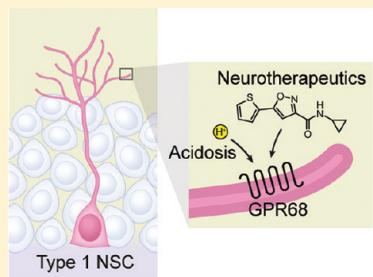


Coupling Hippocampal Neurogenesis to Brain pH through Proneurogenic Small Molecules That Regulate Proton Sensing G Protein-Coupled Receptors

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ABSTRACT: Acidosis, a critical aspect of central nervous system (CNS) pathophysiology and a metabolic corollary of the hypoxic stem cell niche, could be an expedient trigger for hippocampal neurogenesis and brain repair. We recently tracked the function of our isoxazole stem cell-modulator small molecules (Isx) through a chemical biology-target discovery strategy to GPR68, a proton (pH) sensing G protein-coupled receptor with no known function in brain. Isx and GPR68 coregulated neuronal target genes such as Bex1 (brain-enriched X-linked protein-1) in hippocampal neural progenitors (HCN cells), which further amplified GPR68 signaling by producing metabolic acid in response to Isx. To evaluate this proneurogenic small molecule/proton signaling circuit *in vivo*, we explored GPR68 and BEX1 expression in brain and probed brain function with Isx. We localized proton-sensing GPR68 to radial processes of hippocampal type 1 neural stem cells (NSCs) and, conversely, localized BEX1 to neurons. At the transcriptome level, Isx demonstrated unrivaled proneurogenic activity in primary hippocampal NSC cultures. *In vivo*, Isx pharmacologically targeted type 1 NSCs, promoting neurogenesis in young mice, depleting the progenitor pool without adversely affecting hippocampal learning and memory function. After traumatic brain injury, cerebral cortical astrocytes abundantly expressed GPR68, suggesting an additional role for proton-GPCR signaling in reactive astrogliosis. Thus, probing a novel proneurogenic synthetic small molecule's mechanism-of-action, candidate target, and pharmacological activity, we identified a new GPR68 regulatory pathway for integrating neural stem and astroglial cell functions with brain pH.



KEYWORDS: Small molecules, acidosis, G protein-coupled receptors, hippocampal neurogenesis, traumatic brain injury

INTRODUCTION

Chemical biology provides a versatile discovery platform for identifying new functions of cells, proteins, and biochemical pathways, leading to new disease targets and possibly new pharmaceuticals.¹ Identifying novel disease targets through small molecule science is particularly important in the central nervous system (CNS), where few, if any, drugs or other effective treatment options exist for some of mankind's most debilitating degenerative, ischemic, and traumatic diseases.

Brain function is critically dependent on pH.² Protons, as signaling molecules, have never been directly linked to neurogenesis, but a number of hippocampal neurogenesis-evoking pathophysiological conditions such as epileptic seizures, ischemic or embolic stroke, tissue inflammation, and traumatic brain injury are associated with regional or global brain acidosis.² These conditions cause acidosis by producing lactate from anaerobic cellular metabolism due to compromised circulatory function or by producing glutamate from excessive synaptic signaling.² Most intriguingly, coupling cardiovascular and brain function, vigorous voluntary physical exercise (the most powerful stimulus of hippocampal neurogenesis³) causes systemic lactic acidosis.⁴ Additionally, many well-established proneurogenic chemical agents are, in fact, acids (e.g., valproic and retinoic).⁵ The hippocampal dentate gyrus (DG) is

hypothesized to be a hypoxic/acidic microenvironment⁶ and, in this regard, hydrogen ions (H^+) could be important regulators of progenitor function and neurogenesis. Although acid sensing ion channels (ASICs) (H^+ -gated Ca^{2+} channels) have been described in hippocampal neurons,⁷ they have never been functionally linked to progenitors or neurogenesis.⁸ Acid sensing G protein-coupled receptors (GPCRs) comprise a second family of ambient pH biosensing signaling molecules that includes G2A, GPR4, TDAG8, and GPR68 (OGR1).^{9,10} Although these GPCRs play roles in acid-induced pain sensation in dorsal root ganglia,^{11,12} and GPR68 is highly expressed in the central nervous system¹³ and specifically localized to hippocampus by GENSAT and BGEM, pH sensing GPCRs have never been mechanistically coupled to neural progenitor biology or neurogenesis.

Originally discovered in a P19CL6 embryonal carcinoma cell screen,¹⁴ we have more recently extended our analysis of *N*-cyclopropyl-5-(thiophen-2-yl)-isoxazole-3-carboxamide (Isx) to adult rat hippocampal neural (HCN) progenitors,¹⁵ glioma cells,¹⁶ pancreatic β -cells,¹⁷ and Notch-activated epicardium-

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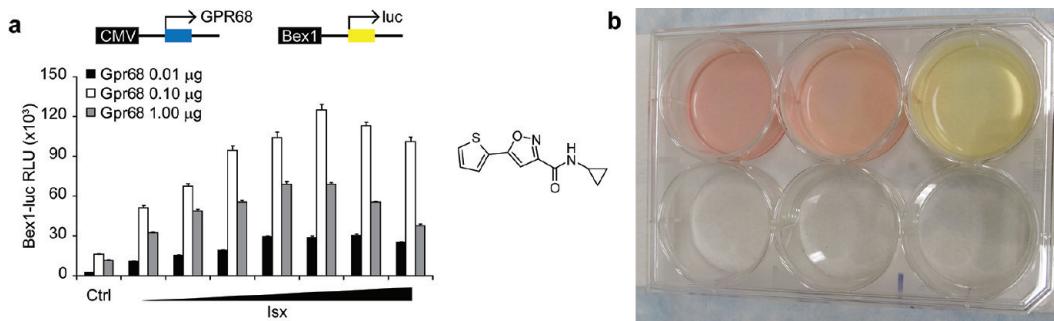


Figure 1. GPR68 Isx and acid-amplifying pathway for regulating target genes in NSCs. (a) Coregulation of Bex1-luc by GPR68 and Isx small molecules. Clonal adult rat hippocampal neural (HCN) progenitors¹⁵ coelectroporated with increasing amounts of flag-tagged Gpr68 expression plasmid and pGL3-Bex1 promoter/enhancer-luciferase construct containing -648 to +177 of Bex1 mouse genomic DNA, evaluated for luciferase activity after DMSO-vehicle or Isx (*N*-cyclopropyl-5-(thiophen-2-yl)-isoxazole-3-carboxamide) dose-response treatment for 24 h. Each data point represents 12 replicate cultures from the same electroporation (+ standard error of the mean). (b) Acidification of HCN media by Isx. Within 24 h of changing to differentiation media (no growth factors) containing Isx (20 μ M), subconfluent HCN cells entered a rapid growth phase¹⁵ that acidified the media to pH < 7.0 (right-hand well). Media in control (left-hand well) and DMSO-vehicle (center well) treated HCN wells, under the same conditions, remained buffered at pH ~ 7.4.

derived cells (NECs), adult cardiac-resident progenitors.^{18,19} In each of these studies, Isx profoundly altered transcriptional programs in a cell context-dependent manner; progenitor-type cells seem particularly vulnerable to this compound's gene regulating effects. Working backward toward a mechanism and target, we recently demonstrated that Isx triggered Ca^{2+} release from endoplasmic reticulum through G_q PCR activation and we identified GPR68 in a functional target screen.¹⁹ We also recently confirmed Isx's *in vivo* efficacy in the heart.^{18,19}

Here, we refocused on neural systems. First, we set out to explore GPR68's role in Isx's neuronal gene activating function in hippocampal neural progenitors.¹⁵ We asked the intriguing questions whether GPR68, based on its expression pattern in mouse brain, could function as a proton (pH) biosensor for hippocampal neurogenesis, and, if so, whether Isx, our candidate GPR68 ligand, targeted GPR68 expressing brain cells *in vivo*. To gain a genome-wide understanding of the Isx-mediated neuronal phenotype, we evaluated Isx's neurogenic activity in primary cultures, side-by-side with retinoic acid/forskolin, the gold standards of the field, by microarray. We next asked whether Isx-mediated hippocampal structural changes had behavioral correlates in mice. Finally, we asked whether GPR68 gene expression dynamically responded to traumatic brain injury in mice, a pathophysiological event that involves brain acidosis. Our results demonstrate that Isx small molecule ligands are highly versatile and valuable mechanistic probes for GPR68, which is a new CNS disease target. Moreover, our results suggest that GPR68-expressing cells are components not only of the hippocampal stem cell niche but also of cerebral cortical regions involved in injury repair, and that GPR68 functions to integrate brain cellular responses to acidosis, a universal sign of serious tissue injury.

