replacing S179, S208, or both with alanine almost completely abolished Neurog1’s ability to stimulate NeuroD2-luciferase in HEK293 cells. D, E, Western analysis demonstrating equal expression of wt Neurog1 and Neurog1 mutants in HEK293 cells. F, Replacing S179, S208, or both with alanine greatly attenuates Neurog1’s ability to stimulate NeuroD2-luciferase activity in E16 cortical neuron cultures.

doi:10.1371/journal.pone.0005204.g003
Figure 4. Activation of ERK5 is sufficient to induce Neurog1 phosphorylation. 

A, ERK5 activation in HEK293 cells leads to an electrophoretic mobility shift of Neurog1, indicative of Neurog1 phosphorylation (p-Neurog1). HEK293 cells were transiently transfected with either vector control (V) or Flag-Neurog1. Cells were also co-transfected with HA-tagged caMEK5 and Flag-wtERK5 to activate ERK5 signaling in transfected cells. Cells co-transfected with Flag-dnERK5 were used as a control. Cell lysates were analyzed by anti-Flag Western blotting.

B, The electrophoretic mobility shift of Neurog1 was abolished after treatment with CIP.

C, ERK5 activation does not induce an electrophoretic mobility shift of the mutant SA179/208.
phenotype of the transfected cells (GFP+) was identified by immunostaining for PCNA (Fig. 7 A), a marker for cells in early G1/S phase, or the T-box brain (Tbr) 2 (Fig. 7 B), a transcription factor and marker for cells actively proliferating in the upper layer of the ventricular zone (VZ) and the sub-ventricular zone (SVZ) [22,23]. The slices were also stained with Tbr1 (Fig. 8 A) or NeuN (Fig. 8 B), markers for post-mitotic neurons in the cortical plate during development [22,24,25] and mature neurons, respectively. Co-labeling of cells immunopositive for GFP and PCNA (Fig. 7 C), Tbr2 (Fig. 7 D), Tbr1 (Fig. 8 C), or NeuN (Fig. 8 D) was confirmed using de-convolution imaging under high magnification.

In control, vector-transfected cells, most of the transfected cells (GFP+) were still proliferating (47% PCNA+ or 66% Tbr2+)
ERK5 Regulation of Neurogenin1

A

<table>
<thead>
<tr>
<th>GFP/Hoechst</th>
<th>β-III tubulin/Hoechst</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt Neurog1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>GFP/Hoechst</th>
<th>MAP-2/Hoechst</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt Neurog1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

% β-III tubulin⁺ cells in GFP⁺ population

D

% MAP-2⁺ cells in GFP⁺ population
ERK5 Regulation of Neurogenin1

Figure 6. Neurog1-induced neurogenesis is regulated by phosphorylation on S179 and S208 in monolayer cortical progenitor cultures. Cells were cultured as in Figure 5. A, B, Representative deconvolution images of cells infected with either GFP control virus or wt Neurog1, and immunostained for β-III tubulin (A) or MAP-2 (B) (red). Cells that were co-labeled with GFP and β-III tubulin, or MAP-2 were orange. Images were captured using 40× objective lens. Scale bar: 25 μm. C, D, Quantification of the data showing percentage of β-III tubulin+ neurons (C), or MAP-2+ mature neurons (D), in GFP+ population. Three independent experiments were done, each in triplicate and >2000 GFP+ cells were counted for each data point.

doi:10.1371/journal.pone.0005204.g006

(Fig. 7, E and F) and localized to SVZ/VZ. Only a small fraction of the cells had differentiated (8% Tbr1+ or 14% NeuN+) (Fig. 8, E and F) and migrated to the cortical plate (CP) after 40 h in culture. However, more of the wt Neurog1-transfected cells were found in the CP layer and were Tbr1+ (37%) or NeuN+ (45%). This supports our in vitro cell culture data shown in Fig. 1, 5, and 6 and demonstrates a role for Neurog1 in promoting neurogenesis in slice cultures. In contrast, cells transfected with the Neurog1 SA179/208 mutant behaved like vector control-transfected cells; most remained proliferative (54% PCNA+, 68% Tbr2+) and only a few expressed the post-mitotic neuron markers Tbr1 (12%) or NeuN (18%). Because the Neurog1 SA179/208-transfected cells found in the SVZ/VZ expressed PCNA and layer specific marker Tbr2 but did not express post-mitotic neuron markers Tbr1 or NeuN, we conclude that Neurog1 phosphorylation at S178 and S208 does not affect neuronal migration. These data suggest that mutations at the putative ERK5 phosphorylation sites S179 and S208 suppress the pro-neural activity of Neurog1.

