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Neurochemistry

Imidazole-Based Small Molecules that Promote Neurogenesis in Pluripotent Cells**

*Gun-Hee Kim, Debasish Halder, Jinhong Park, Wan Namkung, and Injae Shin**

Abstract: *Reported herein are two imidazole-based small molecules, termed neurodazine (Nz) and neurodazole (Nzl), which induce neuronal differentiation of pluripotent P19 cells. Their ability to induce neurogenesis of P19 cells is comparable to that of retinoic acid. However, Nz and Nzl were found to be more selective neurogenesis inducers than retinoic acid owing to their unique ability to suppress astrocyte differentiation of P19 cells. Our results also show that Nz and Nzl promote production of physiologically active neurons because P19-cell-derived neurons induced by these substances have functional glutamate responsiveness. The present study suggests that Nz and Nzl could serve as important chemical tools to induce formation of specific populations of neuronal cell types from pluripotent cells.*

Neurodegenerative diseases, including Parkinson's, Alzheimer's, and Huntington's diseases, result from the loss of the structure and function of neurons which cause progressive and irreversible deterioration of the nervous system. These diseases, which represent an area of unmet medical need, will become more serious in the near future because of the increase in the age of the world's population. When damaged or destroyed, neurons are rarely regenerated in the body under normal physiological conditions. Recently, a strategy (we termed this small-molecule-based cellular alchemy) to employ small molecules which promote changes of the fate of cells to produce specific cell types has received great attention for biomedical applications because small molecules have several advantages, such as high temporal control (or rapid, reversible regulation of target function), facile adjustment of their effects, easy development of therapeutic agents, and suppression of cancer or other diseases, over genetic methods.[1]

Over the last decade, a number of attempts have been made to identify small molecules which enhance neurogenesis in vitro.[1a,b,2] However, only a few compounds induce neuronal differentiation of pluripotent cells or progenitors into neurons with physiological activities.[2g] The search for small molecules, which serve as molecular therapeutic agents to treat neurodegenerative diseases, is guided by the requirement that these substances induce differentiation of cells into neurons which possess physiological functions. As part of an intense effort to find small molecules that promote neurogenesis to produce physiologically functional neurons, we recently conducted an investigation focused on screening of an imidazole library to identify substances which enhance neurogenesis in pluripotent cells. Herein, we describe the results of this effort, which has led to the development of imidazole-based small molecules that convert pluripotent P19 cells into functional neurons.

P19 embryonic carcinoma cells are pluripotent in their ability to differentiate into various cell types, including neuronal, glial, cardiac, and skeletal muscle cells, by different specific treatment.[2c,3] Because P19 cells can develop into neurons which have functional synapses and can be integrated into the brain under certain conditions,[4] these cells were utilized to identify neurogenesis-inducing small molecules in this investigation. A promising method for identification of neurogenesis inducers employs cell-based high-throughput screening with small-molecule libraries. To rapidly identify chemical inducers of neuronal differentiation in P19 cells, we used the fluorescent dye FM1-43, which has been employed to detect depolarization-induced synaptic vesicle recycling, a physiological property of neurons.[1a,b,5] This dye is internalized into neurons from the culture media in the presence of a high extracellular concentration of KCl when the synaptic vesicles are recycled back into the neurons after depolarization. As a consequence, the fluorescence-based screening protocol, utilizing FM1-43, enables the rapid identification of bioactive compounds which promote the development of neurophysiological properties.

Owing to the fact that imidazole derivatives display a broad range of biological activities, an imidazole library containing amine-terminated diethylene gycol linkers was constructed using a solid-support method (see Figure 1 A and Figure S1 in the Supporting Information).[1a,6] In the screening process, P19 cells in a 96-well plate containing differentiation media were treated with these imidazole derivatives (3 μM final concentration). As a positive control, P19 cells were incubated with 0.5 μM retinoic acid, which is known to be an inducer of neuronal differentiation in P19 cells.[2b] After incubation for seven days, the culture medium was changed to Ringer buffer containing 2 μM FM1-43 and 100 mM KCl.

