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A Phenotypic Small-Molecule Screen Identifies an Orphan Ligand-Receptor Pair that Regulates Neural Stem Cell Differentiation

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SUMMARY

High-throughput identification of small molecules that selectively modulate molecular, cellular, or systems-level properties of the mammalian brain is a significant challenge. Here we report the chemical genetic identification of the orphan ligand phosphoserine (P-Ser) as an enhancer of neurogenesis. P-Ser inhibits neural stem cell/progenitor proliferation and self-renewal, enhances neurogenic fate commitment, and improves neuronal survival. We further demonstrate that the effects of P-Ser are mediated by the group III metabotropic glutamate receptor 4 (mGluR4). siRNA-mediated knockdown of mGluR4 abolished the effects of P-Ser and increased neurosphere proliferation, at least in part through upregulation of mTOR pathway activity. We also found that P-Ser increases neurogenesis in human embryonic stem cell-derived neural progenitors. This work highlights the tremendous potential of developing effective small-molecule drugs for use in regenerative medicine or transplantation therapy.

INTRODUCTION

The use of small-molecule probes in biological studies has a long, rich history. Such compounds, though often found by chance, are increasingly sought after using high-throughput screening (HTS) efforts [1]. Complicated experimental systems, such as the mammalian brain, remain recalcitrant to systematic approaches [2, 3]. Potentially, cell-based chemical genetic screens, which can be used to identify compounds that produce a phenotype of interest, provide a simple, rapid, and content-rich format for identifying such small molecules [4].

Neural stem cells (NSCs) have the capacity to self-renew and ultimately produce the three major cell types of the brain, namely neurons, astrocytes, and oligodendrocytes. The discovery of neural stem cells has tremendous importance not only for understanding the generation of this cellular diversity but also has vast practical implications for repair of the central nervous system (CNS) following injury. The initial isolation of neural stem cells has led to large, systematic efforts to identify and manipulate molecular mechanisms of NSC proliferation, cell-fate determination, and differentiation. Although many of the genetic and biochemical pathways underlying these processes are beginning to be understood, currently there are few known small-molecule probes for specifically studying and controlling these mechanisms.

The need for such tools has been recognized for some time (reviewed in [5]), and previous studies have utilized cell lines to screen for small molecules that specifically induce differentiation of stem cells of neuroepithelial, and other, origin [6–8]. However, small-molecule probes for examining the intricately related processes of stem cell proliferation and cell-fate choice have remained unidentified, and the advantages of using primary culture stem cells have not been exploited. In the current study, we developed a reliable primary cell screen using neurospheres, ex vivo cultures containing neural stem cells [9]. Neurospheres derived from embryonic day 11.5 (E11.5) mouse telencephalon were screened against libraries of bioactive small molecules (following mitogen withdrawal), including kinase/phosphatase inhibitors and orphan ligands (see Experimental Procedures). Using these primary cultures as a screening platform, we identified phosphoserine (P-Ser), a member of the orphan ligand library and an endogenous serine metabolite, as a regulator of neural progenitor proliferation, fate commitment, and differentiation. Subsequently, we determined that these effects are mediated through activation of metabotropic glutamate receptor 4 (mGluR4). Broadly, these studies demonstrate the utility of relatively simple screening assays for identifying highly useful small-molecule probes which can be used to elucidate novel roles for cell-intrinsic and extrinsic factors in neural stem cell biology.

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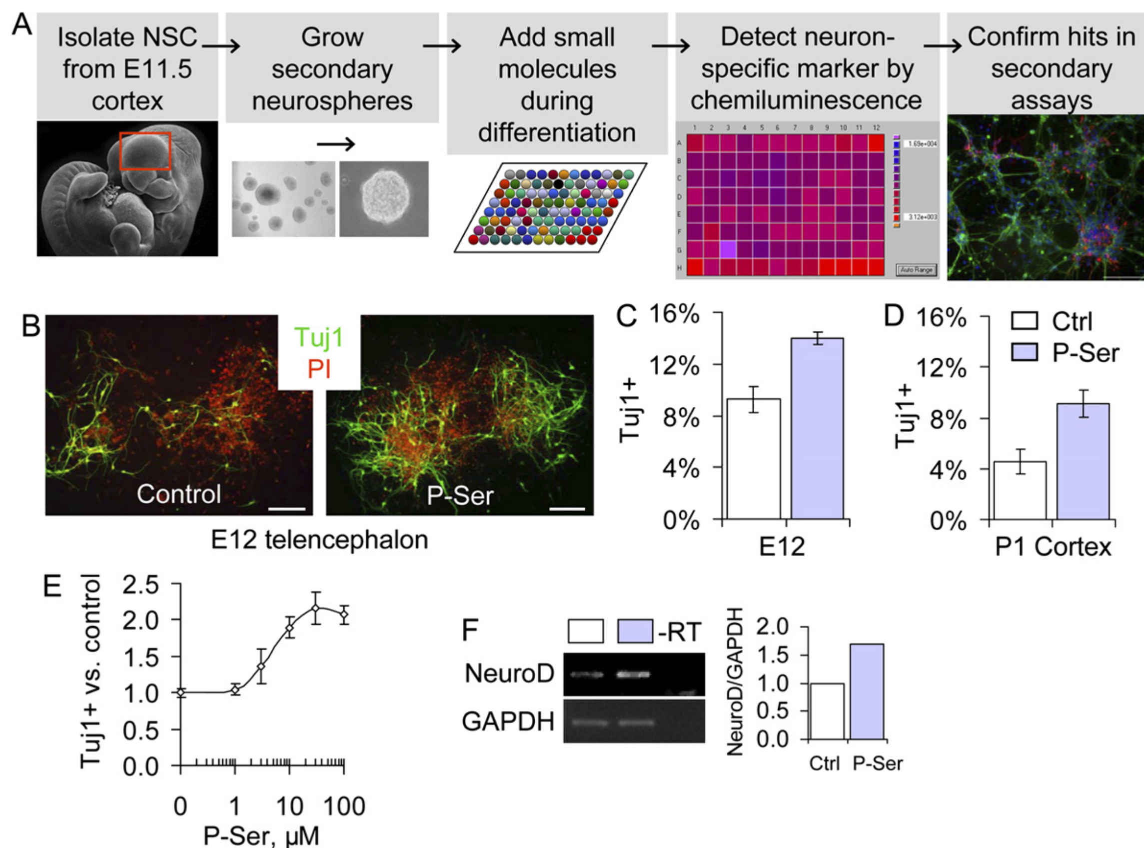

Figure 1. P-Ser Enhances Neuronal Differentiation of Neural Stem Cells

(A) Scheme of small-molecule screen. The scanning electron micrograph (SEM) micrograph is from the embryo images collection at http://www.med.unc.edu/embryo_images/, used with permission.