RESULTS AND DISCUSSION

Isx and Acid-Amplifying Pathway for Regulating Target Genes through GPR68 in NSCs. We recently identified GPR68 as a candidate target protein for proneurogenic Isx small molecules¹⁵ and, by microarray, identified Bex-1 (brain-enriched X-linked protein-1), a ~14 kDa signaling adaptor protein involved in NSC growth and differentiation,²⁰ as one of the top Isx upregulated target gene in NECs, *in vitro* (8.93-fold) and *in vivo* (12.88-fold)^{18,19} (data not shown). To begin evaluating GPR68's possible function in Isx-induced NSC

differentiation, we constructed a flag-tagged mouse GPR68 cDNA expression vector (CMV-GPR68) and a reporter gene containing the Isx-responsive promoter and enhancer of mouse Bex1 (-648 to +177 including exon 1) driving luciferase (pGL3-Bex1-luciferase; Bex1-luc) (Figure 1a). We coelectroporated CMV-GPR68 and Bex1-luc into adult rat HCN progenitors and treated these cultures with increasing concentrations of Isx, using our lead *N*-cyclopropyl-5-(thiophen-2-yl)-isoxazole-3-carboxamide compound (Figure 1a). Isx and GPR68 synergistically coactivated Bex1-luc in HCN cells, displaying both an Isx dose response and regulation by GPR68 expression level (Figure 1a). Notably, at all three GPR68 levels, high micromolar Isx concentrations adversely affected reporter activity and, conversely, at all Isx concentrations, high GPR68 levels adversely affected reporter activity (Figure 1a). We conclude from these results that Isx and GPR68 function in the same mechanistic pathway for activating the Bex1 reporter gene in HCN cells.

We previously observed that rat HCN cells exposed to Isx rapidly acidified their culture media (Figure 1b), correlating with Isx-triggered differentiation from a slow-growing/quiescent starting progenitor population into rapidly expanding transient amplifying progenitors (TAPS), depleting media energy substrates and shifting to lactate-producing metabolism.¹⁵ We observed this effect in Isx-treated PC12 rat adrenal pheochromocytoma cells as well (data not shown). The Isx-triggered extracellular media acidification now acquired new physiological significance after recognizing the possible role of proton (pH) sensing GPR68 in HCN cells. Indeed, within 24 h, Isx drove the HCN differentiation culture media to pH ~ 6.9 (Figure 1b), the optimum pH for GPR68 mediated IP3-kinase signaling activity.^{9,10} In our previous studies, experimentally increasing proton levels (dropping pH) acted synergistically with Isx to activate the Nkx2-5 reporter gene in NECs.¹⁸ Here, Isx induced HCN cells to produce GPR68-activating protons as a natural metabolic byproduct, secondarily amplifying Isx's effect on the Bex1-luc reporter gene through GPR68 (Figure 1a). Next, to begin evaluating this proneurogenic small molecule/proton signaling circuit *in vivo*, we explored GPR68 expression in brain.

Acid Sensing GPR68 Localizes to Hippocampal Type 1-NSC Radial Processes. We used immunohistochemical localization and quantitative polymerase chain reaction

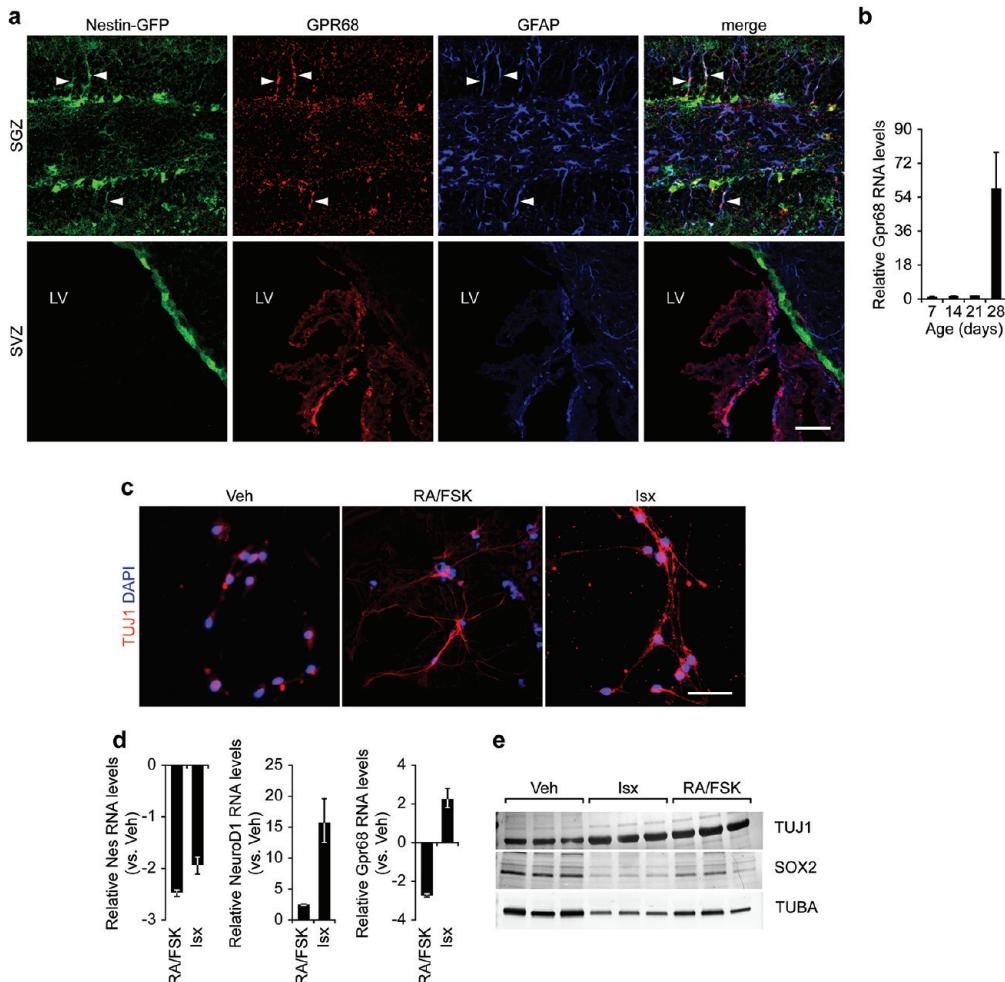


Figure 2. Localization, developmental regulation, and Isx induced neuronal differentiation of GPR68-expressing cells in hippocampus and primary cultures. (a) Localization of GPR68 to SGZ type 1-NSCs and SVZ choroid plexus ependymal cells. Triple immunohistochemistry colocalizing Nestin-GFP (green), GPR68 (red), and GFAP (blue) in adult mouse SGZ (upper panels) and SVZ (lower panels). Arrowheads denote type 1-NSC dendritic process. LV = lateral ventricle. (b) Postnatal developmental regulation of GPR68 in hippocampal progenitors. QPCR measurement of Gpr68 mRNA levels in FACS-purified Nestin-GFP⁺ progenitors from pooled dissected DG from 7, 14, 21, or 28 day old mice. Normalized to Gapdh mRNA. (c) Small molecule-induced morphological differentiation and TuJ1 expression in cultured DG cells. DG cells from week old pups were cultured for 3 days with DMSO (vehicle), RA/FSK, or Isx, fixed, and stained with TuJ1 antibody and DAPI. (d) Confirmation of small molecule-induced neuronal differentiation by QPCR. QPCR measurement of Nestin, NeuroD1, and Gpr68 mRNA levels in 3-day RA/FSK and Isx treated DG cells from week old pups. Relative mRNA levels are presented versus DMSO (vehicle) control. (e) Confirmation of small-molecule induced neuronal differentiation by protein blots. Protein extracts from triplicate DG cultures from week old pups treated for 3 days with DMSO (vehicle), Isx or RA/FSK blotted with TuJ1, Sox2, and α -tubulin antibodies.