The results of fluorescence analysis showed that a few compounds exhibited relatively high depolarization-induced fluorescence intensities in comparison to those of untreated cells. To further select small molecules which induce neuro-

[*] Dr. G.-H. Kim, Dr. D. Halder, Prof. Dr. I. Shin
Department of Chemistry
Yonsei University, Seoul 120-749 (Korea)
E-mail: injae@yonsei.ac.kr
J. Park, Prof. Dr. W. Namkung
Department of Integrated Omics for Biomedical Science
Yonsei University, Seoul 120-749 (Korea)

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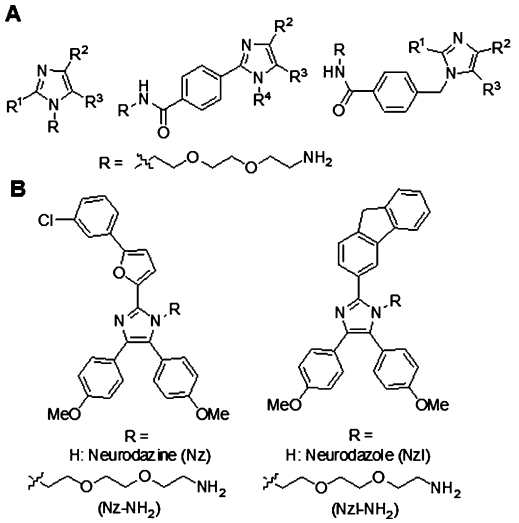

Figure 1. A) Structures of an imidazole library (see the Supporting Information for substituents R^1–R^4) and B) Structures of neurodazine (Nz) and neurodazole (Nzl).

genesis, the P19 cells were treated, with compounds identified in the initial screen, for 10 days and then subjected to immunocytochemical analysis using an antibody against a neuron-specific βIII tubulin (Tuj1). Two compounds, neurodazine-NH_2 (Nz-NH_2) and neurodazole-NH_2 (Nzl-NH_2) (Figure 1B), were observed to have neurogenesis-inducing activities in P19 cells and are comparable to that of retinoic acid. To confirm the neurogenesis-inducing effects of two compounds, the analogues neurodazine (Nz) and neurodazole (Nzl), which lack the diethylene gycol linker, were synthesized and evaluated for their neurogenesis-inducing activities in P19 cells. The results of immunocytochemical analysis showed that both Nz and Nzl promoted neurogenesis of P19 cells with activities which are similar to that of retinoic acid (see Figure S2).

To demonstrate that the P19 cells do indeed develop a neuronal phenotype after treatment with Nz or Nzl, immunocytochemical analyses were conducted with antibodies against neuron-specific markers (retinoic acid was used as a positive control). The results of these analyses show that treatment with 5 μM of Nz and Nzl results in the expression of neuron-specific markers in P19 cells with 40–60% differentiation efficiencies (see Figure 2 and Figures S3 and S4). Dose-dependent study reveals that treatment with 2–10 μM of Nz and Nzl induces neurogenesis of P19 cells to a similar extent (see Figure S5). A confirmation of neuronal differentiation in P19 cells came from an examination of the Tau-promoter activities of Nz- and Nzl-treated P19 cells. Analysis of the Tau-promoter expression pattern of the transfected P19 cells, after treatment with 5 μM of each compound for 10 days, shows that most GFP-positive cells express the neurite-specific protein MAP2 (see Figure S6). This finding provides additional support for the conclusion that both imidazole derivatives induce neuronal differentiation of P19 cells.

The neurogenesis-inducing activities of Nz and Nzl in P19 cells were also examined by western blot analysis of neuron-specific proteins. The results show that both compounds induce the expression of neuron-specific markers in treated