We also examined the effect of blocking ERK5 expression on the pro-neural activity of Neurog1 using the organotypic slice culture assay. To block expression of endogenous ERK5, we constructed a retroviral shRNA vector against ERK5. A non-specific shRNA against dsRed (NS) was used as a control. Specific knockdown of ERK5 expression by shERK5 was confirmed in cultured rat E13 cortical progenitors (Fig. S1). Cells co-transfected with Neurog1 and shERK5 had greatly increased numbers of proliferative PCNA+ or Tbr2+ cells (Fig. 7, G and H) and fewer differentiated Tbr1+ or NeuN+ neurons (Fig. 8, G and H) compared to those co-transfected with Neurog1 and NS control. These data suggest that blocking ERK5 expression and signaling attenuates the pro-neural effect of Neurog1 in cortical slice cultures.

Discussion

The objective of this study was to investigate downstream mechanisms mediating the neurogenic activity of ERK5. We published evidence that ERK5 is highly expressed in proliferating cortical progenitor cells and is both necessary and sufficient to specify cortical progenitor cells towards a neuronal fate [18]. We report here that Neurog1 is a downstream target of ERK5. ERK5 directly phosphorylated Neurog1 in vitro and modulated the transcriptional and pro-neural activity of Neurog1 in cortical progenitors. We also identified S179 and S208 as putative ERK5 phosphorylation sites on Neurog1. These two serine residues are located within the putative transactivation domain of Neurog1 [26]. Intact S179 and S208 were required for Neurog1’s function since replacing each with a non-phosphorylatable alanine greatly attenuated the ability of Neurog1 to initiate transcription and specify neuronal fate. These data identify Neurog1 as a downstream target mediating the pro-neural effect of ERK5 and implicate phosphorylation of Neurog1 as a novel mechanism regulating neuronal fate commitment of cortical progenitors.

During cortical neurogenesis, the pro-neural bHLH transcription factors including Neurog1, Neurog2, and Ascl1 direct cortical progenitors to a neuronal fate [9]. Many signaling pathways have been implicated in stimulating neuronal differentiation including the Wnt/β-catenin pathway [27], PI3K [20], Notch pathway [15,29,30], and the ERK1/2 pathway [31,32]. However, it is not known if these signaling pathways regulate the pro-neural activity of the bHLH transcription factors. Protein phosphorylation has been implicated in regulating the stability and function of bHLH transcription factors during neuronal terminal differentiation, maturation and sub-neuronal phenotype specification. For example, Neurog1 stability is regulated by protein phosphorylation and subsequent ubiquitin-mediated proteolysis [33]. The function of Xenopus NeuroD in retinal neuron differentiation is inhibited by glycogen synthase kinase (GSK) β, presumably via GSK3β phosphorylation of XNeuroD [34]. CaMK II induces the phosphorylation of NeuroD at Ser 336, which regulates granule neuron dendritic morphogenesis during cerebellar development [35]. In addition to their pro-neural activity, Neurog1 and Neurog2 also regulate neuron migration [36,37], and a recent report implicates phosphorylation of Neurog2 in the regulation of neuron migration [37]. Another recent report demonstrates that Neurog2 phosphorylation at Ser231 and Ser234 by GSK3 regulates the specification of motor neuron subtypes but has no effect on the total number of neurons produced per se [38]. There is little published data addressing the role of kinases or phosphorylation in modulating the production of neurons which can be attributed to the function of Neurog1, Neurog2, or Ascl1. Our study is the first to demonstrate that phosphorylation of Neurog1 modulates the total number of neurons produced from cortical progenitors.