(B) Secondary E12.5 spheres differentiated with or without P-Ser and stained for Tuj1 and propidium iodide (PI). The scale bars represent 300 μm.

(C) Neuron counts of differentiated secondary E12.5 spheres, $p = 0.0013$.

(D) Neuron counts of differentiated secondary P1 cortical spheres, $p = 0.0169$.

(E) Dose response of P-Ser concentration versus number of neurons generated from differentiated E12.5 spheres. Data are normalized so that untreated is set to 100%.

(F) RT-PCR analysis of differentiated E12.5 spheres. –RT, no reverse transcriptase control. For densitometry, data are normalized so that untreated is set to 100%. P-Ser (10 μM) was used in all experiments. Data are represented as mean ± SEM in (C)–(E).

RESULTS

Primary neurospheres were passaged one time to expand the limited amount of starting material and to ensure a self-renewing progenitor population while minimizing effects of any differentiated cells that remain after dissection. Secondary neurospheres were differentiated in 96-well plates with one library compound per well for 3 days. Cultures were then processed for immunoreactivity against a commonly used neuron-specific marker, βIII tubulin (Tuj1), using chemiluminescent detection (Figure 1A). Approximately 7%–9% of cells normally differentiate toward a neuronal lineage and express Tuj1; this assay reliably detects molecules which enhance expression of this marker, as shown by the addition of the neurotrophin brain-derived neurotrophic factor (see Figure S1A in the Supplemental Data available with this article online). Importantly, this enhancement in Tuj1 immunoreactivity need not be due to increased neurogenesis; compounds which influence neurite branching or axonal outgrowth would also register as positive hits. Any potential candidates need to be tested in secondary assays to validate functional effects on the phenotype of interest.

For the primary screen using Tuj1 immunoreactivity as a readout, we assayed several hundred bioactive molecules (including kinase/phosphatase inhibitors and orphan ligands; see Experimental Procedures) and identified 64 compounds which were classified into two groups: "enhancers" which increased Tuj1 signals, and "inhibitors" which decreased Tuj1 signals (Table S1). Most of the inhibitors exhibited general cytotoxicity and were not studied further; others have been described as exerting negative effects on neuronal function, e.g., p38 MAP kinase inhibitors [10, 11]. Among the enhancers, several are known to improve neuronal survival, e.g., tobacco smoke constituents [12]. Fourteen compounds were chosen for secondary screening, of which five (four enhancers and one inhibitor; Figures S1B–S1D) were confirmed to

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modulate neuronal differentiation. A novel enhancer identified from the orphan ligand library is L-serine-O-phosphate (phosphoserine; P-Ser). P-Ser increased the number of Tuj1$^+$ cells in differentiating E12.5 cortical cultures (Figures 1B and 1C) in a dose-dependent manner (Figure 1E). Similar effects were observed in cultures derived from mouse postnatal day 1 (P1) cortex (Figure 1D). These results suggest that the effects of P-Ser are not limited to a single developmental stage. The increased neuronal identity of P-Ser-treated cultures was further confirmed by elevated expression of the neurogenic bHLH transcription factor NeuroD in differentiated E12.5 neurospheres, as determined by RT-PCR (Figure 1F).

The neurogenic phenotype of P-Ser could be due to effects on progenitor proliferation, cell-fate commitment, and/or neuronal survival. We first assayed the effects of P-Ser on progenitor proliferation using the neurosphere assay. P-Ser treatment of secondary neurospheres (see below) reduced proliferation, based on cell number measurements (Figure 2A) and neurosphere size (Figure 2B). This finding was confirmed by a 33% decrease in BrdU labeling of P-Ser-treated attached progenitor cultures (Figure 2C).

The findings above could represent an effect on neural stem cells, other progenitors within the culture, or both. Therefore, we also analyzed production of new stem cells during P-Ser treatment. Primary neurospheres can be dissociated and replated, as single cells, to form secondary neurospheres. Neurospheres form aggregates at cell densities of >5000 cells/ml, which can confound analysis of the true proliferative potential of the plated cells [13]. In contrast, at a cell density of 1000 cells/ml, nearly all newly formed neurospheres are clonal in origin, that is, derived from a single plated cell [14] (T.K.K. and H.I.K., unpublished observations). Thus, at this cell density, the neurosphere assay can be used as a reliable estimate of stem cell proliferation (see Discussion). Secondary neurospheres were grown under clonal conditions with or without P-Ser. These spheres were then dissociated to single cells and allowed to form tertiary neurospheres under optimal conditions. The number of tertiary neurospheres formed is a direct measure of stem cell proliferation. We found that there were fewer tertiary spheres formed from P-Ser-treated secondary spheres than from control secondary spheres (Figure 2D). Nearly all spheres were tripotent, giving rise to neurons, astrocytes, and oligodendrocytes (Figure S2A), suggesting that P-Ser inhibits proliferation of multipotent sphere-forming progenitors.

We next measured the effects of P-Ser on neurogenesis in proliferating neurospheres. We found that whereas P-Ser decreased cell number and neurosphere size (Figures 2A and 2B), there was an increase in the percentage of Tuj1$^+$ cells within the proliferating spheres (Figure 2E; Figure S2B). Upon differentiation, secondary spheres grown in P-Ser generated more Tuj1$^+$ cells than control cultures (Figure 2F). As these P-Ser-treated spheres were passaged continually, the relative percentages of Tuj1$^+$ cells generated upon differentiation increased compared to controls (Figure 2G). Furthermore, spheres grown in the presence of P-Ser contained elevated levels of the neuronal fate-determining bHLH transcription factor Neurogenin2 (Ngn2; Figure 2F), as determined by RT-PCR. Together, these results strongly suggest that P-Ser directly enhances commitment of NSCs toward a neurogenic fate.

This possibility was confirmed via clonal analysis. If P-Ser instructs commitment of NSCs toward a particular lineage, then tertiary neurospheres derived from treated secondary spheres should show a predisposition toward generating that fate. Therefore, secondary neurospheres were grown at clonal density in the absence or presence of P-Ser. These neurospheres were then passaged to form tertiary neurospheres at clonal density, in the absence of P-Ser, and subsequently differentiated to determine their neurogenic capacity. We found that P-Ser-treated spheres gave rise to tertiary spheres that were twice as neurogenic as control-derived tertiary spheres (Figure 2H).

We also tested whether P-Ser affects neuronal survival. To this end, we derived neuronal cultures from E14.5 mouse cortex, treated with P-Ser or water, and quantified the neurons at multiple time points. Compared to control conditions in which the number of Tuj1$^+$ cells with neuronal morphology decreased over time (Figures 3A and 3B), P-Ser treatment caused an increase in the number of these cells remaining in the cultures at both time points assayed (Figure 3B). This rescue was especially pronounced by 3 days postplating, suggesting that P-Ser has neuroprotective effects under these conditions (Figures 3A and 3B). Consistent with this possibility, P-Ser treatment resulted in a substantial decrease in the total number of apoptotic nuclei as indicated by TUNEL assay (Figures 3A and 3C), in agreement with the percentage of Tuj1$^+$ cells undergoing apoptosis after 3 days (Figure 3D).