(QPCR) to provide evidence of GPR68's functional importance through regulated expression in brain. We colocalized GPR68 (using a validated antibody) with Nestin-driven green fluorescent protein (GFP) and glial fibrillary acid protein (GFAP) in the subgranular zone (SGZ) of adult Nestin-GFP transgenic mouse brain²¹ (Figure 2a, upper panels). Type 1-neural stem cells (NSCs) expressed GPR68, defined by triple (GPR68/Nestin-GFP/GFAP) colocalization and characteristic treelike neurite morphology (Figure 2a, upper panels, arrowheads). Indeed, we clearly demonstrated proton-sensing GPR68 in type 1-NSC-radial processes, suggesting that one function of these antenna-like structures that penetrate the granule layer may be to sense micro-environmental pH (Figure 2a, upper panels, arrowheads).

We also colocalized GPR68 with Nestin-GFP and GFAP in the subventricular zone (SVZ) of adult Nestin-GFP transgenic mice (Figure 2a, lower panels). Although SVZ Nestin-GFP⁺

NSCs did not express GPR68, choroid plexus ependymal cells bordering the lateral ventricle (LV) robustly expressed this receptor, implying a previously unsuspected role for GPR68 in regulating cerebrospinal fluid pH (Figure 2a, lower panels). Differential GPR68 expression in SGZ but not SVZ NSCs provides a new molecular hallmark distinguishing these two functionally divergent neural progenitor pools. Moreover, these studies localized GPR68 to the cell bodies and processes of SGZ radial type 1-NSCs, critical cells in the hippocampal neurogenic niche.

We also demonstrated developmentally regulated GPR68 expression in Nestin-GFP⁺ neural stem/progenitor cells purified by fluorescence-activated cell sorting (FACS) from dissociated mouse hippocampi. We detected Gpr68 mRNA by QPCR analysis of RNA prepared from Nestin-GFP⁺ cells at days 7, 14, 21, and 28 of postnatal life (Figure 2b). Notably, between days 21 and 28, Nestin-GFP⁺ hippocampal cells

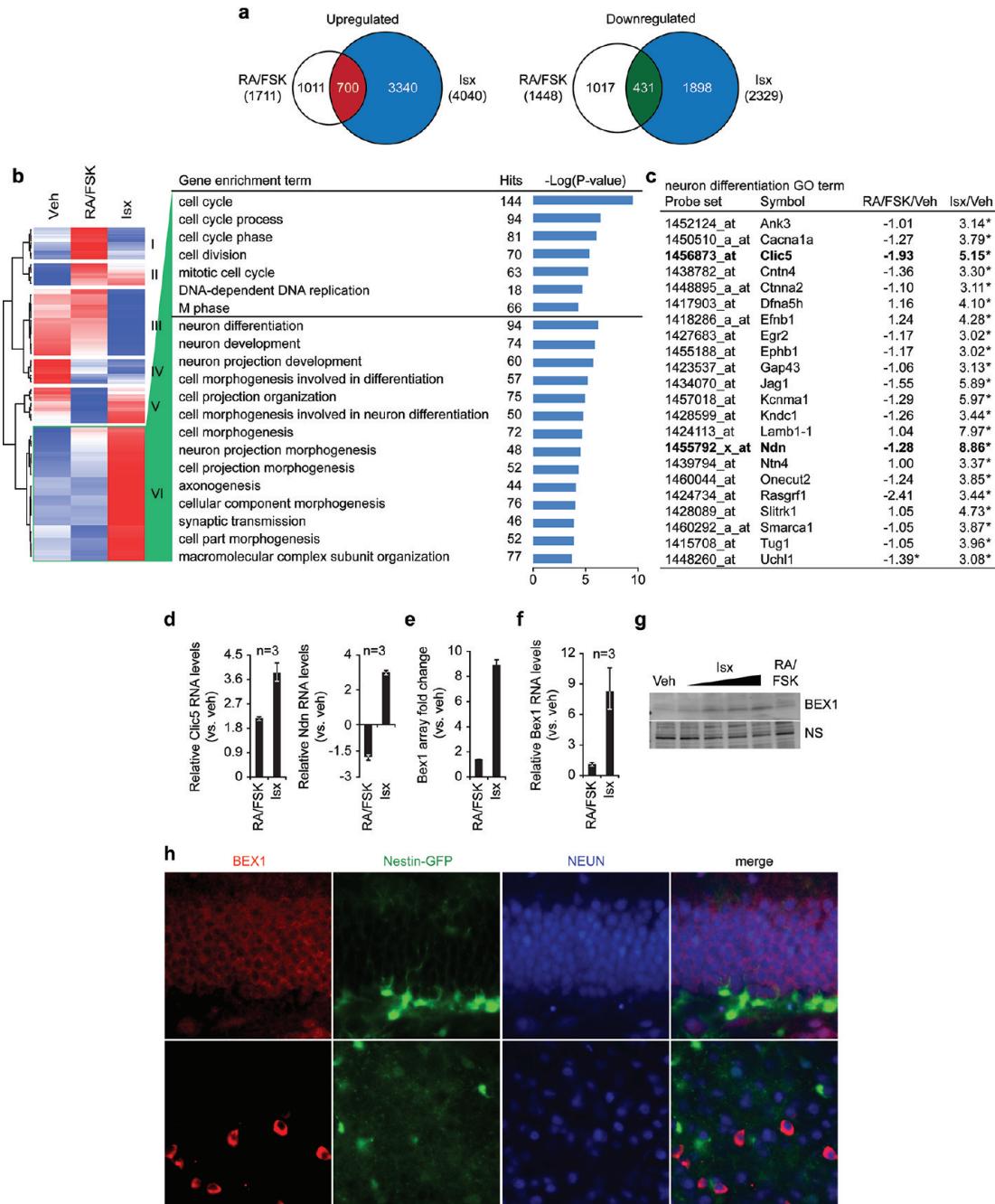


Figure 3. Transcriptome analysis of Isx induced neuronal differentiation in hippocampal progenitors and validation using brain-enriched X-linked protein-1. (a) Venn diagrams of gene numbers dysregulated by RA/FSK or Isx in cultured DG cells. Comparison of genes upregulated (left) or downregulated (right) in Affymetrix array analysis of mRNA from week old pup DG cultures treated for 3 days with RA/FSK or Isx (dysregulated versus vehicle control). (b) Heat map of gene cluster analysis of small-molecule treated hippocampal progenitor transcriptomes. Cluster analysis of DMSO (vehicle), RA/FSK, and Isx treated DG cells defined six subgroups (I–VI) based on relative gene expression levels. Subgroup VI (genes strongly upregulated in Isx compared with RA/FSK and vehicle) was composed of two major related gene enrichment terms: cell cycle (upper grouping) and neuronal differentiation (lower grouping). The number of individual gene hits and the *P*-values (on a scale of 10^{-10} – 10^{-10}) for each gene enrichment term are shown at the right. (c) Comparison of transcript levels from “neuronal differentiation” GO term in RA/FSK and Isx treated DG cells. Neuronal differentiation GO term transcript levels from microarray analysis of RA/FSK and Isx treated DG cells, each normalized versus DMSO (vehicle). Two genes, Clic5 and Ndn (bold), were chosen to validate the microarray by QPCR in (d). (d) Validation of microarray by QPCR of candidate genes. Microarray results for candidate genes, Clic5 and Ndn, independently confirmed by QPCR from triplicate RA/FSK and Isx treated DG cultures. Normalized against DMSO (vehicle). (e–g) Validation of microarray results for Isx target gene, Bex1, by QPCR and protein blot. Isx target gene Bex1 was upregulated >8-fold by microarray (e), confirmed by QPCR in triplicate cultures (f), and confirmed in Isx dose response by protein blot for Bex1 protein (g) in Isx treated DG cells. (h) Localization of BEX1 in hippocampus and cerebral cortex. Triple immunohistochemistry colocalizing BEX1 (red), Nestin-GFP (green), and NEUN (blue) to granule layer neurons in adult mouse hippocampus (upper panel) and small interneurons in cerebral cortex (lower panel).

markedly increased Gpr68 gene expression, more than 50-fold by QPCR (Figure 2b). Thus, GPR68 is developmentally regulated in postnatal hippocampal progenitors, suggesting that proton-GPR68 signaling becomes increasingly important during this transition.