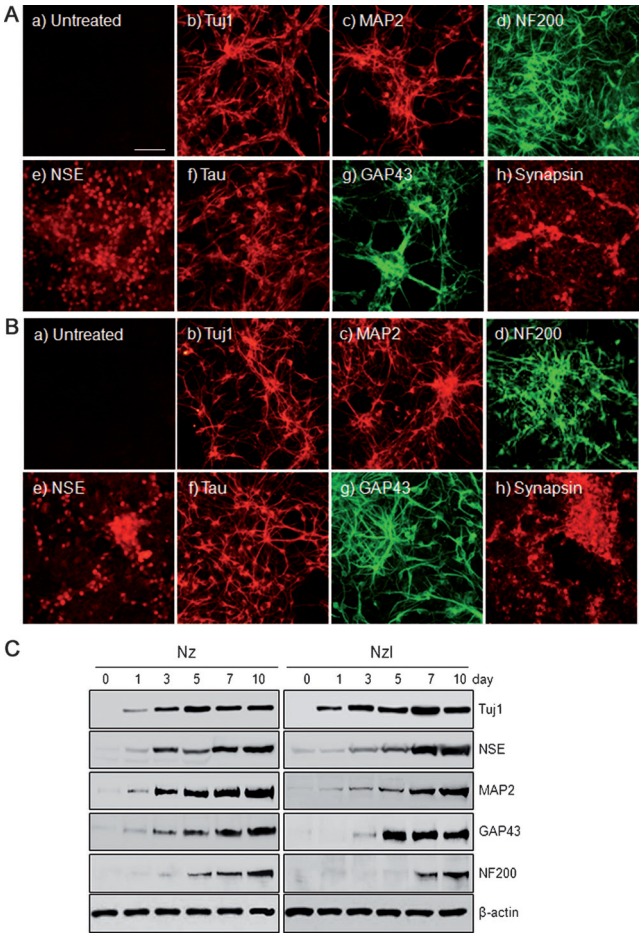

Figure 2. Induction of neurogenesis of P19 cells by A) Nz and B) Nzl. P19 cells were incubated in the absence (a) and presence (b–h) of 5 μM of each compound for 10 days. The cells were fixed and stained with neuron-specific antibodies against (a) Tuj1 and (b–h) Tuj1, MAP2 (microtubule-associated protein 2), NF200 (neurofilament 200), NSE (neuron-specific enolase), Tau, GAP43 (growth associated protein-43), and synapsin (bar: 50 μm). C) Expression of neuron-specific proteins in P19 cells was examined at various times after treatment with 5 μM Nz or 5 μM Nzl by Western blot analysis.

P19 cells (see Figure 2C and Figure S4B). In addition, RT-PCR analyses also show that induction of neuronal differentiation of P19 cells is enhanced by Nz and Nzl (see Figure S7). Expression of Oct4 and Nanog, which are pluripotent stem cell markers,[7] is abolished at the early stage after treatment of P19 cells with Nz or Nzl. In marked contrast to this observation, neuronal basic helix-loop-helix (bHLH) factors, such as NeuroD and Mash1,[8] are expressed in Nz- and Nzl-treated P19 cells. Importantly, glial fibrillary acidic protein (GFAP, an astrocyte marker) and MF20 (a myocardial marker) positive cells are almost not detected under these conditions even though retinoic-acid-treated P19 cells undergo astrocyte differentiation (see Figure S8). The results demonstrate that P19 cells treated with Nz or Nzl become neurogenic cells without either being converted into astrocytes or progressing along the myogenic lineage. Comparative transcriptome analyses using mouse 44 K DNA chips show that Nz preferentially up-regulates genes involved in neurogenesis, in comparison to those participating in other

differentiation pathways such as gliogenesis, cardiogenesis, and myogenesis (see Table S1).

In an effort to understand the structural features responsible for neurogenesis-inducing activities of Nz and Nzl, 29 members of a focused imidazole library (Figure 3) were

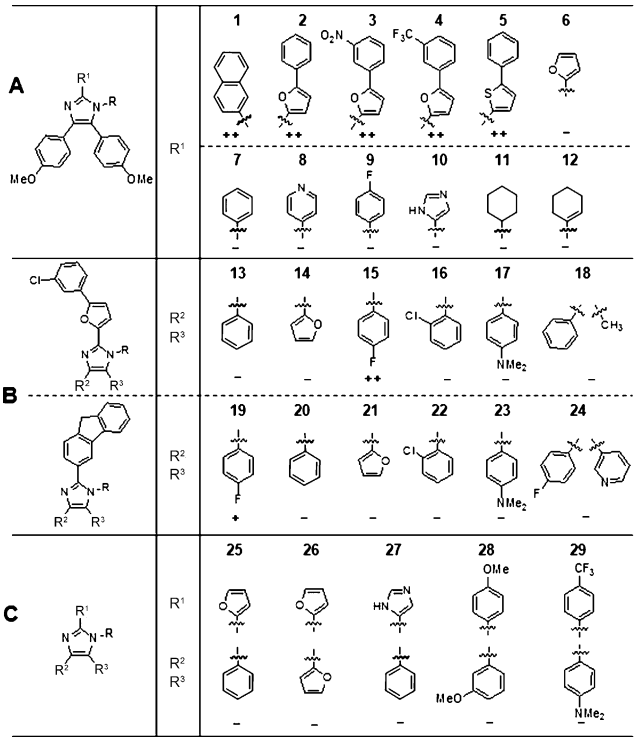

Figure 3. Structure–activity relationship studies. A) Imidazole derivatives modified at C2. B) Imidazole derivatives modified at C4 and C5. C) Imidazole derivatives modified at C2, C4, and C5 (++: modest activity, +: weak activity, −: no activity, see Nz and Nzl: +++).