We then investigated potential molecular mechanisms through which P-Ser could act to modulate these phenotypes. One possibility is that P-Ser influences the serine biosynthesis pathway, which has been postulated to be important for neurogenesis [15, 16]. Another possibility is that the neurogenic effects of P-Ser may be mediated by group III metabotropic glutamate receptors, for which it is an agonist [17]. The latter hypothesis is supported by our findings that another group III receptor agonist (L-AP4) [17] produced similar effects as P-Ser on neurosphere size, whereas a group III receptor antagonist (UBP1112) [18] reversed the effects of P-Ser (Figure 4A).

The effects of these compounds were also observed on differentiating secondary neurospheres derived from P1 cortex (Figure 4B). Here, treatment with either P-Ser or L-AP4 caused a >2-fold increase in the number of Tuj1$^+$ cells generated (Figure 4B). UBP1112 again abolished this increase (Figure 4B). No enhancement of neuronal differentiation was observed in E12 neurospheres following treatment with either 10 μM D-serine-O-phosphate or D-AP4 (Figure S3A), which are >300-fold less potent for this class of receptors than their L enantiomers [19, 20]. Thus, group III metabotropic receptors appear to mediate the effects of P-Ser in both proliferating and differentiating neurosphere cultures.

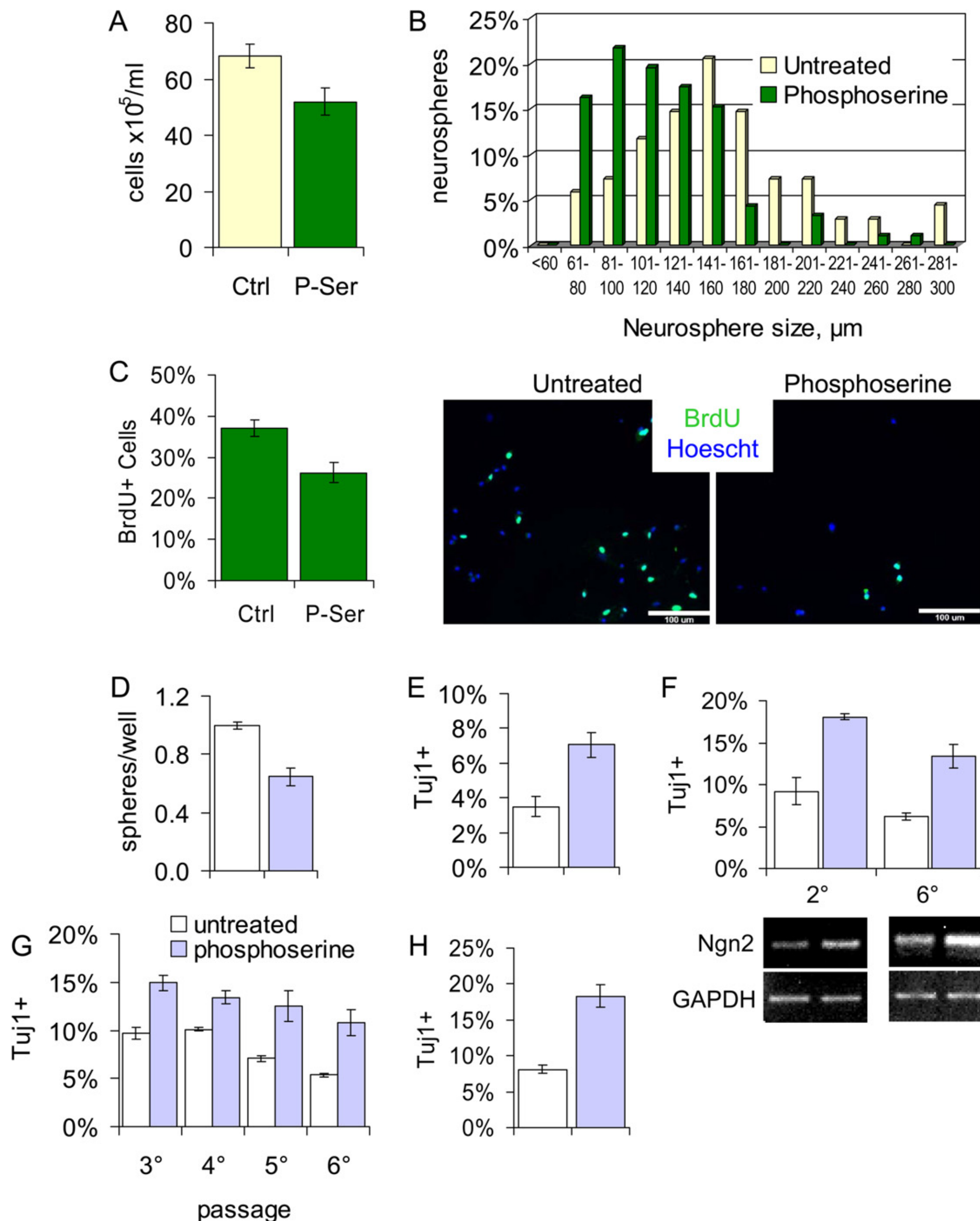

Figure 2. P-Ser Slows Progenitor Proliferation and Enhances Neurogenic Commitment

(A) Cells were plated at 1 k/ml and grown with or without 10 µM P-Ser for 1 week, then trypsinized and counted by trypan blue exclusion, p = 0.0293.

(B) Histogram of clonal neurosphere size after 1 week in culture.

(C) BrdU labeling index; dissociated primary neurospheres were plated on polyornithine-fibronectin-coated coverslips and grown for 4 days with or without 10 µM P-Ser, then pulsed with 5 µM BrdU for 2 hr and fixed, p = 0.009. The scale bars represent 100 µm.

(D) Number of tertiary clonal neurospheres derived from P-Ser-treated (10 µM) or untreated secondary neurospheres. Data are normalized to untreated (100%), $p = 1.91 \times 10^{-5}$.

(E) Analysis of Tuj1$^+$ cells present in acutely dissociated neurospheres grown with or without 10 µM P-Ser, p = 0.00137.

(F) Neurospheres were grown continually with or without 20 µM P-Ser for the indicated number of passages. Neurospheres were then harvested for RNA for RT-PCR analysis of ngn2 expression, or differentiated and stained for Tuj1, p(2°) = 0.0031, p(6°) = 0.00031.

