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Small-molecule activation of neuronal cell fate

Jay W Schneider[1], Zhengliang Gao[2], Shijie Li[3], Midhat Farooqi[3], Tie-Shan Tang[4], Ilya Bezprozvanny[4], Doug E Frantz[5] & Jenny Hsieh[2]

We probed an epigenetic regulatory path from small molecule to neuronal gene activation. Isoxazole small molecules triggered robust neuronal differentiation in adult neural stem cells, rapidly signaling to the neuronal genome via Ca^{2+} influx. Ca^{2+}-activated CaMK phosphorylated and mediated nuclear export of the MEF2 regulator HDAC5, thereby de-repressing neuronal genes. These results provide new tools to explore the epigenetic signaling circuitry specifying neuronal cell fate and new leads for neuro-regenerative drugs.

The transduction of fate signals to the genome of undifferentiated stem cells remains one of the most fundamental problems in stem cell and developmental biology. Despite their immaturity, neural stem cells (NSCs) are receptive to neurotransmission signals regulating neurosphere growth *in vitro*[1]; they are also receptive to those regulating *neuroD* expression and differentiation *in vitro* and *in vivo*[2,3]. However, the mechanism that transduces neurotransmitter-evoked Ca^{2+} signals to the neuronal gene program in stem cells is unknown. The brain-enriched epigenetic regulatory module comprised of class IIa histone deacetylases (HDACs) and myocyte enhancer factor-2 (MEF2) transcription factors has an important role in neuronal maturation and survival[4], but a role for this mechanism in NSCs has been elusive. We previously conducted a screen for cardiogenic small molecules that induce expression of *Nkx2.5*, a homeodomain transcription factor gene, in P19 embryonal carcinoma cells[5]. Unexpectedly, a subset of these *Nkx2.5*-activating small molecules induced a neuronal (and not a cardiogenic) phenotype. Indeed, *Nkx2.5* has reported roles in neural development and differentiation[6,7], which provides biological rationale, in retrospect, for the discovery of neurogenic molecules from the original screen. Thus, we counterscreened our validated hits for neurogenic small molecules and identified several isoxazole compounds that selectively convert NSCs into neurons.

Initial screening efforts identified five hits (**1**, **2**, **3**, **4** and **5**) that all contain a 3,5-disubstituted isoxazole as a defining structural motif. Each was capable of activating transfected *neuroD* and *gluR2* neuronal reporter genes in NSCs in a dose-dependent manner, with activity peaking in the low micromolar range (**Supplementary Fig. 1** online). In an effort to generate a more potent and soluble lead, we synthesized several small, targeted libraries of 3,5-disubstituted isoxazoles (~75 analogs total) using the chemistry outlined in **Supplementary Methods** online. Through these efforts, we identified **6**, **7**, **8** and **9** (**Supplementary Fig. 1**), which displayed not only the highest activity but also adequate aqueous solubility for our downstream mechanistic studies. Thus, we used **9** as our primary chemical probe to delineate the mechanistic pathway for excitation-neurogenesis signaling to the stem cell genome. Studies were done in hippocampal NSCs from adult rat HCN cells—a well-characterized continuous line of multipotent stem and progenitor cells that rarely exhibit spontaneous lineage differentiation in the presence of fibroblast growth factor-2 (FGF-2) (ref. 8). Compound **9** not only induced robust neuronal differentiation, but also dominantly blocked competing astrocyte differentiation inducible by leukemia inhibitory factor (LIF) and bone morphogenetic protein-2 (BMP-2) in HCN cells (**Fig. 1a,b** and **Supplementary Fig. 2** online). Within a few hours of exposure, **9** activated the endogenous neuronal gene program in HCN cells (**Fig. 1c**), which is consistent with the results of neuronal reporter genes (**Fig. 1a**). Taken together, these data indicate that **9** sends a rapid biochemical signal to the nucleus of HCN cells, thereby initiating the neuronal differentiation program while blocking non-neuronal fates in the presence of strong gliogenic signals.

Compound **9** mediated an instructive fate signal in uncommitted HCN cells and increased proliferation of committed progenitor cells (neuroblasts) while causing only a modest amount of cell death (**Supplementary Fig. 3** and **Supplementary Discussion** online). In addition to its effects on HCN cells, **9** induced neuronal differentiation in adult mouse whole brain (MWB), subventricular zone (SVZ) progenitors and P19 embryonal carcinoma cells, thereby establishing a wide range of neurogenic activity for this class of molecules (**Supplementary Fig. 2**).