These results localized GPR68, a pH sensor regulating cell-mediated responses to local tissue acidosis,⁹ to type 1-NSCs in the hippocampal neurogenic niche, a hypoxic (and therefore acidic) brain microenvironment.^{6,22}

Isx is Proneurogenic in Cultured Hippocampal NSCs.

We previously reported Isx's proneurogenic activity in HCN cells, a multipotent clonal line of adult rat hippocampal neural progenitors.¹⁵ Here, we confirmed and extended these findings in DG cultures prepared from 1 week old mice (Figure 2c). First, we confirmed by QPCR that DG primary cultures expressed Gpr68 mRNA (Figure 2d). As previously explored mechanistically in HCN cells¹⁵ and noted above, Isx increased proliferation in mouse DG primary cultures, advancing slowly proliferating NSCs toward TAPS (data not shown). In addition to expanding population cell number, Isx triggered remarkably robust neuronal differentiation in DG primary cultures (Figure 2c–e). We compared Isx to retinoic acid and forskolin (RA/FSK), until now considered the gold standard proneurogenic chemical cocktail in NSCs.^{23,24} Like RA/FSK, Isx induced dramatic morphological differentiation of primary DG cells, demonstrated by immunocytochemical staining for neuron-specific class III β -tubulin (TUJ1) (Figure 2c). Importantly, neuronlike DG cells generated by RA/FSK or Isx had distinctive morphological patterns; compared to complex RA/FSK patterns, Isx induced simpler patterns, commonly bearing only one or two and much thicker neurite extensions (like interneurons) (Figure 2c).

We confirmed reciprocal regulation of Nestin and NeuroD1 mRNAs, markers of early and later NSC neuronal differentiation, respectively, by QPCR in RA/FSK and Isx generated neuronlike DG cells (Figure 2d). Isx induced NeuroD1 mRNA in DG NSCs more strongly than RA/FSK, confirming previous results in HCN,¹⁵ more recently extended to pancreatic β -cells (where NeuroD1 is the β 2 gene).¹⁷ Notably, we also confirmed that Isx increased Gpr68 mRNA in DG cells, implying a feed-forward signaling circuit between this candidate ligand-receptor pair and the receptor's gene.

Finally, we demonstrated reciprocal regulation of SOX2, a marker of type-1 NSCs, and the TUJ1 neuronal differentiation marker by protein blot in triplicate cultures of vehicle, Isx, or RA/FSK generated neuronlike DG cells (Figure 2e). Compared to RA/FSK, Isx more effectively decreased SOX2 in these primary cultures (Figure 2e).

Taken together, in vitro gene expression data demonstrated that Isx strongly activated neuronal differentiation in primary mouse DG cells, significantly out-performing RA/FSK, until now the gold standard, in this task.

Unrivaled Activation of Neurogenic Transcriptional Programs by Isx in Cultured DG Cells. To gain a genome-wide perspective on Isx-regulated neuronal transcriptional programs, we evaluated RNA prepared from vehicle, RA/FSK, and Isx treated DG primary cells by Affymetrix microarray gene expression analysis. As shown in the Venn diagram (Figure 3a), RA/FSK dysregulated 3159 genes (1711 up and 1448 down) and Isx dysregulated 6369 genes (4040 up and 2329 down) in DG primary cells. Comparing these two genes pools, RA/FSK and Isx upregulated a common set of 700 genes and downregulated a common set of 431 genes in DG primary

cells (Figure 3a). To extract a broader biological meaning from these results, we performed hierarchical cluster analysis with all dysregulated genes from each treatment (7804 total genes) and identified 6 clusters (I–VI), shown by heat map (Figure 3b) with certain features highlighted in (Table 1).

Table 1. Six Major Subgroups Defined by Hierarchical Cluster Analysis of All Genes Dysregulated by RA/FSK or Isx (compared with vehicle) in Primary Mouse Dentate Gyrus Cells Using Affymetrix Microarray Gene Expression Analysis

cluster group	no. of genes in group	highly ranked GO term in cluster (hits)	significance (<i>P</i> -value)	impt genes in group
I	793	mitochondrion (70)	3.52×10^{-7}	RA target genes, Rbp1 and Rab
II	536	tube development (15)	7.69×10^{-4}	
III	1650	nucleotide binding (189)	1.55×10^{-9}	
IV	608	sterol biosynthetic process (11)	5.84×10^{-10}	
V	879	actin binding (27)	3.17×10^{-6}	
VI	3338	neuron differentiation (94)	4.68×10^{-10}	See Figure. 1c; Ndn, Bex1

Here, we focused on two key clusters (I and VI). The highest ranked gene ontology (GO) term in cluster I (genes highly expressed in RA/FSK but not in Isx or vehicle treated DG cells) was "mitochondrion," consistent with known regulation of mitochondrial genes by retinoic acid (RA).²⁵ Among group I genes, RA/FSK strongly upregulated RA target genes, as expected, like Rab (retinoic acid receptor- β ; upregulated > 10-fold)²⁶ and Rbp1 (retinol binding protein-1; upregulated > 110-fold)²⁷ (Figure 3b and Table 1). The upregulation of RA-responsive genes and dramatic morphological differentiation (see Figure 2c) confirmed the pharmacological action of RA/FSK. Unexpectedly, when compared with Isx, RA/FSK only weakly activated neuronal transcriptional programs in DG primary cells.

To highlight this observation, we focused on group VI, the genes highly expressed in Isx but not in RA/FSK or vehicle treated DG cells (Figure 3b). We identified the biological themes in group VI by gene enrichment analysis and discovered remarkable enrichment in two major themes: cell cycle progression and neuronal differentiation, two highly integrated aspects of neurogenesis, as slowly proliferating type 1-NSCs first differentiate into highly proliferative transit amplifying progenitors (TAPS) and neuroblasts, before becoming terminally differentiated neurons. Indeed, we identified "cell cycle" and "neuron differentiation" as among the highest ranked group VI gene enrichment terms, comprising 144 and 94 distinct genes, respectively (Figure 3b). Narrowing focus even further onto the genes within the "neuron differentiation" term, Isx strongly increased Necdin (Ndn), an important regulator of neuronal growth, differentiation, and survival,²⁸ but RA/FSK did not (Figure 3c). To validate these microarray results, we evaluated Ndn mRNA levels by QPCR in DG primary cell cultures, comparing vehicle, RA/FSK, and Isx treatment regimens, each in triplicate (Figure 3d). Mirroring the microarray, Isx increased Ndn mRNA levels in all three cultures (Figure 3d). Thus, Isx strongly activated complex

