Effects of Isx-9 and stress on adult hippocampal neurogenesis: Experimental considerations and future perspectives

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ABSTRACT
The development of synthetic small molecules capable of promoting neuronal fate in stem cells is a promising strategy to prevent the decline of hippocampal function caused by several neurological disorders. Within this context, isoxazole 9 (Isx-9) has been shown to strongly induce cell proliferation and neuronal differentiation in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG), while also improving hippocampal function in healthy mice. We have recently demonstrated that Isx-9 is able to restore normal neurogenesis levels after procedural stress. Here, we further discuss these findings highlighting the importance of including a naïve group in studies investigating the effects of either restraint stress or mild chronic unpredictable stress (CUS) on adult hippocampal neurogenesis.

KEYWORDS
Hippocampus; Isx-9; mild chronic unpredictable stress; neurogenesis; restraint stress

Hippocampal neurogenesis appears to have important roles in the adult brain, particularly regarding cognitive and affective processes such as episodic and spatial memory, as well as mood regulation. Adult neurogenesis recapitulates the process of neuronal development that occurs in embryonic stages, where progenitor cells undergo a regulated process of differentiation as well as morphological and physiological maturation. It is well known that a broad range of environmental, physiological, and pathological stimuli can influence the various stages of hippocampal neurogenesis, including neural stem cell proliferation, neuronal differentiation and survival, as well as functional integration into pre-existing neuronal circuitries. Given that a decline in adult hippocampal neurogenesis may contribute to brain dysfunction in several neuropathologies, the development of strategies that stimulate adult neurogenesis may be of therapeutic value for the mitigation of these neuropathological processes.

The development of synthetic small molecules that can mimic the effects of growth factors and/or activate signaling cascades that normally regulate the different stages of the neurogenic process is an attractive means to enhance neurogenesis, and promote cognitive function in the healthy brain. The small molecule compound Isoxazole-9 [Isx-9; N-cyclopropyl-5-(thiophen-2-yl)isoxazole-3-carboxamide], has been shown to stimulate neuronal differentiation in vitro, and in vivo in healthy mice. These effects appear to be mediated by the activation of myocyte enhancer factor-2 (Mef-2) transcription factors, which have been implicated in the regulation of neuronal differentiation. The regulation of Mef-2 transcription factors by Isx-9 seems to involve N-methyl-D-aspartate receptor (NMDAR)-mediated excitatory signaling, which leads to phosphorylation of histone deacetylase 5 (HDAC5) and consequently, de-repression of Mef-2 and other neuronal genes such as the neurogenic differentiation (NeuroD) gene, an helix-loop-helix transcription factor known to be involved in neuronal maturation in the hippocampal dentate gyrus (DG). It is also noteworthy that Isx-9-induced activation of endogenous neuronal gene programs is able to inhibit differentiation of neural progenitors into other cell types, even in the presence of strong gliogenic signals.
We have recently shown that Isx-9 is able to protect adult rats from the decline in hippocampal cell proliferation and differentiation that normally result from procedural stress. In the present work we seek to determine if Isx-9 will have therapeutic benefits for animals exposed to either acute or mild chronic unpredictable stress (CUS) both of which have been reported to impair hippocampal neurogenesis and related behaviors.

To determine whether Isx-9 was able to potentiate adult hippocampal neurogenesis in models of stress, we first evaluated its effects in animals that were exposed to repeated restraint stress (45 min/day, 7 days). Our results showed that intraperitoneal (i.p.) administration of Isx-9 (20 mg/kg, 7 days) induces an increase in cell proliferation, as assessed by immunohistochemistry for the endogenous cell cycle marker Ki-67 \([F(1,24) = 5.57, p < 0.05; \text{Fig. 1A}]\), in all animals. Interestingly, we failed to detect an effect of restraint stress on hippocampal cell proliferation \([F(1,24) = 1.72, p = 0.20; \text{Fig. 1A}]\). Similarly, NeuroD, a marker for neuronal commitment, was not impacted by restraint stress \([F(1,21) = 0.15, p = 0.71; \text{Fig. 1B}]\), although animals that received Isx-9 showed a significant increase in the number of NeuroD-labeled cells \([F(1,21) = 19.71, p < 0.01; \text{Fig. 1B}]\).