Compound **9** has neurotransmitter-like effects in undifferentiated HCN cells—it rapidly activates Ca^{2+} influx through both voltage-gated Ca^{2+} channels and *N*-methyl-D-aspartic acid (NMDA) receptors. We first searched for neurotransmitter-like activity based on previous reports of NSC response to neurotransmitter growth signals[1–3]. Given that both glutamate and $GABA_A$ receptors induce hippocampal neurogenesis through Ca^{2+} signaling[2,3], we first investigated whether **9** triggers increased $[Ca^{2+}]_i$. Indeed, **9** did increase $[Ca^{2+}]_i$ in HCN cells, with a slow but steadily rising current of appropriate magnitude for undifferentiated stem cells with primitive Ca^{2+} handling mechanisms (**Fig. 1d** and **Supplementary Fig. 4** online). We successfully inhibited the **9**-mediated $[Ca^{2+}]_i$ rise in NSCs by blocking all major sources of Ca^{2+} influx in HCN cells with a cocktail of inhibitors (AP5 (**11**), CNQX (**12**) and nifedipine (**13**), targeting NMDA- and AMPA-type glutamate receptors and L-type Ca^{2+} channels, respectively) (**Fig. 1d** and **Supplementary Fig. 4**). Next, we identified the most probable source of Ca^{2+} influx by demonstrating that MK801 (**14**), a specific NMDA receptor antagonist, can block the **9**-mediated Ca^{2+} signal (**Fig. 1d** and **Supplementary Fig. 4**). To confirm the physiological importance of **9**-triggered Ca^{2+} signaling, we demonstrated that the inhibitor cocktail

[1]Department of Internal Medicine, [2]Department of Molecular Biology and Cecil H. and Ida Green Center for Reproductive Biology Sciences, [3]Department of Molecular Genetics, [4]Department of Physiology and [5]Department of Biochemistry, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390, USA. Correspondence should be addressed to J.H. (jenny.hsieh@utsouthwestern.edu).

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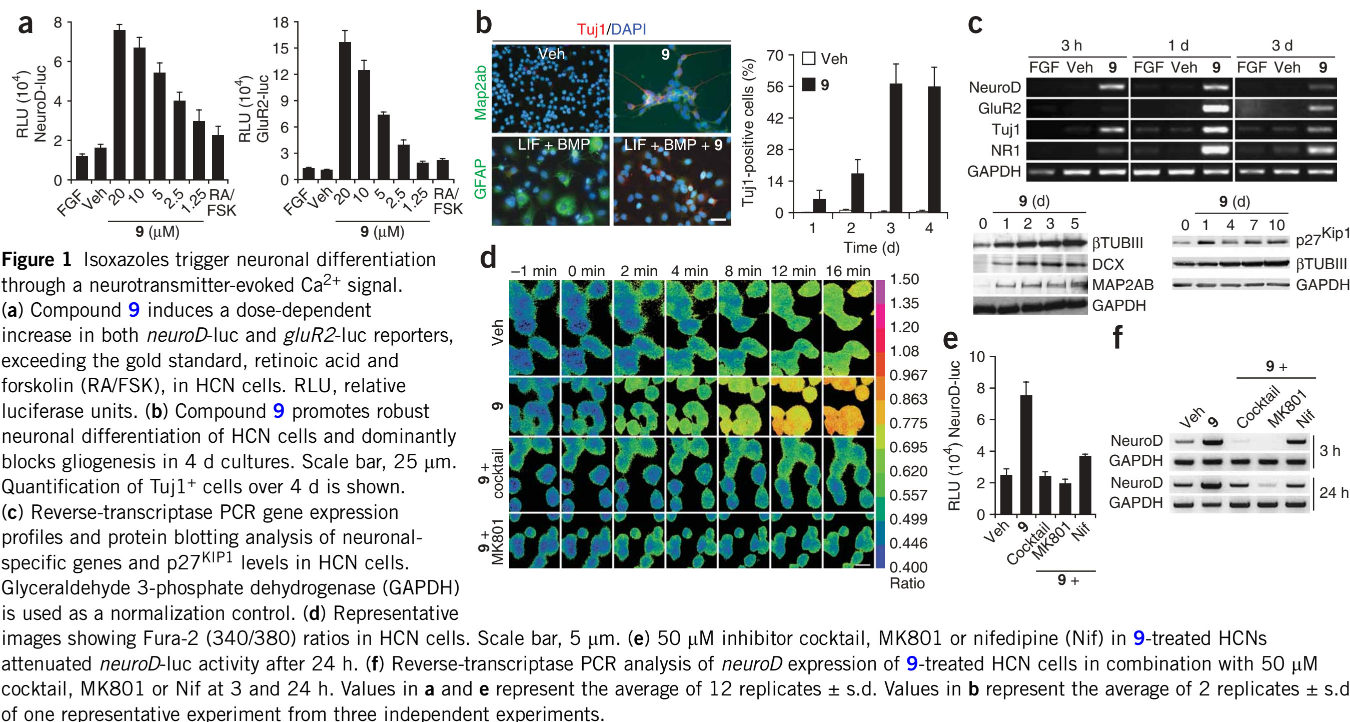