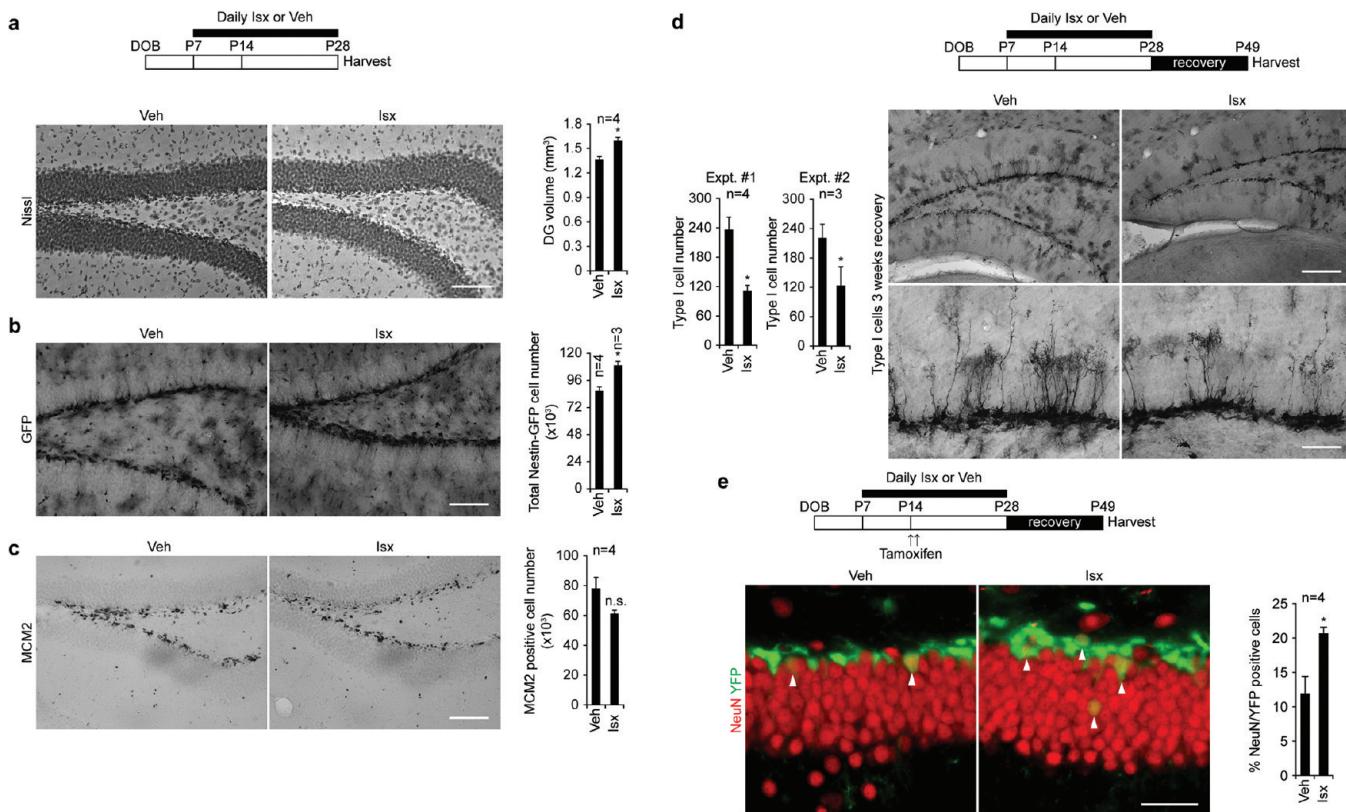


Figure 4. Isx induced hippocampal neurogenesis in young mice. (a) Increased dentate gyrus volume in Isx treated young mice. Mice were injected with Isx (16 mg/kg/day, formulated in 20% hydroxypropyl β -cyclodextrin) or vehicle control (20% hydroxypropyl β -cyclodextrin) daily from days 7 to 28, and DG volume measured by three-dimensional stereology after Nissl staining of serial sections. $N = 4$ for each group, two hippocampi per brain. (b) Increased dentate gyrus neural progenitor cell number in Isx treated young mice. Mice were injected with Isx or vehicle control as above, and total Nestin-GFP⁺ cell number per hippocampus counted after GFP immunohistochemistry and DAB staining. $N = 4$ for vehicle and $n = 3$ for Isx, two hippocampi per brain. (c) Stable dentate gyrus proliferative index in Isx treated young mice. Mice were injected with Isx or vehicle control as above, and total MCM2⁺ cell number per hippocampus counted after MCM2 immunohistochemistry and DAB staining. $N = 4$ for each group, two hippocampi per brain. (d) Decreased subgranular zone type 1-neural stem cell pool in Isx treated young mice. Mice were injected with Isx or vehicle control as above, and Nestin-GFP⁺ type 1-NSC number per hippocampus counted after GFP immunohistochemistry and DAB staining. This experiment was repeated twice: experiment #1 ($n = 4$, each group, two hippocampi per brain) using the same treatment paradigm as in (a–c), and experiment #2 ($n = 3$, each group, two hippocampi per brain), which included a 3 week drug-free recovery period before brain harvest on day 49. (e) Increased production of granular layer neuronal cells in Isx treated young mice. Nestin-Cre^{ERT2}/ROSA26-loxp-stop-loxp-YFP mice were injected with Isx or vehicle control ($n = 4$ each group) for 3 weeks as above, pulsed with tamoxifen on day 14, and then allowed to recover until brain harvest on day 42. After NeuN and YFP double immunohistochemistry, the number of NeuN/YFP double positive cells/hippocampus was counted.

neuronal transcriptional programs in DG primary cells and, importantly, far exceeded RA/FSK in this function. We also validated the array results with Clic5 (chloride internal channel 5), a second gene involved in neuron differentiation,²⁹ more strongly upregulated by Isx than RA/FSK in triplicate cultures (Figure 3d).

Next, we returned to study Bex1, a gene transcriptionally regulated by Isx and GPR68 in HCN cells (see Figure 1) and whose function in brain remains incompletely understood,^{20,30} gene expression in mouse DG primary cultures and mouse brain. Isx strongly induced Bex1 mRNA in mouse DG primary cells, >8-fold by microarray (Figure 3e), and we independently validated this result by QPCR in triplicate cultures (Figure 3f). As further validation, Isx dose-dependently induced BEX1 polypeptide in Isx treated DG cells (Figure 3g). Even further, we localized BEX1 in the mouse brain. Consistent with this gene being a downstream marker of Isx-mediated neuronal differentiation, BEX1 localized to granule layer neurons in the DG and to small interneurons in the cerebral cortex (Figure 3h).

Taken together, the results thus far localized GPR68, the candidate Isx target, to hippocampal type 1-NSCs in vivo and confirmed that, when harvested, cultured, and exposed to Isx in vitro, GPR68-expressing hippocampal type 1-NSCs proliferated and differentiated into neuronlike cells, confirmed at mRNA, protein, and cellular morphological levels. The next important question was whether systemically administered Isx targeted GPR68-expressing type 1-NSCs in the mouse brain in vivo, providing that Isx crossed the blood brain barrier. For these in vivo experiments, we focused on young mice, P7 through P28, encompassing the developmental period of robust hippocampal neurogenesis and GPR68 upregulation (Figure 1b).

Isx Promotes Hippocampal Neurogenesis in Young Mice. There is intense interest in discovering novel proneurogenic small molecules for neurotherapeutics.^{31–34} We evaluated Isx's CNS activity in mice after confirming penetration of the blood brain barrier by mass spectrometry (data not shown). Young mice injected daily with Isx for 3 (and up to 9) continuous weeks demonstrated no unusual behavior. To evaluate Isx's CNS activity, we focused on hippocampal neurogenesis, measuring DG volume, Nestin-GFP⁺ cell

number, cell cycle activity, and type 1-NSC number. Additionally, we did Nestin-CreER^{T2} lineage tracing to confirm increased production of newly generated neuronal cells in Isx treated mice. All of the results are consistent with increased hippocampal neurogenesis in Isx treated young mice.

First, we evaluated Isx's effects on DG volume by Nissl staining and three-dimensional stereology. We injected mice once daily for 3 weeks, starting on day 7 after birth and concluding on day 28, the day of sacrifice. We measured the volumes of 16 hippocampi, 2 each from 4 young mice treated with Isx and 4 vehicle-treated control mice, all littermates (Figure 4a). Mice treated with Isx had significantly increased DG volumes, providing the first evidence of Isx's proneurogenic activity in young mice *in vivo* (Figure 4a).

Focusing on the stem/progenitor cells responsible for increasing DG volume, we counted the number of GFP-expressing neural stem/progenitor cells detected by GFP immunohistochemistry-coupled DAB staining in hippocampi of Isx and vehicle treated Nestin-GFP transgenic mice (Figure 4b). We measured the Nestin-GFP⁺ cell number in 14 hippocampi, 2 each from 3 young mice treated with Isx and 4 vehicle-treated control mice, all littermates. Corroborating the increased DG volumes, we demonstrated statistically significantly increased total number of Nestin-GFP⁺ cells in Isx treated as compared with vehicle treated control hippocampi (Figure 4b). Taken together, the increased number of Nestin-GFP⁺ cells and larger DG volumes confirmed enhanced hippocampal neurogenesis in Isx treated young mice.

We also evaluated neural stem/progenitor proliferative activity in DG from Isx and vehicle treated mice (Figure 4c). Using MCM2 immunohistochemistry-coupled DAB staining, we counted the number of actively cycling cells in DG cells from 16 hippocampi, 2 each from 4 young mice treated with Isx and 4 vehicle-treated control mice, all littermates. We counted fewer MCM2⁺ cells in hippocampi from Isx versus vehicle control brains, approaching but not quite achieving statistical significance (Figure 4c). Thus, despite 3 weeks of daily Isx therapy leading to increased DG volume and Nestin-GFP⁺ cell number, we observed normal net cell cycle activity in the DG.