It is possible that the protocol of repeated restraint stress used was insufficient to produce significant stress. Therefore, we next tested the effects of mild CUS (exposure to 2 daily stressors for a period of 14 days) and Isx-9 treatment on hippocampal cell proliferation and neuronal differentiation. However, 14 days of mild CUS also failed to induce significant changes in cell proliferation, as assessed by immunohistochemistry for the exogenous marker 5-bromo-2’-deoxyuridine (BrdU) \([F(1,23) = 0.02, p = 0.88; \text{Fig. 2A}]\), and the endogenous cell cycle marker Ki-67 \([F(1,21) = 0.06, p = 0.81; \text{Fig. 2B}]\). Similarly, mild CUS also failed to have a significant effect on neuronal commitment, as assessed with immunohistochemistry.

**Figure 1.** Effects of 7 consecutive days of restraint stress (RS) (45 min/day) and/or Isx-9 treatment (20 mg/kg/day) on cell proliferation (as assessed with Ki-67 immunohistochemistry) (A) and neuronal commitment (as assessed with NeuroD immunohistochemistry) (B) in the dentate gyrus sub-region of the hippocampus of young adult male rats. Values are represented as mean ± SEM \((n = 6–8)\). *\(p < 0.05\) and **\(p < 0.01\) as compared with vehicle-treated animals. Representative photomicrographs of Ki-67- and NeuroD-labeled cells present in the hippocampal DG of control or RS-exposed rats (both treated with either vehicle or Isx-9) (C). Scale bar = 100 μm, scale bar (insets) = 10 μm.
against NeuroD \( [F(1,20) = 0.22, p = 0.65; \text{Fig. 2C}] \). Nevertheless, as in our restraint stress experiment, Isx-9 was able to induce a significant increase in cell proliferation and neuronal commitment in all groups (BrdU \( [F(1,23) = 23.91, p < 0.01; \text{Fig. 2A}] \), Ki-67 \( [F(1,21) = 27.53, p < 0.01 \text{ Fig. 2B}] \), and NeuroD \( [F(1,20) = 20.16, p < 0.01; \text{Fig. 2C}] \)).

Despite the fact we were unable to detect an effect of stress (either restraint stress or mild CUS) on hippocampal cell proliferation and neuronal commitment, these results support our previous work showing the pro-neurogenic properties of Isx-9 in rats.19 These findings are in agreement with previous studies, which have also failed to detect significant alterations in adult hippocampal neurogenesis following various types of stress paradigms (including acute restraint stress, acute or subchronic tailshock stress, and acute, subchronic, and chronic resident-intruder stress).21 Together, these results further demonstrate that not all stress paradigms are associated with decreased neurogenic capacity and that multiple complex factors may modulate the neurogenic response to stress.

Our results also highlight the need to take into account the potential impact of procedural stress in studies that evaluate the effects of both stress (such as restraint stress and mild CUS) and pro-neurogenic compounds (such as Isx-9) on adult hippocampal neurogenesis.19 Indeed, the fact that no significant differences in hippocampal cell proliferation and neuronal differentiation were noted between the groups submitted to either restraint stress or mild CUS (and daily injections of Isx-9 and/or BrdU) and the control group (not submitted
to stress but submitted to daily injections of Isx-9 and/or BrdU), may be due to the fact that daily experimental manipulations (i.e., animal handling and repeated i.p. injections), to which all groups were submitted to, could be acting as a potential stressor on hippocampal cell proliferation and neuronal commitment. This idea is in agreement with our previous study, where naïve animals showed an increase in hippocampal cell proliferation and neuronal commitment when compared with vehicle-treated animals (not exposed to stress). It has been shown in previous studies in rats that repeated handling can cause an increase in corticosterone levels and reduce responsiveness to both restraint stress and mild CUS. In rats, the first exposure to restraint stress results in an elevation of glucocorticoids, but animals quickly habituate over subsequent exposures, resulting in a decrease of the stress response. Conversely, it may also be that more severe models of restraint stress (2hrs/day as opposed to 45 min/day for 7 days) may be required to reliably produce significant changes in hippocampal neurogenesis. Here we attempted to use unpredictable stressors to reduce any potential habituation of the stress response to repeated restraint, using a protocol that produced deficits in hippocampal neurogenesis. This protocol failed to induce significant deficits in our hands, which may indicate that either the period of exposure to each of the stressors was too short or that procedural stress (due to repeated injections and daily manipulations) was confounding our results. Other studies have increased the exposure period to similar stressors for longer periods of time (i.e., more than 3 weeks). In addition, it is also worth pointing that there is evidence for sex differences with regards to stress responses, and since the present study only included male rats, we cannot exclude the possibility that a different response to the stress protocols used in this study could be observed in females.

