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# ISX-9 CAN POTENTIATE CELL PROLIFERATION AND NEURONAL COMMITMENT IN THE RAT DENTATE GYRUS

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**Abstract—Adult hippocampal neurogenesis can be modulated by various physiological and pathological conditions, including stress, affective disorders, and several neurological conditions. Given the proposed role of this form of structural plasticity in the functioning of the hippocampus (namely learning and memory and affective behaviors), it is believed that alterations in hippocampal neurogenesis might underlie some of the behavioral deficits associated with these psychiatric and neurological conditions. Thus, the search for compounds that can reverse these deficits with minimal side effects has become a recognized priority. In the present study we tested the pro-neurogenic effects of isoxazole 9 (Isx-9), a small synthetic molecule that has been recently identified through the screening of chemical libraries in stem cell-based assays. We found that administration of Isx-9 for 14 days was able to potentiate cell proliferation and increase the number of immature neurons in the hippocampal DG of adult rats. In addition, Isx-9 treatment was able to completely reverse the marked reduction in these initial stages of the neurogenic process observed in vehicle-treated animals (which were submitted to repeated handling and exposure to daily intraperitoneal injections). Based on these results, we recommend that future neurogenesis studies that require repeated handling and manipulation of animals should include a naïve (non-manipulated) control to determine the baseline levels of hippocampal cell proliferation and neuronal differentiation. Overall, these findings demonstrate that Isx-9 is a promising synthetic compound for the mitigation of stress-induced deficits in adult hippocampal neurogenesis. Future studies are thus warranted to evaluate the pro-neurogenic properties of Isx-9 in animal models of affective and neurological disorders associated with impaired hippocampal structural plasticity.**


**Key words: adult hippocampal neurogenesis, cell proliferation, isoxazole 9 (Isx-9), immature neurons, stress.**

## INTRODUCTION

Neurogenesis in the adult brain results in the production of new neurons from a pool of progenitor cells in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG). This form of structural plasticity can be modulated by a number of factors that include: exercise (van Praag et al., 1999a, 1999b); environmental enrichment (Kempermann et al., 1997); learning (Gould et al., 1999); and stress (Gould et al., 1998) and is thought to play a role in certain aspects of cognition, including hippocampal-dependent learning and memory (Gould, 1999; Kempermann, 2002) as well as affective (i.e., anxiety- and depressive-like) behaviors (Bannerman et al., 2004; Degroot and Treit, 2004; Engin and Treit, 2007).

The hippocampus is one of the most malleable structures in the brain, and it can respond to external stimuli through structural and functional neuroplastic adaptations, a feature that can lead to beneficial or deleterious alterations in brain functioning (Sapolsky, 2003; McEwen and McEwen, 2008). Since this brain region has one of the highest concentrations of receptors for glucocorticoids, the hippocampus is particularly vulnerable to the effects of stress, which in turn may have an inhibitory effect on hippocampal plasticity, namely adult neurogenesis (Kim and Diamond, 2002). Indeed, several studies have demonstrated that chronic exposure of rodents to corticosterone (CORT) inhibits hippocampal cell proliferation and differentiation (Wong and Herbert, 2006; Murray et al., 2008; Brummelte and Galea, 2010), an effect that seems to correlate with cognitive dysfunction (Drapeau et al., 2003; Monje and Dietrich, 2012) and the pathogenesis of depressive disorders (Gregus et al., 2005; Zhao et al., 2008). Compounds that stimulate the generation of endogenous neural progenitors in the hippocampus may counteract the hippocampal neuronal

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*Abbreviations:* BrdU, 5-bromo-2′-deoxyuridine; CORT, corticosterone; DAB, 2,2-diaminobenzidine; DG, dentate gyrus; GR, glucocorticoid receptors; Isx-9, isoxazole 9; Mef2, myocyte enhancer factor-2; MR, mineralocorticoid receptors; NHS, normal horse serum; PFA, paraformaldehyde; SGZ, subgranular zone; TBS, Tris-buffered saline.

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loss and/or the development of cognitive deficits that are associated with certain neuropathological conditions. Thus, neurogenic drugs present potential therapeutic value for treatment of these disorders (Taupin, 2011).

Screening analysis of chemical libraries in stem cell-based assays has identified several pro-neurogenic small molecules with therapeutic potential (Schneider et al., 2008; Pieper et al., 2010; Wurdak et al., 2010). Within this context, isoxazole 9 [Isx-9; *N*-cyclopropyl-5-(thiophen-2-yl)isoxazole-3-carboxamide] was reported to influence stem-cell fate both *in vitro* and *in vivo*, namely by inducing a robust increase in neuronal differentiation. This effect seems to occur through the modulation of myocyte enhancer factor-2 (Mef2) (Schneider et al., 2008; Petrik et al., 2012), a family of transcription factors that plays a key role in the activation of genetic programs that control cell differentiation, proliferation, morphogenesis, survival and apoptosis (Potthoff and Olson, 2007).