To more precisely evaluate Isx's effect on the DG, we counted the number of type 1-NSCs, identifiable by their highly arborized dendritic tree, using GFP immunohistochemistry-coupled DAB staining in hippocampi of Nestin-GFP, employing the same 3 week Isx or vehicle treatment regimen as above. We repeated this experiment twice. In the first experiment, we counted the number of type 1-NSCs in 16 hippocampi, 2 each from 4 young mice treated for 3 weeks with Isx and 4 vehicle-treated control mice, all littermates. Surprisingly, we observed a more than 50% decrease in type 1-NSC number in Isx treated Nestin-GFP mice compared and with their vehicle treated counterparts (Figure 4d, expt #1). To confirm and extend these results, we repeated this experiment with 3 Isx and 3 vehicle treated littermate Nestin-GFP mice; this time, however, we gave the mice a 3 week long drug-free recovery period. Confirming the initial result, we found that the SGZ of Nestin-GFP mice treated for 3 weeks with Isx remained depleted of type 1-NSCs, again by ~50%, even after a 3 week drug-free interval (Figure 4d, expt #2). Thus, Isx pharmacologically promoted hippocampal neurogenesis but concomitantly depleted the hippocampal type 1-NSC pool and this effect persisted for at least 3 weeks post-therapy.

Finally, we used genetic fate mapping to confirm Isx's proneurogenic activity *in vivo*. To generate lineage-tracing

mice, we crossbred Nestin-CreER^{T2} transgenic mice³⁵ with ROSA26-YFP reporter mice.³⁶ We employed the same paradigm of treating young mice with Isx or vehicle (4 littermate mice in each group) daily for 3 weeks starting on day 7 after birth and coupled this tamoxifen injection on days 14 and 15, 1 week into the Isx pharmacological study (Figure 4e, time map). This experiment allowed us to compare the number of Nestin-YFP⁺ cells born in Isx and vehicle treated hippocampi and, more importantly, by superimposing NeuN immunohistochemistry, allowed us to count the number of NeuN/YFP double positive newborn neurons in granular layer (Figure 4e). Evaluating a total of 16 hippocampi (8 Isx treated and 8 control), we observed a statistically significant increase in (almost doubling of) the number of NeuN/YFP double positive newly generated neurons in Isx versus vehicle treated mice (Figure 4e). Taken together, these results provided conclusive fate mapping evidence that Isx pharmacologically increased hippocampal neurogenesis in young mice, correspondingly depleting the type 1-NSC pool.

Hippocampal Learning and Memory Function Is Normal in Isx Treated Young Adult Mice. The functional significance of hippocampal neurogenesis remains highly controversial.^{37,38} Although we observed significant hippocampal structural changes in Isx treated young mice, their behavioral immaturity precluded Morris water maze testing. Therefore, we tested hippocampal memory function at the earliest suitable time point, starting Isx therapy at day 7 after birth and continuing until 9 weeks old. After a 2 week hiatus, Isx treated mice trained for Morris water maze testing. Empirically, mice chronically exposed to Isx had no obvious behavioral abnormalities. Experimentally, Isx treated young adult mice ($n = 16$) and vehicle treated controls ($n = 13$) scored equivalently on Morris water maze testing, providing evidence of normal hippocampal learning and memory function (Figure 5). Thus, despite Isx's ability to enhance hippocampal neurogenesis, at least in young mice, this did not translate into

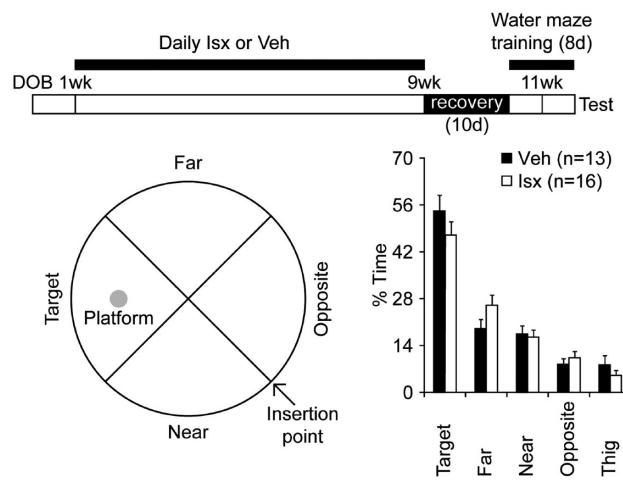


Figure 5. Preserved hippocampal learning and memory function in Isx treated young adult mice. Mice were treated with Isx ($n = 16$) or vehicle control ($n = 13$) from day 7 after birth for 8 weeks, allowed a 2 week long drug-free recovery interval, and then trained and tested in a Morris water maze (% time spent in platform-containing target, far, near or opposite quadrants) and anxiety testing by thigmotaxis in an open field (Thig).

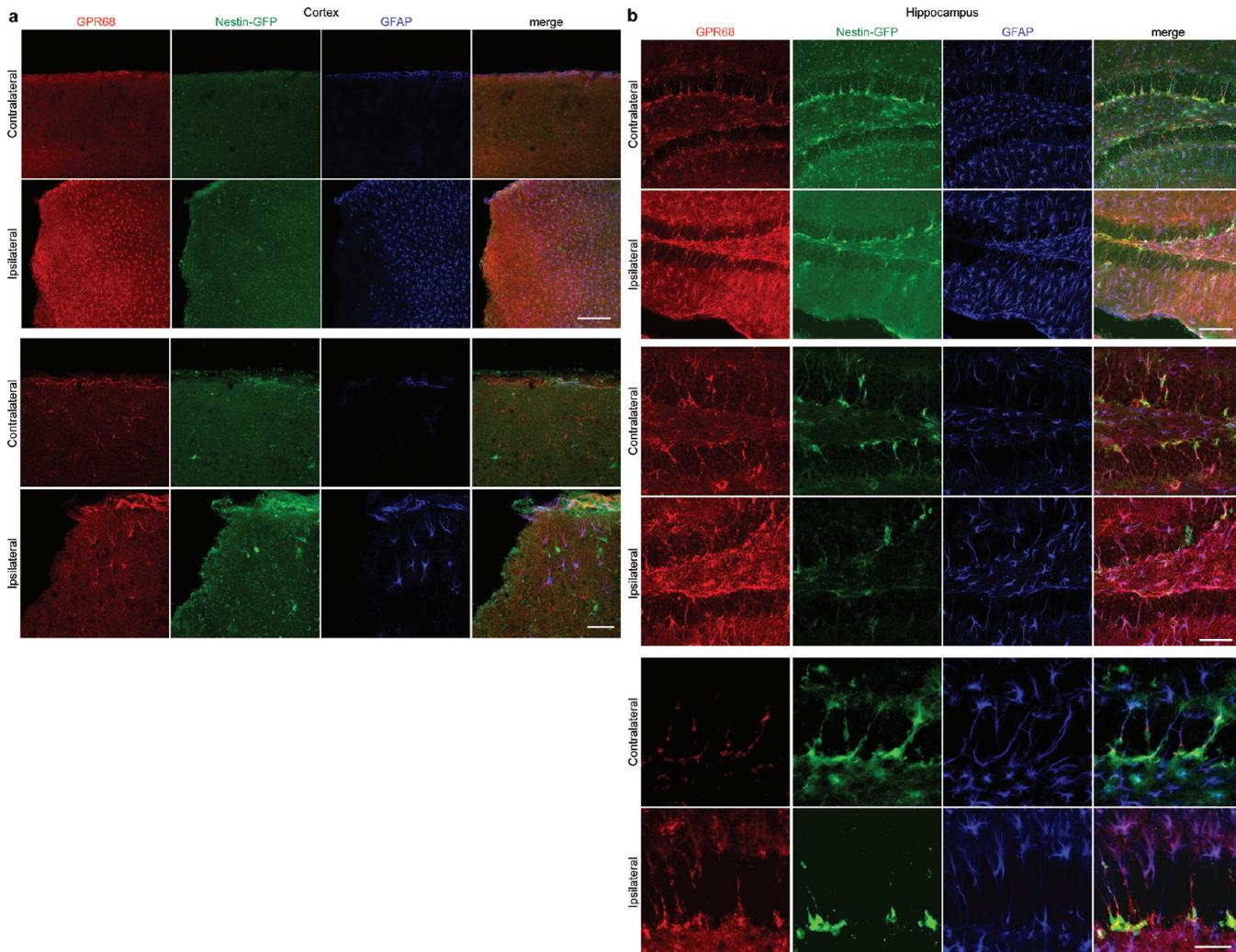


Figure 6. Increased cerebral cortical and hippocampal GPR68 after traumatic brain injury in mice. Adult mice were sacrificed 3 days after controlled pneumatic-compression injury of the cerebral cortex and perfusion fixed brain slices tripled immunostained for GPR68 (red), Nestin-GFP (green), and GFAP (blue). (a) Localization of GPR68 to reactive astrogliosis in ipsilateral cerebral cortex. Increasing magnification of ipsilateral and contralateral cerebral cortex immunohistochemistry for GPR68 after traumatic brain injury. (b) Localization of GPR68 to type 1-neural stem cells in ipsilateral hippocampus. Increasing magnification of ipsilateral and contralateral hippocampus immunohistochemistry for GPR68 after traumatic brain injury.

learning or memory enhancement or deficits in young adult mice, the earliest age for reliable formal behavior testing.