The putative ERK5 phosphorylation sites S179 and S208 are evolutionarily conserved among mouse, rat, and human sequences of Neurog1 (Fig. S2). A putative phosphorylation site similar to S208 is also found in the Neurog1 sequence of non-mammalian vertebrates zebrafish and xenopus. Furthermore, two putative phosphorylation sites comparable to S179 and S208 exist in the Neurog2 sequence (Fig. S3). Therefore, protein phosphorylation of the pro-neural bHLH transcription factors may be a common mechanism by which extrinsic factors in the neurogenic niche regulate the neuronal fate specification of neural progenitor cells. A large body of evidence suggests that environmental cues such as the microenvironment surrounding progenitor cells play an important role in cell fate determination of neural progenitor cells [16,17,39–42]. Since ERK5 is activated by neurotransmitters, growth factors, and neurotrophins including NT3/4 and BDNF [43,44], it seems likely that environmental cues may instruct cortical progenitors to become neurons by activating the ERK5-Neurog1 pathway.

Interestingly, the Neurog1-NeuroD axis bears significant similarity to the myogenic MyoD-Myogenin pathway of muscle differentiation [45]. There is evidence that the myogenic bHLHs, MyoD and Myogenin, are phosphoproteins [46,47] and that MyoD and Myogenin can be directly phosphorylated by ERK5 [48]. Besides its high level of expression in the nervous system, ERK5 is also highly expressed in muscle and is required for muscle differentiation [48]. Thus, phosphorylation of the bHLH transcription factors may be a conserved mechanism by which ERK5 regulates muscle and neuron differentiation. Neurog1 also confers
Figure 7. Mutations of Neurog1 at S179 and S208 and inhibition of ERK5 signaling retains Neurog1-transfected cells in proliferating state in organotypic slice cultures. Plasmid DNA encoding control vector, wt Neurog1, SA179/208 Neurog1, shRNA against dsRed (NS) or ERK5 (shERK5) was electroporated ex vivo into the dorsolateral telencephalon of rat E15 brain as indicated. A GFP expression vector was co-electroporated to identify transfected cells. Cortical slices were sectioned coronally, cultured for 40 h, and cryosectioned for immunostaining. A, B. Representative deconvolution images of cortical slices immunostained for GFP (green) and PCNA or Tbr2 (red), respectively. Images were captured using a 20× objective lens. Scale bar: 50 μm. C, D. Representative high magnification (63×) images of GFP+ cells co-labeled with PCNA or Tbr2, respectively. E–H. Quantification of cells double-immunostained for GFP and PCNA (panels E and G) or Tbr2 (panels F and H) in total GFP+ cells. Vector: vector control. The data were obtained from at least three sections each from three independent experiments. doi:10.1371/journal.pone.0005204.g007
Figure 8. Neurog1-induced neurogenesis is suppressed by mutations at S179 and S208 and inhibition of ERK5 signaling in organotypic slice cultures. Cortical slices were transfected and cultured as in Figure 7. A, B, Representative deconvolution images of cortical slices immunostained for GFP (green) and Tbr1 or NeuN (red), respectively. Images were captured using a 20x objective lens. Scale bar: 50 μm. C–D, Representative high magnification (63x) images of GFP+ cells co-labeled with Tbr1 or NeuN, respectively. E–H Quantification of cells double-immunostained for GFP and Tbr1 (panels E and G) or NeuN (panels F and H) in total GFP+ cells. Vector: vector control. The data were obtained from at least three sections each from three independent experiments. doi:10.1371/journal.pone.0005204.g008
anti-gliogenic activity independent of its pro-neural activity in the nervous system [7]. It would be interesting to examine if ERK5 phosphorylation also modulates the anti-gliogenic properties of Neurog1.

A number of ERK5 substrates have been identified, including myocyte enhancer factor (MEF) 2C, Sap 1a, c-myc, SGK (serum- and glucocorticoid-inducible kinase), the pro-apoptotic Bcl-2 family protein BAD, and pp90Rsk [20,49–51]. ERK5 has been implicated in many aspects of cellular and physiological function including apoptosis, cell cycle, muscle differentiation, cardiovascular function, neuronal survival, and neuronal cell fate specification [18,50–52]. Data presented here identify Neurog1 as a new substrate for ERK5 and implicate ERK5 in the regulation of the pro-neural bHLH transcription factors.

In summary, we identified a novel mechanism during cortical neurogenesis in which the pro-neural and transcriptional activity of Neurog1 is regulated by ERK5 through phosphorylation. Similar kinase phosphorylation mechanisms may also regulate the pro-neural activities of other bHLH family transcription factors including Neurog2 and Ascl1.