(G) Neurospheres were continually grown with or without 20 µM P-Ser over multiple passages. Aliquots of each passage were differentiated and stained for Tuj1, p(3°) = .00295, p(4°) = 0.0163, p(5°) = 0.0111, p(6°) = 0.0075. Multiple comparisons across passages were not made.

(H) Tertiary neurospheres were derived from P-Ser-treated (20 µM) or untreated secondary spheres, differentiated for 5 days, and stained for Tuj1, $p = 2.86 \times 10^{-5}$. Data are represented as mean ± SEM in (A) and (D)–(H).

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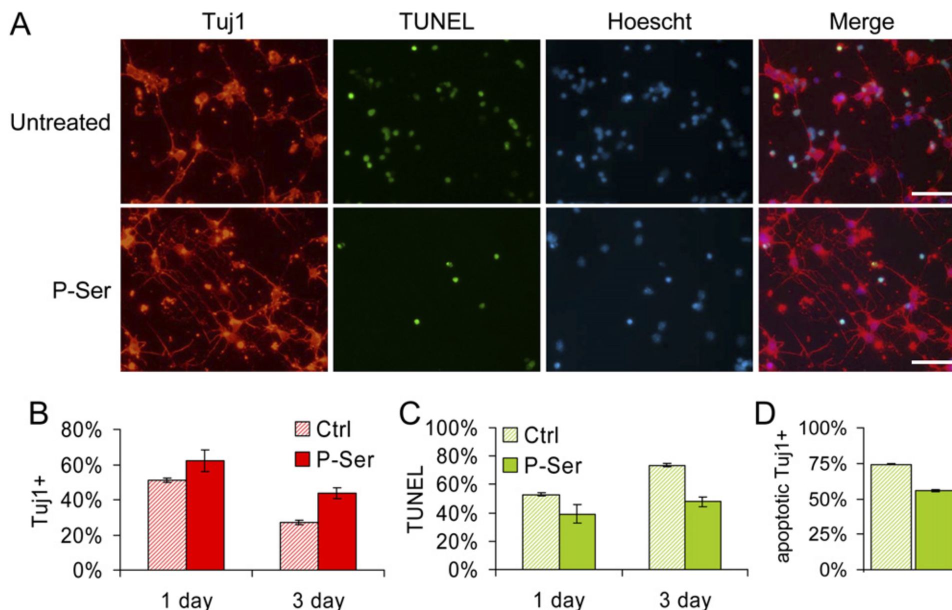

Figure 3. P-Ser Improves Survival in Differentiating Neuron Cultures

(A) Three day old E14.5 cortical neurons cultured with or without 10 μM P-Ser. TUNEL assay (green) was performed on cultures, followed by staining for Tuj1 (red). The scale bars represent 25 μm.

(B) Number of Tuj1$^+$ cells in 1 or 3 day E14 cortical neuron cultures untreated or with 10 μM P-Ser. 1 dayCtrl versus 1 dayP-Ser, p = 0.0931; 3 dayCtrl versus 3 dayP-Ser, p = 0.0346; 1 dayCtrl versus 3 dayCtrl, p = 0.0136; 1 dayP-Ser versus 3 dayP-Ser, p = 0.0284.

(C) Number of TUNEL$^+$ cells in 1 or 3 day E14 cortical neuron cultures untreated or with 10 μM P-Ser. 1 dayCtrl versus 1 dayP-Ser, p = 0.0719; 3 dayCtrl versus 3 dayP-Ser, p = 0.0138; 1 dayCtrl versus 3 dayCtrl, p = 0.025; 1 dayP-Ser versus 3 dayP-Ser, p = 0.1877.

(D) Number of TUNEL$^+$ Tuj1$^+$ double-positive cells as a percentage of total Tuj1$^+$ cells in 3 day E14 cortical neuron cultures untreated or with 10 μM P-Ser, p = 0.0013. All data are presented as mean ± SEM.

In order to examine the physiologically relevant receptor species, we analyzed the expression of the various members of the group III mGluRs by RT-PCR. In primary neurospheres, mGluR7 and 8 were present at very low levels, whereas mGluR4 was moderately abundant (Figure 4C). In secondary neurospheres, only mGluR4 was detected (Figure 4C). The primer sets used were equally capable of detecting equimolar quantities of appropriate template in control reactions (Figure S3B), suggesting that observed differences in mRNA quantity were not due to inefficient amplification by different primer sets. mGluR4 mRNA was present in the positive and negative fractions of secondary neurospheres fluorescence activated cell (FAC)-sorted for the NSC marker LeX [21] (Figure S3C), indicating the receptor is present in progenitors as well as more differentiated or committed cells. As a control, the progenitor-specific marker nucleostemin [22] was correctly detected only in the LeX(+) fraction (Figure S3C). According to the Allen Brain Atlas (http://www.brain-map.org/) [23], mGluR4 was expressed by a subset of cells within the subventricular zone proximal to the lateral ventricles (one of two neurogenic germinal zones within the adult brain [24]; Figure S3D), as shown by nonisotopic in situ hybridization. A specific probe for mGluR8 did not detect any positive cells within this region (Figure S3D). Thus, mGluR4 expression patterns suggest an important role for this receptor in mediating neural progenitor proliferation.

To confirm that mGluR4 is the target of P-Ser for progenitor proliferation, we used a loss-of-function approach by RNA interference. Two siRNAs (Supplemental Experimental Procedures) were designed to target mGluR4; both effectively reduced mGluR4 mRNA levels (Figure 4D). Dissociated primary spheres were transfected with mGluR4 siRNA versus control siRNA and replated to form secondary spheres in the presence of either P-Ser or vehicle. As shown in Figure 4E, although P-Ser inhibits proliferation of cells transfected with control siRNA (as expected), this inhibition by P-Ser is lost in mGluR4 knockdown cells, indicating that the effect of P-Ser is indeed mediated through mGluR4. Interestingly, we found that mGluR4 knockdown produced larger spheres than control cells (Figure 4E), suggesting that mGluR4 may serve to restrict neural progenitor growth. Consistent with this hypothesis, mGluR4 knockdown led to hyperactivation of mTOR, a major regulator of cell growth and proliferation [25], as indicated by the greatly elevated levels of phospho-S389 S6K1 (Figure 4F).