Confirming the behavioral neutrality of Isx's pharmacological action in the CNS, we also tested "fear conditioning" in 9 week-old mice treated with Isx or vehicle for 3 weeks followed by a 3-week drug respite before training and testing. As in the Morris water maze, Isx treated mice failed to distinguish themselves from their control counterparts, including the "context" portion of the fear conditioning study that evaluates hippocampal memory function (data not shown). Thus, in normal mice, despite driving irrefutable hippocampal neurogenesis (coupled with type 1-NSC depletion), Isx neither positively nor adversely affected memory function, even after 8–9 weeks of continuous daily dosing, as defined by rodent behavioral testing.

A Possible Role of GPR68 in Cortical Astrogliosis and Hippocampal Neurogenesis after Traumatic Brain Injury. We previously reported that GPR68 was markedly upregulated in mouse heart after myocardial infarction, specifically localized to cardiomyocytes and other cells in the infarct's border zone.¹⁹ Here, we explored GPR68 expression in

a mouse traumatic brain injury model. After controlled cortical concussion injury of the adult mouse brain, we observed markedly increased number of GFAP⁺ reactive astrocytes in the cerebral cortex ipsilateral to the brain injury (astrogliosis) (Figure 6a). Almost all GFAP⁺ reactive astrocytes strongly coexpressed GPR68, detected by immunohistochemistry (Figure 6a). Upregulation of GPR68 during astrogliosis could be a supportive injury response to microenvironmental acidosis. On the other hand, this might explain the heightened vulnerability of astrocytes, as compared with neurons, to hypoxia and acidosis occurring in the context of traumatic or ischemic brain injury.³⁹ We also focused on the hippocampus after controlled cortical injury. Compared to the contralateral side, the ipsilateral hippocampus demonstrated increased GPR68 staining as well, largely localized to type 1-NSCs (Figure 6b). Taken together, these immunohistochemistry data demonstrated highly regulated GPR68 expression in the context of traumatic brain injury, providing additional evidence for this acid sensing receptor's role in the brain's injury response.

CONCLUSION

We tracked the function of our proneurogenic Isx small molecules to GPR68, proton (pH)-sensing GPCRs abundantly expressed by type 1-NSCs in the hippocampal neurogenic niche, a highly specialized (and presumptively) hypoxic microenvironment within the mouse brain.⁶ Quite unexpectedly, GPR68 emerged as the single agonist hit from an unbiased functional target screen of 158 candidate GPCRs regulated by Isx in heterologous cells.¹⁹ Until now, protons, in their functional capacity as signal transducing molecules, have not been mechanistically linked to NSC biology or neurogenesis, yet, in retrospect, this connection seems highly plausible and probable, for four major reasons (Figure 7): (1) if the SGZ

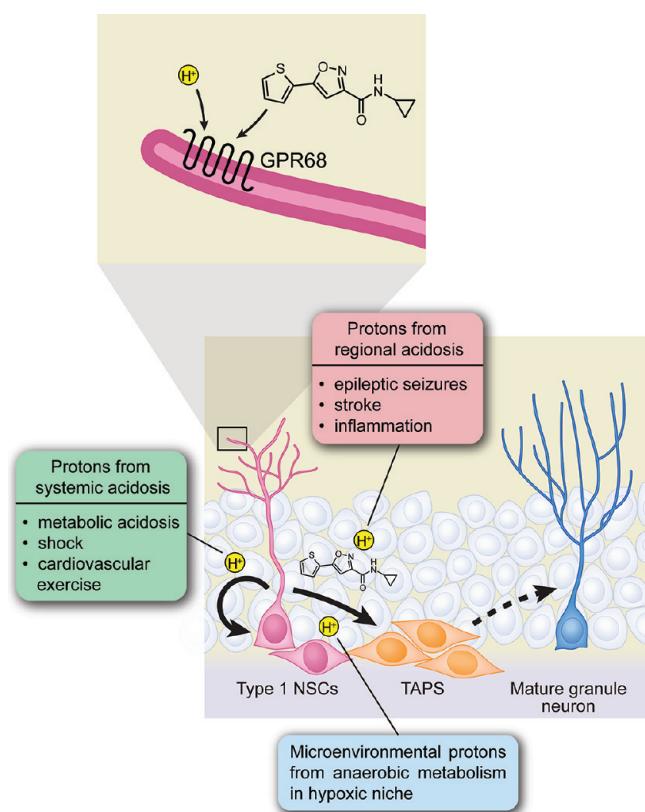


Figure 7. Acid and Isx regulation of hippocampal neurogenesis through GPR68-expressing type 1-NSCs. GPR68-expressing type 1-NSCs in the SGZ of the DG are regulated by protons, derived from three sources: (1) regional acidosis, as occurs, e.g., from glutamate accumulation after epileptic seizure synaptic overactivity; (2) systemic acidosis, as occurs, e.g., from lactic acid generating cardiovascular exercise; or (3) microenvironmental acidosis, as occurs from anaerobic metabolism in the hypoxic stem cell niche. Isx pharmacologically activates hippocampal neurogenesis through GPR68 receptors in type 1-NSCs.

microenvironment is hypoxic (as presumed), it is also acidic; acidosis from anaerobic metabolism is an inevitable byproduct of tissue hypoxia;⁶ (2) acidosis is an important aspect of brain function and pathophysiology; pathophysiological triggers of hippocampal neurogenesis like epileptic seizures, traumatic or ischemic injury all cause brain acidosis;² (3) transient mild lactic acidosis accompanies cardiovascular exercise, the strongest physiological stimulus for hippocampal neurogenesis;⁴⁰ and finally, (4) even the antenna-like processes of type 1-NSCs that penetrate through the granule layer into the

hilus of the hippocampus displayed GPR68 suggested that these structures may function as biosensors of microenvironment pH. Indeed, by promoting hippocampal neurogenesis through NSC GPR68, we speculate that lactic acidosis contributes to the beneficial effects of cardiovascular exercise on Alzheimer's disease.⁴¹ Even further, proton signaling through GPR68 receptors could explain the beneficial hippocampal cellular effects of treadmill exercise after traumatic brain injury.⁴² Pharmacologically, all of our experimental results indicate that Isx is a (neutral) small molecule proxy for the proton that drives neurogenesis by activating pH-sensing GPR68 in SGZ type 1-NSCs.

Certainly, many issues remain unresolved. For example, we do not yet know whether Isx produced hippocampal neurons contribute to brain function *in vivo* (or whether Isx induced NSCs become functional neurons *in vitro*). Isx treated young adult mice failed to distinguish themselves in behavioral tests, but this may reflect their already high baseline hippocampal function. Importantly, our results are distinctive from studies done with our molecule in older adult mice, where a shorter Isx treatment protocol promoted adult neurogenesis without depleting the type 1-NSC pool but improving hippocampal learning and memory function (Petrik, Hsieh, and Eisch, unpublished data). These differences could reflect differences in drug metabolism or hippocampal maturity and functional reserve comparing young and older adult mice, or changes in experimental technique.

If, as originally intended, the goal is to develop Isx as a cardio-regenerative therapeutic, it would be reassuring that “off-target” effects in the brain are behaviorally neutral. On the other hand, we speculate that Isx might therapeutically enhance neurogenesis in the context of injury or other pathophysiological or neurodegenerative conditions or in aged adult mice with normally diminished hippocampal functional reserve. Additionally, targeting GPR68-expressing reactive astroglial cells after traumatic brain injury suggests that Isx therapy might fundamentally change the biology of gliosis in the cerebral cortex, perhaps even pharmacologically redirecting these cells toward a neuronal fate.