Materials and Methods
Reagents
The following plasmids have been described: the lentiviral transfer vector pRLI-exPPT-CMV-X-PRE-SIN [53], a kind gift from Dr. W. Osborne (University of Washington); NeuroD2-Luc reporter [3]; expression vectors for dnMEK5, caMEK5, wtERK5 and dnERK5 [20]. The Neurog1 expression vector (pCS2-NeuroD3) and the 3xE-box-Luc reporter (pCS2-EB7-Luc) were obtained from Dr. Jim Olson [3]. The cDNA sequence of Neurog1 was sub-cloned into pcDNA3 with a Flag-tag added to its N-terminus. For the truncated wt GST-Neurog1 and SA179/208 mutant, the cDNA sequences corresponding to residues 151–244 were subcloned into the pGEX vector. The rabbit polyclonal anti-ERK5 antibody [43] and the polyclonal Tbr2 and Tbr1 antibodies have been described [22]. The rabbit polyclonal anti-Neurog1 antibody was generated by immunizing rabbits (Cocalico Biologicals, Reamstown, PA) with GST-Neurog1 fusion protein. The following antibodies for immunostaining were purchased commercially: mouse monoclonal (M1) anti-Flag antibody (Sigma, St. Louis, MO); mouse monoclonal anti-nestin (Becton Dickinson, Bedford, MA); mouse monoclonal anti-NeuN (Chemicon, Temecula, CA); rabbit polyclonal anti-GFP (Molecular Probes, Eugene, OR); mouse monoclonal anti-GFAP (Molecular Probes, Eugene, OR); mouse monoclonal anti-β-III tubulin (Promega, Madison, WI); mouse monoclonal anti-MAP-2 (Sigma); mouse monoclonal anti-LeX (CD15 FITC) (Becton Dickinson); mouse monoclonal anti-PCNA (Chemicon). The following inhibitors were purchased commercially: Proteasomal Inhibitor Cocktail Set (Calbiochem, San Diego, CA), pan-caspase inhibitor ZVAD-FMK (R&D Systems, Minneapolis, MN).

Lentivirus constructs
We have constructed lentiviral transfer vectors as previously described [18]. All genes of interest were N-terminal Flag-tagged and inserted into the multiple cloning site upstream from an IRES-directed marker protein eGFP (enhanced green fluorescent protein).

Retrovirus constructs
The shRNA sequences against mouse ERK5 and dsRed [54] were cloned into the BamHI/XbaI sites of the multiple cloning site of the pSIE dual promoter retroviral vector [55]. The shRNA expression is under the control of human U6 promoter and GFP expression is under the control of the EF1α promoter. The targeted sequences used were as follows: ERK5 (aa 106–111) acactcaacagcaacat; dsRed-C1 agttccagtacggctccaa.

Rat E13 cortical progenitor cell cultures
These were prepared as described and cells were maintained in culture medium containing 10 ng/ml bFGF (Invitrogen, Inc) [18]. For the adherent culture monolayer assay or progenitor cell clonal assay, cortical progenitors were enriched by magnetic activated cell sorting (MACS) after labeling with an antibody against LeX (anti-CD15), a cortical progenitor marker [56]. For the neurosphere assay, freshly dissociated E13 cortical progenitor cells were plated at a clonal density of 2000 cells/ml in petri dishes without any coating.

Ex vivo electroporation and organotypic slice culture
Plasmid DNA was injected into the lateral ventricles of E15 rat brain and electroporated into the cortex using a CUY21 Edit square pulse electroporator (Bex Co. Ltd., Japan). Plasmid was targeted to the dorsal region of the telencephalon by placing the positive electrode directly superior to the telencephalon and the negative electrode ventral to the head. Following electroporation, dissected cortices were immobilized in a 4% agarose mold and sliced into 300 μm slices using a vibrating microtome, transferred to permeable membranes and placed in growth medium for 40–50 h. Organotypic slice cultures were fixed and cryosectioned into successive 20 μm slices for immunostaining. For Western analysis, regions with GFP expression (green) were micro-dissected out under a fluorescence microscope and homogenized with a syringe in lysis buffer followed by vortexing to prepare cell lysates.