We then tested the effects of mGluR4 knockdown on neuronal differentiation. Following transfection with control or receptor-specific siRNA duplexes, progenitors were differentiated in the absence or presence of P-Ser. As expected, cells transfected with control siRNA generated more Tuj1$^+$ cells when treated with P-Ser (Figure 5A). Consistent with previous results, knockdown of mGluR4

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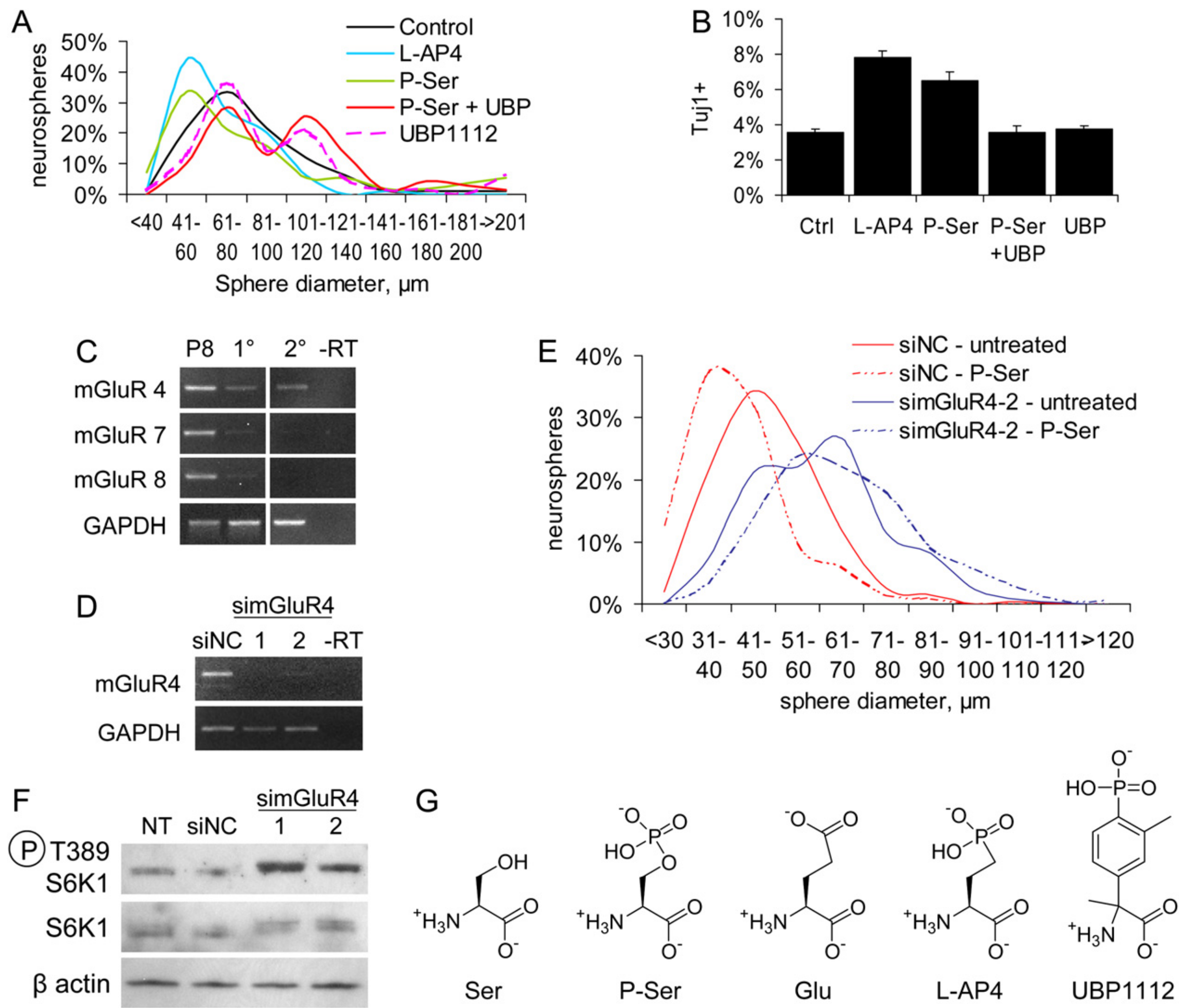

Figure 4. The Effects of P-Ser Are Mediated by Metabotropic Glutamate Receptor 4

(A) Histogram of secondary sphere sizes; cells were plated at 1 k/ml and grown untreated or with 10 μM P-Ser, 20 μM L-AP4, 15 μM UBP1112, or 10 μM P-Ser + 15 μM UBP1112.

(B) P1 secondary cortical spheres, differentiated untreated or with 20 μM L-AP4, 10 μM P-Ser, 10 μM P-Ser + 15 μM UBP1112, or 15 μM UBP1112, then stained for Tuj1 expression. Ctrl versus L-AP4, $p = 0.0034$; Ctrl versus P-Ser, $p = 0.0041$; P-Ser versus P-Ser + UBP, $p = 0.0065$. Presented as mean ± SEM.

(C) RT-PCR analysis of group III metabotropic glutamate receptor expression in primary (1°) and secondary (2°) neurospheres, and postnatal day 8 mouse brain (P8; positive control). The retina-specific mGluR6 was not detected in any experiments (data not shown). –RT, no reverse transcriptase.

(D) RT-PCR analysis of cells transfected with negative control siRNA (siNC) or duplexes targeting mGluR4 (simGluR4).

(E) Histogram of neurosphere size following transfection with siNC or simGluR4 and treatment with water or 10 μM P-Ser. Similar results were obtained with both duplexes; for clarity, only results from duplex 2 (simGluR4-2) are shown.

(F) Western blot analysis of E12.5 adherent progenitors cultured for 3 days posttransfection with either siNC, simGluR4-1, or simGluR4-2, or no transfection (NT).

(G) Chemical structures of L-serine, P-Ser, L-glutamate, L-AP4, and UBP1112.

abrogated the effect of P-Ser on neuronal differentiation (Figure 5A). Interestingly, we consistently observed an increase in O4$^+$ cells under the receptor knockdown conditions, regardless of P-Ser treatment (Figures 5B and 5C). These data show that mGluR4 mediates the effects of P-Ser on neuronal differentiation and also suggests an unexpected role for mGluR4 in oligodendrocyte differentiation.

The experiments described above suggest that treatment with P-Ser provides a simple method to enhance neuronal production from mouse neural progenitors. To determine whether such is also the case for human neural progenitors, and to investigate potential therapeutic application of these studies, we determined the effects of P-Ser on differentiating neural progenitors derived from human embryonic stem (hES) cells. As shown in Figures 6A–6C,