To conclude, although the mechanistic connections between Isx, GPR68, and hippocampal neurogenesis need to be confirmed through direct genetic experiments, and even more importantly, through development of pharmacological antagonists, our initial chemical biology data provide a compelling starting point. Most importantly, our results validate chemical biology’s promise to discover completely new and unexpected CNS disease targets by probing brain structure and function with novel small molecules, setting the stage for future drug discovery and development.

METHODS

Mice. All the mice were humanely housed and cared for by the UT Southwestern Medical Center’s Animal Resource Center, and all mouse protocols were preapproved and conducted under the guidelines of the Institutional Animal Care and Use Committee. For *in vivo* experiments, mice were injected with vehicle (20% hydroxypropyl β -cyclodextrin in sterile water) or Isx formulated in 20% hydroxypropyl β -cyclodextrin, once daily, ip, at a final dose of 16 mg/kg/day, beginning on postnatal day 7 and continuing as described in each figure’s time map. Nestin-GFP mice, Nestin-Cre^{ERT2}, and ROSA26-YFP mice have been maintained on a C57Bl6 background (Jackson Laboratories) as previously described.²¹

Cell Culture and Immunocytochemistry. For primary cell cultures, DG was dissected from 600 μ m coronal sections of 1 week

old mouse brain, digested with active papain solution and then grown in serum-free NSC growth medium (DMEM/F12, Invitrogen) containing 1% N2 supplement (Gibco), 1× B27 (Gibco), 1% penicillin/streptomycin, 10 µg/mL heparin, 20 ng/mL epidermal growth factor (EGF, Invitrogen), and 20 ng/mL fibroblast growth factor-2 (FGF-2, Sigma). Cells were expanded through at least three passages and then plated onto glass chamber slides. For neuronal differentiation, cells were treated for 72 h with Isx (generally, 10 µM, or escalating dose response) or retinoic acid (1 µM)/forskolin (5 µM) in growth factor-free NSC medium as previously described.²¹ For immunocytochemistry, the chamber slides were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature, washed in phosphate buffered saline (PBS), blocked for 1 h in 10% normal donkey serum in PBS with 0.3% Triton X-100, followed by overnight incubation primary antibody (rabbit anti-Tuj1 antibody, Covance, 1:2000) at 4 °C. Chamber slides were washed with 0.3% Triton X-100 in PBS and then incubated with the fluorescently conjugated secondary antibody (donkey anti-rabbit cy3, Jackson ImmunoResearch, 1:400) and 4',6-diamidino-2-phenylindole (DAPI) for 2 h and covered with mounting solution (Vector).

RNA Isolation and Real-Time PCR. Total RNA was isolated from the primary cell cultures using Trizol (Aldrich), purified and reverse transcribed into cDNA with iScript cDNA Synthesis Kit (Bio-Rad). Suitable primers and iTaq Fast SYBR Green Supermix with Rox (Bio-Rad) were mixed together with cDNA into a total 20 µL volume for QPCR using the CFX96 system (Bio-Rad). All gene expression levels were normalized to Gapdh and all primer sequences are available upon request.

Microarray/Gene Enrichment Analysis. All samples were prepared as previously described.⁴³ Briefly, total RNA was extracted with Trizol (Invitrogen) and submitted to the UT Southwestern Microarray Core Facility for Bioanalyzer (Agilent) quality evaluation, labeling, hybridization, and scanning using Affymetrix chips. Samples were normalized and compared using dChip software 2010 as described.⁴⁴ Change was specified as significant if the lower 90% confidence bound of fold change was greater than or equal to 2 and less than or equal to -2, and the absolute difference of the mean expression was greater than 100. Gene-enrichment analysis of upregulated genes was determined by a modified Fisher Exact P-Value and presented as the -log(P-value) as described.⁴⁵ Unsupervised hierarchical clustering of genes was performed with Cluster 3.0 software centroid linkage using default options.⁴⁴ The gene list for cluster analysis consisted of all dysregulated genes from each sample.

Transfections/Luciferase Assays. HCN cells were electroporated with pGL3-Bex1 promoter plasmid and pcDNA3.1-FLAG-GPR68 plasmids. After electroporation, HCN cells were seeded into a 96-well plate at the concentration of 50 000 cells/well in growth medium (DMEM/F12 containing 1× N2 and FGF-2). Twenty-four hours after electroporation, cells were treated with either DMSO or Isx in differentiation medium (DMEM/F12 containing 1× N2) at increasing concentrations (0.78, 1.56, 3.13, 6.25, 12.5, 25, 50 µM). Twenty-four hours after Isx treatment, medium was removed and cells were lysed with luciferase substrate solution (Invitrogen) for 5 min at room temperature and the luciferase activity was read with FLUOstar OPTIMA plate reader.

Immunohistochemistry. After tribromoethanol/Avertin deep anesthesia, mice were transcardially perfused with ice-cold PBS, followed by 4% PFA in PBS. Whole mouse brain was dissected and postfixed in 4% PFA in PBS overnight. Brains were embedded in 3% agarose in PBS and sectioned on a Vibratome at 50 µm intervals. Serial sections containing the entire hippocampus were collected sequentially in a 12-well plate. For side-by-side comparisons of Isx versus vehicle treated brains, anatomically similar sections were chosen for immunohistochemistry and stereological quantification. Brain sections were permeabilized and blocked in PBS containing 10% normal donkey serum and 0.3% Triton X-100 at room temperature for at least 1 h. Brain section were incubated with primary antibodies at 4 °C overnight, washed with PBS containing 0.3% Triton X-100, and then incubated with corresponding secondary antibodies diluted in 5% normal donkey serum in wash buffer for 2 h at room temperature.

Primary antibodies used in this study were chicken anti-GFP (Aves, 1:500), rabbit anti-MCM2 (Cell Signaling, 1:100), mouse anti-NeuN (Chemicon, 1:500), rabbit anti-GFAP (DAKO, 1:1000), and fluorescent tag-conjugated secondary antibodies for confocal microscopy (Jackson ImmunoResearch, 1:200) and biotinylated antispecies IgG with avidin-peroxidase/diaminobenzidine (DAB) staining for stereology analysis (Jackson ImmunoResearch, 1:500).

Confocal Microscopy and Stereology. For protein expression and localization of immunohistochemical markers, a Zeiss LSM 510 confocal multicolor microscope with argon 488, helium 543, and helium 633 lasers was used. To determine specific marker-expression cell number, we used an unbiased stereological approach (Stereoinvestigator, Microbrightfield) for quantification with an Olympus BX51 system Microscope with a MicroFIRE A/R camera. A series of sections harboring the entire hippocampus in its rostrocaudal extension was immunostained with primary and horseradish peroxidase-coupled secondary antibodies, developed with DAB, and then visualized under a light microscope. Quantifications were performed under the 100× immersion lens using an unbiased counting frame. The GFP-stained sections were also used for counting type 1-NSC number according to characteristic cell morphology, a highly arborized dendritic tree extending into the DG molecular layer.²¹ Nissl stained sections were used for DG volume quantification and estimated with Cavalier Estimator program as described.²¹

Tamoxifen Treatment in Vivo. Tamoxifen (Sigma) was prepared as described previously.²¹ Briefly, tamoxifen was dissolved in sunflower oil (Sigma) and ethanol mixture (9:1) at a concentration of 6.7 mg/mL and injected ip at a final concentration of 0.83 mg/kg twice a day for 2 consecutive days.

Behavior Testing. Morris water maze and fear conditioning behavior testing was done in the Rodent Behavior Core of the Department of Psychiatry at UT Southwestern Medical Center. Briefly, for water maze, 1 week old mouse pups were injected ip with Isx (16 mg/kg/day) ($n = 16$) or vehicle control ($n = 13$) each day for a total of 9 weeks, allowed to "recover" from Isx therapy for 2 weeks, and then subjected to 1 week of water maze training followed by testing.

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Author Contributions

J.W.S. conceived, designed, and directed the experiments with assistance from S.C.G., X.L., S.M.L., J.L.R., C.P.Y., and Q.J.Z.; J.W.S. wrote the manuscript; S.C.G. did the bioinformatics and made the figures and graphics.

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Notes

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