prepared on a solid support. P19 cells were incubated with each member of this library (5 μM final concentration), and then subjected to immunocytochemical and western blot analyses. The imidazole derivatives **1**–**5**, which contain fused aromatic or biaromatic substituents at C2 of the imidazole ring (see Figure 3 and Figure S9), were observed to display high neurogenesis-inducing activities which are slightly less than those of Nz and Nzl. However, the analogues **6**–**12**, which possess monocyclic substituents at C2, display very low activities. The imidazole derivatives **13**–**24**, in which the 4-methoxyphenyl groups present at C4 and C5 in Nz and Nzl, respectively, are replaced by other aromatic moieties, have very low neurogenesis-inducing activities in P19 cells. The sole exceptions to this trend are the bis(4-fluorophenyl) analogues **15** and **19**, which have higher activities than those of other members of this group. Finally, substances that are modified at the 2-, 4-, and 5-positions of the imidazole ring (e.g., **25**–**29**) have remarkably attenuated neurogenesis-inducing activities. This structure–activity relationship study suggests that incorporation of relatively sterically bulky aromatic moieties at the C2-position of the imidazole ring enhances neurogenesis-inducing activity, and that the presence of the 4-methoxy group at the C4- and C5-positions might be critical for this activity.

One challenge in developing useful neurogenesis-inducing agents is the requirement that the substances promote differentiation of cells into physiologically functioning neurons. Because glutamate is a principal excitatory neurotransmitter present in most mammalian neurons, the glutamate-induced calcium influx property, one of the functional neuronal properties, in P19-cell-derived neurons induced by Nz and Nzl was evaluated. The results of RT-PCR assays show that several glutamate receptors, including mGluR3, 5, and 7, are expressed in the differentiated P19 cells (see Figure S10). We then examined if the glutamate receptors in differentiated P19 cells are functionally expressed. For this purpose, glutamate-induced calcium influx through mGluRs was measured using the fluorescent calcium indicator Fluo-3AM.[9] In these experiments, P19 cells were incubated in the absence and presence of 5 μM Nz and Nzl for 10 days and then treated with Fluo-3AM. The results of fluorescence microscopy analysis show that emission from untreated P19 cells is not altered by the addition of L-glutamate (Figure 4).

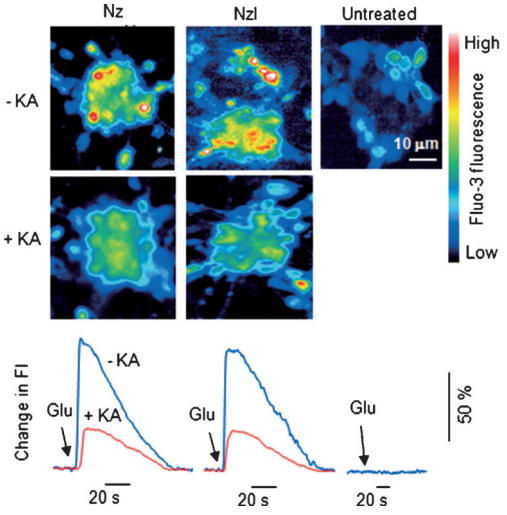

Figure 4. Nz- or Nzl-promoted differentiation of P19 cells into physiologically active neurons. Glutamate-induced calcium influx property of P19 cells was measured using Fluo-3AM. P19 cells differentiated by 5 μM Nz or 5 μM Nzl for 10 days were stimulated with 1 mM L-glutamate (Glu) in the presence or absence of 1 mM kynurenic acid (KA). FI = fluorescence intensity.