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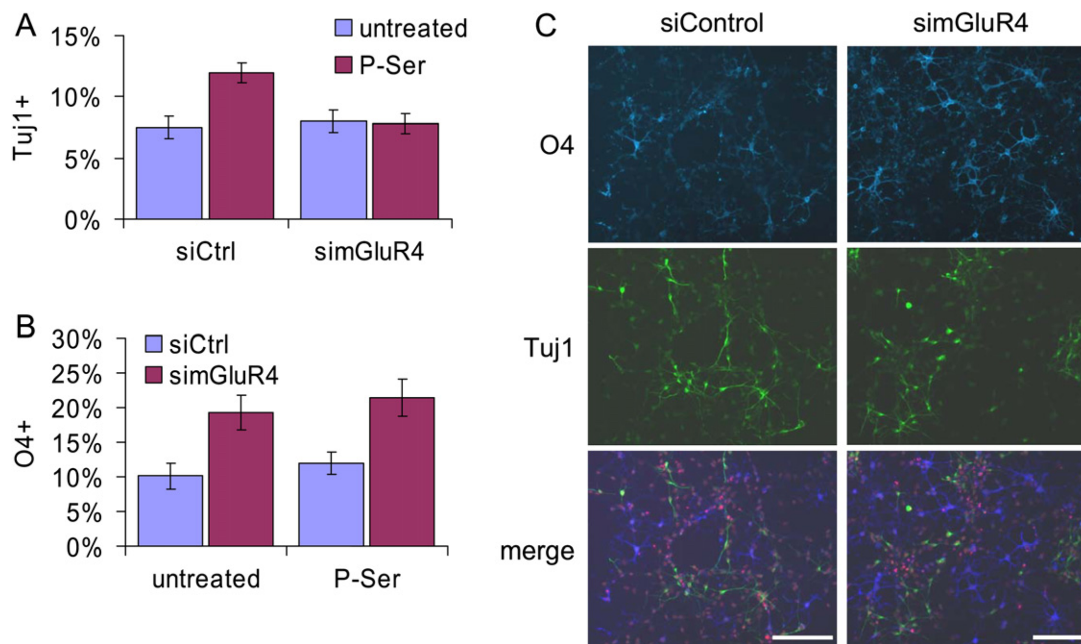

Figure 5. mGluR4 Mediates the Effects of P-Ser on Differentiation

(A) mGluR4 knockdown abolishes P-Ser-induced neuronal differentiation. E12 progenitors were transfected with negative control siRNA (siControl) or duplex targeting mGluR4 (simGluR4) and differentiated for 3 days in the absence or presence of 20 μM P-Ser, and stained for Tuj1. siCtrl untreated versus siCtrl P-Ser, $p = 0.0018$; siCtrl P-Ser versus simGluR4 P-Ser, $p = 0.0021$.

(B) mGluR4 knockdown increases oligodendrocyte differentiation. Cells were treated as in (A) and stained for the oligodendrocyte marker O4. siCtrl untreated versus simGluR4 untreated, $p = 0.0374$; siCtrl P-Ser versus simGluR4 P-Ser, $p = 0.0184$.

(C) E12 progenitors transfected with siControl or simGluR4, differentiated in the absence of P-Ser, and stained for Tuj1 (green) and O4 (blue), counterstained with propidium iodide (red). The scale bars represent 50 μm.

Data are represented as mean ± SEM in (A) and (B).

treatment with P-Ser doubled the number of neurons produced from two hES cell lines, HSF-1 (National Institutes of Health [NIH] UC01; karyotype XX) and HSF-6 (NIH UC06; karyotype XY). These results demonstrate that the neurogenic effects of P-Ser are neither species nor gender specific, and thus should prove broadly useful in directing hES cells for neural regeneration.

DISCUSSION

In this paper, we describe high-throughput screening using primary cells to identify small molecules that regulate complex phenotypes. There are clear advantages in being able to use primary cells from animals of defined genetic makeup and developmental stage, as well as patient-derived cells, as opposed to cell lines that may contain unknown genetic abnormalities or adaptations. We utilized a simple method for the propagation of multipotent neural progenitors that yielded reliable and reproducible data. This culture system has proven invaluable for determining the roles of both cell-intrinsic and extrinsic regulators (i.e., PTEN, LIF, and BMPs) of NSC proliferation, commitment, and differentiation [26–29].

When dissected neural tissue is plated as single cells under nonadherent, permissive conditions, floating balls of cells termed neurospheres [9] are formed. These highly heterogeneous structures contain neural stem cells as well as more restricted (i.e., bi- and unipotent) progenitors and differentiated progeny. The presence of stem cells can be confirmed by dissociating these neurospheres into single cells and replating them. A small percentage (generally ~3%) of these cells will re-form neurospheres, demonstrating self-renewal. Multipotency of all of these spheres can be determined post hoc, by differentiating them and performing immunocytochemistry for common markers of all three lineages.

To truly test the self-renewal potential of cells within the neurosphere cultures, dissociated single cells need to be plated at a density at which a single cell gives rise to a single neurosphere. This is termed clonal analysis, and recent reports [13, 30] have demonstrated that, when cultured together under medium to high cell densities, small neurospheres can adhere to each other and combine to form larger neurospheres. Clonal analysis is impossible under these conditions, and the true proliferative capacity of the cells cannot be determined.

We have previously determined culture conditions which allow for true clonal analysis [14]. Neural stem cells from GFP^{+} animals were dissected and mixed with comparable cells from a wild-type animal, and the resulting mixture was plated at varying cell densities. These different cell densities were then analyzed for the presence of

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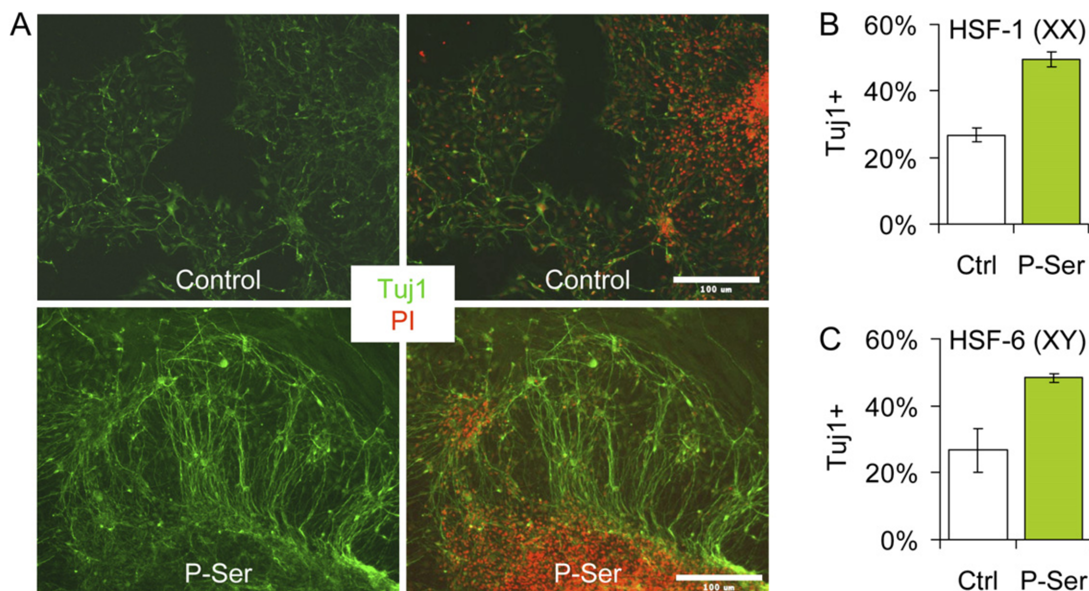

Figure 6. P-Ser Increases Neuronal Differentiation of Human Embryonic Stem Cells

(A) Human embryonic stem cell-derived neural progenitors (hES-NPCs) derived from line HSF-1 were differentiated with or without 10 μM P-Ser for 6 days, then stained for Tuj1 expression. Left panels: Tuj1, green; right panels: Tuj1, green; propidium iodide, red. The scale bars represent 100 μm.