Figure 1 Isoxazoles trigger neuronal differentiation through a neurotransmitter-evoked Ca^{2+} signal. (**a**) Compound **9** induces a dose-dependent increase in both *neuroD*-luc and *gluR2*-luc reporters, exceeding the gold standard, retinoic acid and forskolin (RA/FSK), in HCN cells. RLU, relative luciferase units. (**b**) Compound **9** promotes robust neuronal differentiation of HCN cells and dominantly blocks gliogenesis in 4 d cultures. Scale bar, 25 μm. Quantification of $Tuj1^+$ cells over 4 d is shown. (**c**) Reverse-transcriptase PCR gene expression profiles and protein blotting analysis of neuronal-specific genes and $p27^{KIP1}$ levels in HCN cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is used as a normalization control. (**d**) Representative images showing Fura-2 (340/380) ratios in HCN cells. Scale bar, 5 μm. (**e**) 50 μM inhibitor cocktail, MK801 or nifedipine (Nif) in **9**-treated HCNs attenuated *neuroD*-luc activity after 24 h. (**f**) Reverse-transcriptase PCR analysis of *neuroD* expression of **9**-treated HCN cells in combination with 50 μM cocktail, MK801 or Nif at 3 and 24 h. Values in **a** and **e** represent the average of 12 replicates ± s.d. Values in **b** represent the average of 2 replicates ± s.d. of one representative experiment from three independent experiments.

and MK801 block **9**-mediated *neuroD* reporter gene induction (**Fig. 1e**). Nifedipine alone also attenuated the *neuroD* reporter, and L-type Ca^{2+} channel agonists Bay K 8644 (**15**) and FPL 64176 (**16**) increased *neuroD* activity in a dose-dependent manner, which indicates that Ca^{2+} influx via L-type Ca^{2+} channels is sufficient to promote *neuroD* expression leading to neuronal differentiation (**Fig. 1e** and **Supplementary Fig. 4**). To further confirm the contribution of Ca^{2+}/NMDA signaling to **9**-mediated excitation-neurogenesis, we applied the inhibitor cocktail and MK801 to HCN cells, which inhibited basal and enhanced *neuroD* mRNA levels elicited by **9** within 3 h (**Fig. 1f**). FPL 64176 alone was able to stimulate *neuroD* expression and neuronal differentiation, although to a lesser extent compared with **9** (**Supplementary Fig. 4**). Importantly, NMDA receptor and Ca^{2+} channel blockers MK801 and nifedipine strongly attenuated the **9**-induced increase in *neuroD* expression, but failed to completely block phenotypic differentiation. Breakthrough of the neuronal phenotype could be due to kinetic or timing effects of **9**, MK801 and nifedipine actions at their respective targets, or it could indicate that **9** acts as well through a second, Ca^{2+}-signaling–independent pathway to the genome.

So far, there has been no evidence that Ca^{2+}-regulated MEF2 transcription factors play a role in the early steps of embryonic or adult NSC differentiation. In fact, despite overall high-level expression in the central nervous system, MEF2 mRNA and protein expression is notably absent from the proliferative zones of the brain under basal conditions[9]. Yet in our gene expression studies, **9** strongly induced the endogenous *NMDA receptor subunit-1* (*NR1*) gene (**Fig. 1c**), one of few validated MEF2 target genes in mature neurons[10]. To provide evidence that MEF2 is involved in **9**-mediated induction of *NR1*, we confirmed that **9** signaling activates a MEF2 response element (MREx3) reporter gene in a dose-dependent manner in HCN cells (**Fig. 2a**). The rapid and strong activation of MREx3 reporter gene by **9** suggests that MEF2 transcription factor activity is available but negatively regulated in these undifferentiated NSCs. The failure to demonstrate MEF2 transcriptional activity on reporter genes in undifferentiated HCN cells, despite the presence of MEF2 proteins and DNA binding activity (**Supplementary Fig. 5** and **Supplementary Discussion** online), suggests that MEF2 is actively repressed in undifferentiated NSCs and that **9** can relieve this repression.