In contrast, stimulation of glutamate receptors in Nz- or Nzl-treated P19 cells with L-glutamate triggers great Ca^{2+} influx, a phenomenon which mimics that seen in retinoic acid treated cells (see Figure 4 and Figure S11). Finally, pretreatment of the Nz- and Nzl-differentiated cells with the glutamate receptor antagonist kynurenic acid (1 mM) causes a substantial reduction in Ca^{2+} influx promoted by addition of L-glutamate. The combined observations show that differentiated P19 cells by Nz or Nzl have functional glutamate responsiveness.

Finally, to understand the underlying molecular mechanism for neuronal differentiation of P19 cells by Nz and Nzl, we examined the effects of these substances on signaling pathways associated with neurogenesis. We initially evaluated

whether these substances affect the Wnt/β-catenin signaling pathway, which is believed to be a key regulator of neuronal differentiation.[10] Accordingly, P19 cells were independently incubated for 10 days with 5 μM Nz or 5 μM Nzl in the presence or absence of the two Wnt pathway inhibitors, NSC668036 (25 μM)[11] and PKF118-310 (25 nM).[12] The results of immunocytochemistry, western blot, and RT-PCR analyses show that the neurogenesis-inducing activities of Nz and Nzl in P19 cells are greatly attenuated in the presence of each of these inhibitors as compared to those of cells treated only with Nz and Nzl (see Figure 5 and Figure S12). Another

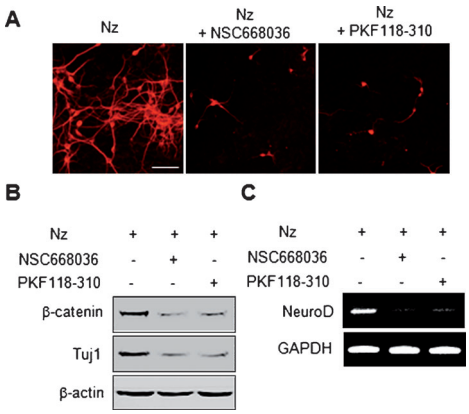

Figure 5. Effect of inhibitors of the Wnt pathway on neurogenesis of P19 cells induced by Nz. A) P19 cells were incubated with 5 μM Nz in the absence (left) and presence of 25 μM NSC668036 (middle) and 25 nM PFK118-310 (right) for 10 days. The cells were stained with Tuj1 antibody (bar: 50 μm). B) Expression of the Wnt pathway (β-catenin) and neuronal marker (Tuj1) proteins in treated P19 cells was examined by western blot analysis. C) Expression of the Wnt pathway-related neuronal gene NeuroD in treated P19 cells was examined by RT-PCR.

key regulatory pathway in neuronal differentiation is the Shh signaling pathway.[13] To test whether this pathway is involved in neuronal differentiation of P19 cells induced by Nz and Nzl, P19 cells were incubated for 10 days with Nz and Nzl in the presence and absence of Cur61414 (25 μM), which is an inhibitor of the Shh signaling pathway.[14] The results of immunocytochemistry and Western blot analyses show that the neurogenesis-inducing activities of our compounds are markedly reduced in the presence of Cur61414 (see Figure S13). However, the presence of MK0752, an inhibitor of γ-secretase that suppresses the neurogenesis related Notch pathway,[15] has no inhibitory effect on neuronal differentiation of P19 cells induced by Nz and Nzl (see Figure S14). Additionally, ingenuity pathway analysis of DNA chip data shows that several genes, with more than twofold changes after treatment of P19 cells with Nz, are crosslinked with Wnt and Shh pathways (see Figure S15). Collectively, these findings suggest that Nz and Nzl promote neurogenesis by activating Wnt and Shh signaling pathways.

In conclusion, we have demonstrated that the imidazole derivatives, Nz and Nzl, serve as small-molecule promoters of neurogenesis in pluripotent cells. The propensities of Nz and Nzl to induce neuronal differentiation are comparable to that of the known neurogenic factor retinoic acid. Moreover, Nz and Nzl are more selective inducers of neuronal differentiation because, unlike retinoic acid, they suppress astrocyte differentiation of P19 cells. In addition, P19 cells differentiated by utilizing Nz and Nzl express neurotransmitter receptors and display a functional neuronal property such as glutamate-induced Ca^{2+} influx. Nz and Nzl will serve as important tools in methods used to produce specific populations of neuronal cell types from P19 cells.