Luciferase reporter gene assays
Rat E13 cortical progenitor cells were transiently transfected at days in vitro (DIV) 2 using the Nucleofector® electroporation method per manufacturer’s instruction (Amaxa Biosystems, Inc.). Briefly, the cells were grown as a monolayer in coated plates for 1–2 d, trypsinized at room temperature for 5 min and centrifuged at 750 g for 5 min. Cells were resuspended in Rat Neural Stem Cell Nucleofector® Transfection Reagent (Amaxa Biosystems, Inc.) at a density of 3×10⁶ cells/100 μl. For each transfection, 3×10⁶ cells were transfected with total plasmid DNA not exceeding 10 μg using the A31 (low toxicity) protocol. Following electroporation, cells were resuspended in pre-warmed (37°C) regular culture medium and incubated at 37°C for 20 min. Cells were then resuspended in regular culture medium and plated onto 24-well plates coated with laminin and poly-D-lysine (PDL). Cell lysates were prepared 36–48 h later for reporter gene assay as described [43].

For E16 cortical neuron luciferase reporter gene assays, neurons were prepared and transfected using LipofectAMINE 2000 (Invitrogen) as described [43,52].

Calf intestinal alkaline phosphatase treatment
Protein lysates were homogenized in protein lysis buffers lacking phosphatase inhibitors [57]. One hundred micrograms of protein lysates were treated with 10 units of CIP (Fermentas, Inc.) and 10 mM MgCl₂ for 60 min at 37°C. For control, protein lysates were homogenized in regular protein lysis buffers containing phosphatase inhibitors.

In vitro kinase assays
In vitro ERK5 kinase assays were performed as described [43,58]. Briefly, whole cell lysates (1000 μg protein) were incubated at 4°C for 2.5 h with 6 μl of polyclonal anti-ERK5
antibody. Protein A-Sepharose beads (60 µl) were then added, and the mixture was incubated at 4°C for an additional hour. The activity of ERK5 in the immunoprecipitates was quantified by a kinase assay using 30 µg recombinant wt GST-Neurog1 (151–244) or GST-Neurog1 SA179/208 (151–244) as the substrates. Relative radiolabeled Neurog1 and ERK5 was quantified using autoradiography and normalized to total wt GST-Neurog1 and GST-Neurog1 SA179/208 protein levels.

Microscopy and image acquisition

Representative images were generated by a Marianas imaging system (Intelligent Imaging Innovations, Inc.) incorporating a microscope (Axiovert 200M; Carl Zeiss MicroImaging, Inc.) with an X.Y motorized stage, shuttered 175 W xenon lamp coupled with a liquid light guide, a digital camera (CoolSNAP HQ; Roper Scientific), and 20× or 63× objective lenses (Axiovert; Carl Zeiss MicroImaging, Inc.) as indicated. Slidebook software package was used for system control and image processing. Adobe Photoshop was used to uniformly optimize images.

To capture images for quantification of organotypic slice cultures, images were generated with an inverted fluorescence microscope (Leitz DMIRB; Leica) using a 40× objective lens (Leitz; Leica). MagnaFire digital microscope camera and MagnaFire software (Optronics, Inc.) were used for system control and image processing.

Cell counting of organotypic slice sections

For quantification, 20 µm sections were chosen that expressed comparable levels of GFP within the same region of the dorsolateral telencephalon. For each condition, photographs were generated from three separate transfected regions using a 20× or 40× objective. To quantify the number of transfected cells that co-labeled with the cell-specific markers, each digital image was segmented into one-inch horizontal bins. The total number of cells per bin were recorded by Hoechst (blue channel) staining, the total transfected cells per bin were recorded by GFP (green channel) immunostaining, the total number of cells labeled for the cell-specific markers (PCNA, Tbr2, Tbr1, or NeuN) per bin were recorded (red channel), and the total transfected cells which co-labeled with the cell-specific markers (red and green channel) were recorded and cross-referenced with the Hoechst.

To ensure that cells were within the same plane in the digital images, each co-labeled cell was confirmed by toggling back and forth between the blue, red, green, and red-green channels in Adobe Photoshop. To validate cell counting acquired from 20× or 40× images, co-labeled (green/red) cells were visualized at higher magnification (63×) using a Marianas Imaging system and deconvolution scope.