Thus, while we can confirm the pro-neurogenic properties of Isx-9, further studies are warranted to determine any therapeutic potential for Isx-9 to reverse the impact of stress on the brain. Specifically, it will be important to investigate the pro-neurogenic properties of this molecule using more robust stress protocols such as those consisting of exposure to environmental stressors for an increased period of time (3 weeks or more) and/or following chronic administration of corticosterone. In addition, considering that Isx-9 is able to increase dendritic complexity in the hippocampus, future studies are also warranted to investigate whether this small synthetic molecule is able to restore other types of stress-related deficits in structural and functional (i.e., synaptic) plasticity such as dendritic atrophy and suppressed hippocampal long-term potentiation (LTP), as well as stress-induced alterations in hippocampal-dependent behaviors.

The current work showed that it is also important to consider different vehicles and/or routes of drug administration/delivery and how they may influence results. In both our previous study and the current one, Isx-9 was dissolved in 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), a vehicle commonly used to solubilize and stabilize drugs. HP-β-CD is considered well-tolerated when compared with other vehicles. However, some studies have raised concerns regarding its safety when chronically administered at high doses. Thus, while it is likely that procedural stress has an impact, we cannot rule out the possibility that vehicle-induced toxicity might underlie, at least in part, the impairment in cell proliferation and/or neuronal commitment that we observed in vehicle-treated (non-stressed) animals. Again, the potential confounding effects associated with vehicle administration further highlight the importance of including a naïve group (not treated with vehicle) when studying adult hippocampal neurogenesis.

In conclusion, given the importance of adult hippocampal neurogenesis to the modulation of cognitive and affective processes and its contribution to the pathophysiology of several neurologic conditions, Isx-9 shows promise as an agent that may reverse deficits in hippocampal-dependent processes. Future studies will elucidate whether this molecule, or other synthetic compounds structurally and functionally related to Isx-9 with extended half-lives (thus reducing the frequency of administration) and better solubility, can restore neurochemical, morphological and behavioral impairments in animal models of neurodegenerative diseases and psychiatric disorders. Within that context, it is worth pointing that a very recent study has shown that systemic Isx-9 treatment can prevent compulsive-like context-driven methamphetamine relapse in Wistar rats and this is associated with modulation of hippocampal structural plasticity and neurogenesis. In addition, given that its pro-neurogenic properties have been correlated with an
improvement in learning and memory in healthy mice, the possibility that Isx-9 may act as a nootropi- 
ical agent should also be further investigated.

**Methods**

**Animals and treatments**

Seventy-day-old male Sprague–Dawley rats (300 g; Charles River Laboratories, Montreal, Canada) were housed in isolation and maintained at 21 °C on a 12-h light/dark cycle, with lights on at 7:00 AM. All rats were given *ad libitum* access to a regular chow diet (Lab Diets 5001; LabDiet, Richmond, IN, USA) and tap water. All protocols were performed in accordance with the Canadian Council for Animal Care and were approved by the Animal Care Committee of the University of Victoria.

Isx-9 was prepared according to the method of Schneider et al. (2008) with minor modifications. Isx-9 solution was prepared using HP-β-CD (Sigma, St. Louis, MO, USA) as vehicle with a final concentration of 4 mg/ml Isx-9 and 30% (w/v) vehicle in sterile milliQ-purified H2O (Millipore Corp., Billerica, MA, USA). Vehicle or Isx-9 (20 mg/kg) were injected intra-peritoneally (i.p.) once daily for 7 days (restraint stress) or 14 days (CUS). Rats submitted to the mild CUS protocol received 2 doses (12 h apart; at 8:00 AM and 8:00 PM) of BrdU (150 mg/kg/injection; i.p.) on the 15th day. On the 16th day, all animals were sacrificed by transcardial perfusion with 0.9% NaCl followed by 4% paraformaldehyde and their brains removed and processed for immunohistochemical analyses of hippocampal cell proliferation and number of immature neuroblasts.

**Restraint stress**

Restraint stress was performed 2 h following Isx-9 administration. Each animal was individually placed in a clear plastic tube (diameter: 7 cm; length: 19 cm) under a bright light as described previously, for a period of 45 min. Restraint stress was conducted in a procedure room separated from the housing area and repeated every day for a period of 7 consecutive days, always at the same time of the day.