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[1] a) D. R. Williams, M. R. Lee, Y. A. Song, S. K. Ko, G. H. Kim, I. Shin, *J. Am. Chem. Soc.* **2007**, *129*, 9258; b) D. R. Williams, G. H. Kim, M. R. Lee, I. Shin, *Nat. Protoc.* **2008**, *3*, 835; c) S. Ding, P. G. Schultz, *Nat. Biotechnol.* **2004**, *22*, 833; d) T. Xu, M. Zhang, T. Laurent, M. Xie, S. Ding, *Stem Cells Transl. Med.* **2013**, *2*, 355.
[2] a) H. Wichterle, I. Lieberam, J. A. Porter, T. M. Jessell, *Cell* **2002**, *110*, 385; b) E. M. Jones-Villeneuve, M. W. McBurney, K. A. Rogers, V. I. Kalnins, *J. Cell Biol.* **1982**, *94*, 253; c) S. Ding, T. Y. H. Wu, A. Brinker, E. C. Peters, W. Hur, N. S. Gray, P. G. Schultz, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 7632; d) J. W. Schneider, Z. Gao, S. Li, M. Farooqi, T. S. Tang, I. Bezprozvanny, D. E. Frantz, J. Hsieh, *Nat. Chem. Biol.* **2008**, *4*, 408; e) M. Warashina, K. H. Min, T. Kuwabara, A. Huynh, F. H. Gage, P. G. Schultz, S. Ding, *Angew. Chem.* **2006**, *118*, 605; *Angew. Chem. Int. Ed.* **2006**, *45*, 591; f) H. Wurdak, S. Zhu, K. H. Min, L. Aimone, L. L. Lairson, J. Watson, G. Chopiuk, J. Demas, B. Charette, R. Halder, E. Weerapana, B. F. Cravatt, H. T. Cline, E. C. Peters, J. Zhang, J. R. Walker, C. L. Wu, J. Chang, T. Tuntland, C. Y. Cho, P. G. Schultz, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 22360; g) J. Ladewig, J. Mertens, J. Kesavan, J. Doerr, D. Poppe, F. Glaue, S. Herms, P. Wernet, G. Kogler, F. J. Muller, P. Koch, O. Brustle, *Nat. Methods* **2012**, *9*, 575.
[3] M. W. Mcburney, *Int. J. Dev. Biol.* **1993**, *37*, 135.
[4] M. F. A. Finley, N. Kulkarni, J. E. Huettner, *J. Neurosci.* **1996**, *16*, 1056.
[5] W. J. Betz, G. S. Bewick, *Science* **1992**, *255*, 200.
[6] a) A. Golebiowski, S. R. Klopfenstein, D. E. Portlock, *Curr. Opin. Chem. Biol.* **2003**, *7*, 308; b) D. R. Williams, S. K. Ko, S. Park, M. R. Lee, I. Shin, *Angew. Chem.* **2008**, *120*, 7576; *Angew. Chem. Int. Ed.* **2008**, *47*, 7466; c) H. J. Cho, H. Y. Gee, K. H. Baek, S. K. Ko, J. M. Park, H. Lee, N. D. Kim, M. G. Lee, I. Shin, *J. Am. Chem. Soc.* **2011**, *133*, 20267.
[7] S. Constantinescu, *J. Cell. Mol. Med.* **2003**, *7*, 103.
[8] L. C. Lo, J. E. Johnson, C. W. Wuenschell, T. Saito, D. J. Anderson, *Genes Dev.* **1991**, *5*, 1524.
[9] A. Takahashi, P. Camacho, J. D. Lechleiter, B. Herman, *Physiol. Rev.* **1999**, *79*, 1089.
[10] H. Clevers, *Cell* **2006**, *127*, 469.
[11] J. F. Shan, D. L. Shi, J. M. Wang, J. Zheng, *Biochemistry* **2005**, *44*, 15495.
[12] P. C. Leow, Q. A. Tian, Z. Y. Ong, Z. Yang, P. L. R. Ee, *Invest. New Drugs* **2010**, *28*, 766.
[13] P. Vanderhaeghen, *Nat. Neurosci.* **2009**, *12*, 1079.
[14] H. Suh, W. Deng, F. H. Gage, *Annu. Rev. Cell Dev. Biol.* **2009**, *25*, 253.
[15] J. A. Williams, C. M. Guicherit, B. Zaharian, Y. Xu, L. Chai, H. Wichterle, C. Kon, C. Gatchalian, R. Nusse, J. A. Porter, L. L. Rubin, F. Y. Wang, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 8607.