Statistical Analysis

All data are expressed as the mean±standard error of means (SEM) from at least three independent experiments (n≥3). Pairwise comparisons between means were tested by a Student’s t-test, two-tailed analysis. n.s. not significant; *p<0.05; **p<0.01; and ***p<0.001.

Supporting Information

Figure S1

Found at: doi:10.1371/journal.pone.0005204.s001 (0.36 MB TIF)

Figure S2

Found at: doi:10.1371/journal.pone.0005204.s002 (5.26 MB TIF)

Figure S3

Found at: doi:10.1371/journal.pone.0005204.s003 (1.64 MB TIF)

Acknowledgments

We thank Dr. William Osborne for providing the lentiviral transfer vector pRRL-C-PPT-CMV-X-PRE-SIN; Dr. Hans-Peter Kiem and his core facility (supported by NIH DK56465) for assisting us in the initial preparation of lentivirus; and Dr. Jim Olson for providing us with the plasmids for Neurog1, 3xE-box-Luc, and NeuroD2-Luc. We also would like to thank Drs. Daniel Storm, Thomas Reh, and Nephi Stella for helpful discussions.

Author Contributions

Conceived and designed the experiments: PC ZX. Performed the experiments: PC LL YW JZ YWP GA. Analyzed the data: PC. Contributed reagents/materials/analysis tools: XD GM CE RH. Wrote the paper: PC ZX.
dimerization motif in globin enhancer binding, daughterless, MyoD,
BMPK/ERK5 regulates serum-induced early gene expression through
Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells,
subpopulation of Tbr1-expressing deep layer neurons in the developing cerebral
protein in vertebrates. Development 116: 201–211.
neurogenin1 induces neurite outgrowth in F11 neuroblastoma cells. Exp Mol
Med 34: 469–473.
basic helix-loop-helix transcription factor-coactivator complex formation and
critical for asymmetric cell division of mouse cortical stem cells and
inactivation of Notch signaling synchronizes differentiation of neural progenitor
a MEK-C/EBP pathway during growth factor-regulated cortical
Detection of ERK2 mitogen-activated protein kinase identifies its key roles in
Regulation of neurogenin stability by ubiquitin-mediated proteolysis. Biochem J
34. Moore KB, Schneider ML, Vetter ML (2002) Posttranslational mechanisms
neurogenin2 specifies the migration properties and the dendritic morphology of
generate neurons after transplantation in the adult dentate gyrus. J Neurosci 20:
6727–6735.
and differentiation of adult neuronal progenitor cells transplanted to the adult
41. Tsai RY, McKay RD (2000) Cell contact regulates fate choice by cortical stem
stimulate self-renewal and expand neurogenesis of neural stem cells. Science 304:
1388–1390.
regulation of mitogen-activated protein kinases ERK1/2 and ERK5 by
44. Wang X, Tourrier C (2006) Regulation of cellular functions by the ERK5
45. Jan YN, Jan LY (1993) HLH proteins, fly neurogenesis, and vertebrate
kinase II-dependent phosphorylation of myogenin contributes to activity-
dependent suppression of nACHR gene expression in developing rat myotubes.
inactivates myogenic helix-loop-helix proteins through phosphorylation of a
Extracellular signal regulated kinase 5 (ERK5) is required for the differentiation
and regulators of mitogen-activated protein kinase ERK5 (ERK5) using chimeric protein
G2-M cell cycle progression by the ERK5-NF{kappa}B signaling pathway. J Biol Chem
277: 253–264.
activation of MEF2-mediated gene expression plays a critical role in
BID-dependent promotion of survival during development but not mature cortical
neurons. Proc Natl Acad Sci USA 100: 8532–8537.
vectors encoding both central polypurine tract and posttranscriptional regulatory
element provide enhanced transduction and transgene expression. Hum Gene
Ther 12: 1105–1108.
synaptic integration of newly generated neurons in the adult brain. Nature 439:
589–593.
Schizophrenia 1 regulates integration of newly generated neurons in the adult
cells, identifying them as nonependymal. Neuron 34: 805–815.
duced changes in acetyl-CoA carboxylase and 5′-AMP-activated kinase in
Effects of ERK and JNK-p38 MAP Kinases on Apoptosis. Science 270:
1326–1331.