(B) hES-NPCs derived from line HSF-1 were differentiated as above and the percentage of $Tuj1^+$ cells was determined, p = 0.000862.

(C) hES-NPCs derived from line HSF-6 were differentiated as above and the percentage of $Tuj1^+$ cells was determined, p = 0.0158. Data are represented as mean ± SEM.

chimeric neurospheres. Under high-density conditions, nearly all spheres contained both GFP^+ and GFP^- spheres, mirroring the results of Singec et al. [13] and Jessberger et al. [30]. However, no chimeric neurospheres were observed at a low cell density of 1000 cells/ml. We have recently reconfirmed these data, observing <5% chimeric neurospheres when cells were plated at 1000 cells/ml in 96-well plates (T.K.K. and H.I.K., unpublished observations). These results are important for proper interpretation of the clonal analysis and sphere size data reported here (although not for the primary screen, which was performed at high density).

In our initial screen, we utilized neurospheres passaged one time to assay for cell differentiation and/or fate commitment effects. These neurospheres contained very few differentiated cells, as judged by TuJ1 immunocytochemistry (Figure 2E and data not shown), at the time they were exposed to the chemical libraries, ensuring that observed effects were not due to the presence of neurons that were present at the time of initial culturing. It is important to note that this screen does not require that each neurosphere is derived from a single stem or progenitor cell. Furthermore, the general methodology described here is widely applicable. As effects on differentiation were determined via antibody-based detection [31], this assay should be easily adaptable to studying any process of interest so long as one has a suitable antigenic marker. This methodology is highly advantageous for screens utilizing delicate cell types such as primary culture stem cells (although nearly any cell type may be used); specifically, no genetic manipulation, such as introduction of a reporter gene, is necessary.

Here we show that our method has great potential for drug discovery and functional studies. Specifically, we identified the orphan ligand P-Ser as a neurogenic enhancer, and established group III metabotropic glutamate receptor 4 as its target. Metabotropic glutamate receptors are generally located presynaptically, where they act to modulate neuronal excitability and play roles in learning and memory, depression, and other behaviors [32]. Recent reports have demonstrated a role for group I (G_q-linked) and II (G_s-linked) receptors in stem cell biology in response to glutamate; mGluR3 and 5 have been shown to promote proliferation of embryonic stem cells [33] and neural stem cells [34, 35], and a positive role in neural stem cell proliferation has been suggested for group II receptors in response to high extracellular concentrations of glutamate [36]. We found a negative role for group III receptors (G_i-linked) in inhibiting neural stem cell/progenitor proliferation and in enhancing neuronal survival. mGluR4 has previously been shown to inhibit proliferation of unipotent cerebellar granule cell precursors [37]; in contrast to this report, we highlight effects of activation of this receptor by an endogenous ligand which act together to regulate proliferation, self-renewal, and neuronal lineage commitment of multipotent progenitors, as well as survival of their differentiated progeny, derived from multiple developmental time points.

Our data support the hypothesis that neural progenitor proliferation, cell fate, and terminal cell differentiation and survival are coupled. For example, we and others have demonstrated that the phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10)

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negatively regulates neural progenitor proliferation, as well as neuronal differentiation and survival [14, 29] (for a review, see [38]). Others have shown that the length of the neural progenitor cell cycle is directly coupled to cell-fate choices, as factors that shorten the cell cycle inhibit differentiative divisions, whereas those that lengthen the cell cycle promote differentiative divisions [39]. Thus, the data presented here are consistent with a model whereby mGluR4 activation by P-Ser promotes neurogenesis through enhanced neurogenic commitment (possibly a result of increased expression of bHLH transcription factors; Figures 1F and 2F) by slowing progenitor proliferation.

As mentioned above, BMP, LIF, Wnt, PTEN/PI3K-Akt, and other signaling events act to regulate NSC proliferation and differentiation. Known downstream effectors of these regulators include the MAPK, STAT, and mTOR pathways (which display extensive crosstalk) [40–42]. PTEN is a negative regulator of the PI3K-Akt-mTOR pathway [42]. Activation of Akt results in enhanced neural progenitor proliferation [43], neuronal differentiation [44], and neuronal survival [45]. Treatment with rapamycin, an mTOR inhibitor, results in decreased neurosphere formation [43]. Consistent with these observations, we found that, in addition to abrogating the effects of P-Ser, siRNA knockdown of mGluR4 also results in a large enhancement of mTOR activity (Figure 4F). This is likely due to loss of constitutive (ligand-dependent) activity upon receptor knockdown. Although P-Ser enhances neuronal differentiation and survival, activation of mGluR4 by P-Ser moderately inhibits Akt and mTOR activity (data not shown). The nature of this complexity could arise from differential activation thresholds for these processes, disparate effects of divergent downstream effectors, modulation by unexamined pathways, or other possibilities.

Although the neurogenic activity of P-Ser was discovered in vitro, this molecule likely has direct relevance for in vivo neurogenesis. The ''phosphorylated'' serine biosynthetic pathway seems to be the main mechanism through which the brain manufactures L-serine. The enzyme 3-phosphoglycerate dehydrogenase (3-PGDH) catalyzes the unidirectional production of a pyruvate intermediate, which is further metabolized to P-Ser by phosphohydroxypyruvate aminotransferase. P-Ser is then irreversibly dephosphorylated by phosphoserine phosphatase (PSP) to form L-serine (for a discussion, see [46]). 3-PDGH is specifically expressed by radial glia and the astrocytes derived from them [47]. Furthermore, genetic deletion of 3-PGDH results in midembryonic lethality accompanied by severe neurodevelopmental defects [16]. PSP was identified by a genetic screen to be differentially expressed in neural progenitors versus their differentiated progeny [48], and is expressed in the germinal zones during embryonic and postnatal development [49].