**Mild Chronic unpredictable stress (CUS)**

The mild CUS paradigm used in the present study was conducted over a period of 14 days, as described previously. Briefly, 2 distinct stressors were used in a variable sequence at random times of the day. The following stressors were used: vinegar water (drinking bottle replaced with a solution of water containing 10% vinegar for 6 h), tail clip (an hair clip was placed at the base of the animal’s tail for 10 min), strobe light (for 2 h), predator odor (cotton ball containing 2.5 ml of fox urine was placed in front of each cage for 6 h), tilted cage (cages were tilted on a 45-degree angle for 6 h), damp bedding (400 ml of tap water was placed in the bottom of the cage for 4 h), predator sounds (a record of predator sounds was played for 1 h), altered light cycle (lights off for 6 h during light phase), open field (for 5 min), novel stimulus (a toy was placed in the home cage for 5 min), and white noise (for 4 h). Animals submitted to mild CUS were maintained in a separated room from the vehicle-injected group, to discard the influence of stress odor.

**Immunohistochemistry**

Brains were left in 4% paraformaldehyde overnight at 4 °C and then transferred to 30% sucrose. Following saturation in sucrose, serial coronal sections were obtained on a vibratome (Leica VT1000S, Nussloch, Germany) at 30 μm thickness. Sections were collected in a 1/6 section-sampling fraction and stored in a cryoprotectant solution [0.04 M Tris-buffered saline (TBS), 30% ethylene glycerol, 30% glycerol] at 4 °C.

All immunohistochemistry protocols were conducted as described previously. Briefly, for BrdU staining, one series of free-floating brain sections was thoroughly rinsed and subsequently incubated in 2 N HCl at 65 °C for 30 min to denature the DNA. Sections were then pre-incubated for 1 h in normal horse serum (NHS) and 0.25% Triton X-100 and then incubated for 48 h at 4 °C with a mouse monoclonal antibody against BrdU (1:60, M0744; Dako, Glostrup, Denmark) followed by an incubation with a biotinylated horse anti-mouse IgG secondary antibody (1:200, BA-2001; Vector Laboratories, Burlingame, CA, USA) for 2 h.

An adjacent series of brain sections was processed for detection of the endogenous proliferative marker Ki-67. Briefly, after thorough rinsing, sections were incubated 2 times for 5 minutes each in 10 mM sodium citrate buffer (pH = 6.0) at 95 °C to fully denature the DNA. After quenching with 3% H2O2/
10% methanol for 15 min and pre-incubating with 5% normal goat serum (NGS) for 1 h at room temperature, the sections were incubated for 48 h at 4 °C with a rabbit polyclonal primary antibody against Ki-67 (1:500, VP-K451; Vector Laboratories). After thorough rinsing, the sections were incubated for 2 h with the secondary antibody (biotin-conjugated goat anti-rabbit IgG, 1:200, BA-1000; Vector Laboratories) in 5% blocking solution.

Finally, an additional series of brain sections was processed for NeuroD. Briefly, after quenching and pre-incubation with NHS at room temperature, the sections were incubated for 48 h at 4 °C with a goat anti-NeuroD primary antibody (1:200, SC-1084; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were then incubated for 2 h with the secondary antibody (biotin-conjugated horse anti-goat IgG, 1:200, BA-9500; Vector Laboratories) in 5% blocking solution.

For all immunostainings, the bound antibodies were visualized using an avidin-biotin-peroxidase complex system (Vectastain ABC Elite kit PK4000; Vector Laboratories) with 2,2-diaminobenzidine (DAB, DAB kit SK 4100; Vector Laboratories) as the chromogen. The sections were mounted onto 2% gelatin-coated microscope slides, dehydrated in a series of ethanol solutions of increasing concentrations followed by a 5 min incubation with a xylene substitute (CitriSolv; Fisher Scientific, Pittsburgh, PA, USA), and coverslipped with Permount mounting medium (Fisher Scientific).

**Morphological quantification by conventional microscopy**

All morphological analyses were performed on coded slides with the experimenter blinded to the identity of the brain sections (i.e., the animal), using an Olympus BX51 microscope equipped with 10 ×, 40 ×, and 100 × objectives (Olympus, Center Valley, PA, USA). The total number of BrDU-, Ki-67- or NeuroD-immunopositive cells present in the SGZ of the entire DG (from Bregma -2.30 to -6.04; approximately 20 coronal sections per brain); were quantified by manually counting all DAB-positive cells present with in 2–3 cell diameters of the SGZ. Results were expressed as the total number of labeled cells in the DG hippocampal sub-region of each individual brain by multiplying the average number of labeled cells/DG section by the total number of 30-μm-thick sections obtained from that respective animal and containing the entire DG (125 sections).

**Statistical analyses**

Statistical analyses were performed using the Statistica 7.1 analytical software (StatSoft Inc., Tulsa, OK, USA). Differences among experimental groups were compared using analysis of variance (ANOVA) followed by Tukey’s multiple range post-hoc test when appropriate. A p value of less than 0.05 was considered to be statistically significant.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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