The class IIa HDAC-MEF2 epigenetic/transcriptional regulatory network is the next logical target mechanism for the neurogenic effects of **9** in HCN cells. MEF2-dependent gene expression at the level of HDAC nuclear transport has been demonstrated in multiple cell types including heart, skeletal muscle and mature cells of the brain, including hippocampal neurons[11]. A nodal point in HDAC-MEF2 network regulation is activation of HDAC kinases. Nuclear MEF2 transcription factors are liberated from HDAC repression through 14-3-3–chaperoned nuclear export of phosphorylated HDAC (ref. 12). We first observed that **9** indeed activates an HDAC kinase in HCN cells, which leads to hyperphosphorylation of HDAC5 at Ser259 and Ser498, two 14-3-3 docking sites (**Supplementary Fig. 6** online). To correlate **9**-induced HDAC5 hyperphosphorylation with export of this transcriptional repressor protein from the NSC nucleus, we fractionated control and **9**-treated HCN cells into nuclear and cytoplasmic components for blotting experiments (**Fig. 2b**). Compound **9** had no effect on the subcellular distribution of MEF2A, MEF2C or cAMP response element–binding protein (CREB), a second class of neuronal Ca^{2+}-regulated nuclear transcription factors, in differentiated HCN cells (**Fig. 2b**). Indeed, cytoplasmic accumulation of phosphorylated HDAC5, with a concomitant decrease in the nucleus, is the only substantial **9**-mediated change we observed in these extracts (**Fig. 2b**). Compound **9** did not significantly affect phosphorylated HDAC4 levels (**Fig. 2b**), which is consistent with the previous finding that HDAC5 and HDAC4 are differentially regulated by neural inputs in hippocampal neurons[13]. We confirmed **9**-mediated nuclear export of HDAC5 in NSCs using a green fluorescent protein (GFP)-HDAC5 fusion protein delivered by adenovirus (**Fig. 2c** and **Supplementary Fig. 6**). GFP-HDAC5 (S-A), a signal-resistant mutant of GFP-HDAC5 bearing mutated 14-3-3 chaperone protein docking sites (Ser259 and Ser498), was predominantly nuclear in NSCs, even after **9** treatment (**Fig. 2c**). We also confirmed cytoplasmic enrichment of

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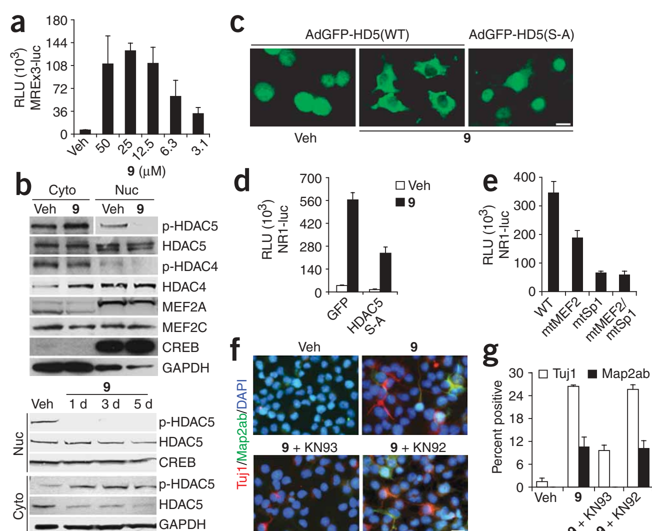