Provocatively, PSP is specifically expressed by GFAP-expressing progenitors within the subventricular zone (a region of ongoing adult neurogenesis [24]) and is present in only a subset of 3-PGDH-expressing cells within this area [50]. This suggests that whereas many cells within the neurogenic regions may synthesize P-Ser, only some of these cells are capable of further metabolizing it to L-serine and removing P-Ser from their local microenvironment. Following along this reasoning, P-Ser may be released and then act in an autocrine or paracrine manner, whereas PSP-expressing cells may be able to ''avoid'' P-Ser's effects by converting it to L-serine. Furthermore, as P-Ser has 10- to 100-fold higher potency for mGluR4 than glutamate [51] and is active only at group III (but not at group I or II) receptors [17], a model can be envisioned where interplay between P-Ser and glutamate contributes to drive neural progenitor behavior in vivo.

The identification of this P-Ser/mGluR4 pathway has implications not only for neural development but also for neural repair. A great deal of effort has been invested in identifying methods of efficient neuronal production from both embryonic and neural stem cells, in vitro and in vivo. In the current study, we demonstrate that P-Ser treatment dramatically enhances neuronal production from human embryonic stem cells, as well as from murine CNS-derived neural progenitors, making it a highly cost-effective reagent to potentially ''prime'' transplantable cells for differentiation toward a neuronal cell fate. These studies also raise the possibility that P-Ser or other mGluR4 effectors could be used to promote neurogenesis following stroke or other focal CNS injury.

Surprisingly, we also found a potential role for mGluR4 in oligodendrocyte differentiation. In mGluR4 knockdown cells, western blot analysis of Stat3 (another mTor substrate [52]) revealed the presence of lower molecular weight bands (Figure S4), similar to those previously shown to arise through proteolytic cleavage of Stat3 mediated by nonapoptotic caspase activity [53, 54]. As Stat3 signaling is important in astrogliogenesis [55], regulated degradation of Stat3 in mGluR4 knockdown cells may promote generation of oligodendrocytes at the expense of astrocytes, although the details of this mechanism are currently under investigation.

In summary, we have demonstrated a powerful, easily adaptable, and robust method to screen molecular libraries for compounds that enhance neuronal production from ex vivo stem and progenitor cells. This method was used to identify P-Ser (phosphoserine), an endogenous serine metabolite, as a regulator of neural stem cell proliferation and differentiation. Use of P-Ser as a tool to probe these processes at the molecular level led to the identification of metabotropic glutamate receptor 4 as the mediator of P-Ser effects, and revealed a novel role for this receptor in neural stem cell biology.

SIGNIFICANCE

Neural stem cells (NSCs) are capable of producing new neurons, and are thus of great interest to those studying the means to repair the central nervous system. Enhancing the generation of neurons from NSCs is an important therapeutic goal, and an understanding of the mechanisms by which neural stem cells proliferate and produce neurons will be critical to these efforts. Small molecules that regulate neural stem cell

proliferation and differentiation would be highly useful as tools for exploring and controlling the mechanisms underlying these processes, and could serve as candidate lead compounds for therapy of a variety of disorders including stroke and spinal cord injury. Here we report the development of a chemical genetic screen using cells isolated from embryonic mouse forebrain. We used this screen to identify the orphan ligand phosphoserine (P-Ser) as a regulator of NSC differentiation. P-Ser inhibits NSC proliferation and promotes survival of nascent neurons. P-Ser also promotes neuronal differentiation of human embryonic stem cell-derived neural stem cells. Thus, P-Ser has potential therapeutic value in addition to its basic utility as a probe for dissecting molecular mechanisms underlying neurogenesis.

This endogenous amino acid metabolite is a ligand for group III metabotropic glutamate receptors (mGluRs), and we show that the group III mGluR4 subtype mediates the effects of P-Ser on NSC proliferation and differentiation (a previously unknown role for mGluR4). Furthermore, we found that the effects of mGluR4 are mediated, at least in part, via the mTor pathway, a pathway known to mediate neural stem cell proliferation and neuronal survival. The current findings not only specifically establish a role for P-Ser and mGluR4 in neural stem cell biology but also demonstrate the potential usefulness of cell-based chemical genetic screens to identify molecules for use in both basic and translational neural stem cell research.

EXPERIMENTAL PROCEDURES

Small-Molecule Screen

Four days after passaging primary spheres to single cells, the resultant secondary spheres were resuspended in differentiation media (750 spheres in 200 μl) and cultured in white 96-well plates (Corning Life Sciences, Corning, NY, USA) coated with 10 μg/ml poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA). Libraries of orphan ligands and kinase/phosphatase inhibitors were obtained from Biomol (Plymouth Meeting, PA, USA). Compounds were transferred to assay plates using 96-pin replicators (Genetix Limited, Hampshire, UK). Spheres were incubated for 3 days and used for antibody probing. Antigen detection was similar to cytoblotting protocols [31, 56], with the following modifications. Following incubation with Tuj1 antibody (1:500; Covance, Princeton, NJ, USA) overnight at 4°C, HRP-conjugated goat anti-mouse secondary antibody (Pierce, Rockford, IL, USA) was added for 2 hr at room temperature, and chemiluminescence was developed with Supersignal ELISA Pico substrate (Pierce). Signal was quantified on a plate reader (Analyst HT; Molecular Devices, Sunnyvale, CA, USA).

Human Embryonic Stem Cell Culture

hESC lines HSF6 (46, XX) and HSF1 (46, XY) were maintained in irradiated CF1 mouse embryonic fibroblast (MEF) cells in high-glucose DMEM supplemented with 1 mM L-glutamine, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol, and 20% knockout serum replacement (all from Invitrogen, Carlsbad, CA, USA). Culture medium was changed and supplemented with 10 ng/ml bFGF daily. Confluent hESC cultures were passaged weekly by incubation in 1 mg/ml dispase/collagenase IV (Invitrogen) for 20 min at 37°C.

For directed differentiation into neural progenitor cells (NPCs), hESCs were passaged onto dishes coated with Matrigel (1:10 dilution; BD Biosciences, San Jose, CA, USA) in the absence of irradiated MEFs. hESC medium was replaced with NPC medium (DMEM/F12 with B27 supplement and 500 U/ml penicillin/streptomycin, all from Invitrogen) 4 days after passaging. Partially differentiated hESCs were cultured for an additional 4 days, on Matrigel, in NPC medium with daily addition of 10 ng/ml bFGF. hESC-NPCs were then continuously passaged mechanically and cultured on PO/FN-coated culture dishes (polyornithine and fibronectin; Sigma-Aldrich). For differentiation of hESC-NPCs, cells were washed with Dulbecco's PBS, and fresh NPC medium, without bFGF, was added. Cells were incubated for 6 days, with one intermediate medium change. P-Ser (20 μM) was added to appropriate wells. The UCLA Institutional Review Board has approved all experiments involving hESCs.

Other procedures are described in the Supplemental Data.

Supplemental Data

Supplemental Data include four figures, one table, and Supplemental Experimental Procedures and are available at http://www.chembiol.com/cgi/content/full/14/9/1019/DC1/.

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