Figure 2 Isoxazole signaling leads to activation of CaMK, phosphorylation/export of HDAC5 and MEF2-dependent gene expression in NSCs. (**a**) MEF2 reporter gene (*3XMRE-luc*) activation in **9**-treated HCN cells. (**b**) Accumulation of phosphorylated HDAC5 in the cytoplasm of 2-d **9**-treated HCNs (top). Compound **9** led to decreased phosphorylated HDAC5 in the nucleus over time, normalized to total HDAC5 and CREB levels (bottom). (**c**) Shown are representative fields of live-cell GFP fluorescence in vehicle or 20 μM **9**-treated HCN cells expressing wild-type GFP-HDAC5 (AdGFP-HD5 (WT)) or S259A S498A mutant GFP-HDAC5 (AdGFP-HDAC5 (S-A)). Scale bar, 5 μm. (**d**) Expression of signal-resistant mutant HDAC5 (S-A) normalized to a control GFP plasmid. (**e**) Activation of *NR1* is dependent on MEF2 and Sp1 binding. (**f**) Blocking CaMK with 2.5 μM KN93, and not KN92, resulted in an inhibition of **9**-mediated neuronal differentiation in HCN cells in 2 d cultures. Scale bar, 5 μm. (**g**) Quantification of Tuj1 and Map2ab$^+$ cells is shown. Values in **a**, **d** and **e** represent the average of 12 replicates ± s.d. Values in **g** represent the average of two replicates ± s.d. of one representative experiment from two independent experiments.

phosphorylated HDAC5 in response to **9** by immunoprecipitation and protein blotting of nuclear and cytoplasmic extracts from **9**-treated and control NSCs using adenovirus encoding a FLAG-tagged HDAC5 (**Supplementary Fig. 6**). Finally, we confirmed that the HDAC-MEF2 axis regulates the *NR1* MEF2 target gene in HCN cells by demonstrating that the signal-resistant mutant HDAC5 (S-A) (**Fig. 2d**) and a dominant-negative MEF2C-engrailed repressor fusion protein (**Supplementary Fig. 5**) completely block the ability of **9** to activate the *NR1* reporter gene. In addition to a MEF2 site, the *NR1* promoter also contains tandem binding sites to the ubiquitously expressed transcription factor Sp1. Indeed, activation of *NR1* triggered by Isx was dependent, in part, on MEF2 binding, as well as binding of Sp1, although MEF2 and Sp1 did not appear to have synergistic effects in HCN cells[10] (**Fig. 2e**). However, neither HDAC5 (S-A) nor MEF2C-engrailed proteins completely blocked **9**-induced neuronal differentiation in HCN cells, which implicates the involvement of other pathways.

Regulatory phosphorylation of class II HDAC-MEF2 activity has been mainly attributed to CaM kinases (CaMK) and protein kinase D (PKD), which is phosphorylated and activated by protein kinase C (PKC)[14,15]. Therefore, we tested inhibitors of CaMK and PKC for their ability to block **9**-induced HDAC5 phosphorylation and neuronal differentiation (**Supplementary Fig.** 7 and **Supplementary Discussion** online). KN93 (**17**), a specific inhibitor of CaMK, and not the analog KN92 (**19**), which blocks potassium channels but not CaMK, effectively suppressed **9**-mediated differentiation (**Fig. 2f,g**). These data suggest that CaMK is the main HDAC kinase activated by **9** mediating the neurogenic signal in HCN cells.

Our experiments with **9** provide compelling evidence that a neurotransmitter-like excitation signal in NSCs can regulate the neuronal genome through an epigenetic transcriptional network involving HDAC5 and MEF2, thus providing the first evidence that the HDAC-MEF2 circuitry participates in early neural cell fate events, at least *in vitro*. Isoxazoles trigger a Ca^{2+} signal, involving voltage-gated Ca^{2+} channels and NMDA receptors, that activates CaMKII, the major HDAC kinase in NSCs. Compound **9**–induced phosphorylation of HDAC5 leads to export of this chromatin-modifying enzyme and repressor protein from the NSC nucleus, thereby de-repressing MEF2 and other transcription factors to directly activate MEF2 target genes such as *NR1* and indirectly activate *neuroD* and other neuronal genes, which together promote early phenotypic differentiation. Though our data demonstrate that NSC Ca^{2+} channels and NMDA receptors play a major role in mediating the neurogenic isoxazole response, we cannot exclude alternative, more direct, pathways for electrochemical epigenetic signaling to the neuronal genome. Future studies will explore whether de-repression of MEF2 by nuclear export of HDAC5 in NSCs underlies the brain's neurogenic response to pathological stress that involves excitation (for example, drugs, ischemia or seizures), and whether **9**-mediated nuclear export of HDAC5 modulates NRSF/REST, an important HDAC5 binding partner and co-regulator of *NR1* and many other neuronal genes.

Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

J.W.S., I.B., D.E.F. and J.H. designed experiments. J.W.S., Z.G., S.L., M.F., T.-S.T., D.E.F. and J.H. performed experiments. J.W.S. and J.H. wrote the paper.

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