In a previous *in vivo* study, it was demonstrated that Isx-9 crosses the blood–brain barrier and is a safe pharmacological approach to increase neurogenesis in the SGZ of the hippocampal DG in adult mice (Petrik et al., 2012). However, despite being a promising synthetic neurogenic compound, our current knowledge on its *in vivo* properties is still limited. Thus, in the present study we confirmed and expanded the results initially reported by Petrik et al. (2012) and demonstrated the neurogenic effects of Isx-9 on cell proliferation and neuronal commitment in the adult rat hippocampal DG following repeated exposure to a commonly used laboratory procedure that is potentially associated with increased levels of stress (repeated intraperitoneal, i.p., injections). We found that Isx-9 was able to reverse the reduction in hippocampal cell proliferation and neuronal commitment found in vehicle-treated animals, further highlighting the neurogenic properties of this compound. Thus, the development of synthetic molecules structurally and functionally related to Isx-9 that possess a greater half-life than this compound (thus reducing the frequency of administration) may prove to have therapeutic value for the treatment of conditions associated with an increase in stress levels.

## EXPERIMENTAL PROCEDURES

### Synthesis of Isx-9

Isx-9 was prepared according to the method of Schneider et al. with minor modifications (Fig. 1). All reactions were performed in oven- or flame-dried glassware, under a positive pressure of argon, unless otherwise indicated. Organic solutions were concentrated between 35 and 40 °C by rotary evaporation under vacuum. All reagents were used as received from commercial suppliers unless otherwise indicated. Commercial solvents were used as received with the following exceptions. Dichloromethane, tetrahydrofuran and toluene were dried by passage through a column of alumina in a commercial solvent purification system. Methanol was dried over activated 4 Å MS. $^{1}$H chemical shifts are reported in parts per million (ppm, $\delta$ scale) downfield from tetramethylsilane, and are referenced to residual protium in the NMR solvent ($CDCl_3$: $\delta$ 7.26; $(CD_3)_2CO$: $\delta$ 2.05). Likewise, $^{13}$C chemical shifts are referenced to the carbon resonances of the solvent ($CDCl_3$: $\delta$ 77.16; $(CD_3)_2CO$: $\delta$ 29.85). Infrared spectra were collected using an FT-IR spectrometer.

*(Z)-Methyl 4-hydroxy-2-oxo-4-(thiophen-2-yl)but-3-enoate (**1**).* To a stirred solution of 2-acetylthiophene (2.57 mL, 23.8 mmol) and dimethyloxylate (3.72 g, 31.5 mmol) in 125 mL dry toluene was added 30 mL of a solution of *t*-BuOK (1.0 M in THF, 30.0 mmol) dropwise at room temperature. Once addition was complete, the resultant slurry was stirred overnight at room temperature (~16 h). The reaction was quenched with 60 mL of 1 N HCl (aq) and the two phases were separated. The organic layer was washed with water and then brine, dried over anhydrous $Na_2SO_4$, filtered and concentrated *in vacuo*. The crude product was recrystallized from hexanes to afford compound **1** as a bright yellow powder in 86% yield. $^{1}$H NMR (300 MHz, $CDCl_3$): $\delta$ 7.85 (dd, $J$ = 3.8, 1.2 Hz, 1H), 7.72 (dd, $J$ = 5.0, 1.2 Hz, 1H), 7.19 (dd, $J$ = 5.0, 3.8 Hz, 1H), 6.93 (s, 1H), 3.94 (s, 3H); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 186.2 (C), 164.6 (C), 162.7 (C), 142.2 (C), 135.4 (CH), 132.8 (CH), 128.9 (CH), 99.8 (CH), 53.3 ($CH_3$); IR (KBr, $cm^{-1}$) 3114 (m), 3092 (m), 3076 (w), 2964 (m), 1751 (m), 1738 (s), 1611 (s), 1426 (s), 1250 (s), 1228 (s), 1121 (s), 1063 (m), 968 (m), 826 (m), 776 (s), 732 (s), 682 (m).

*Methyl 5-(thiophen-2-yl)isoxazole-3-carboxylate (**2**).* *N*-Hydroxylamine hydrochloride (1.67 g, 24.0 mmol) was added to a stirring solution of ketone **1** (4.21 g, 19.8 mmol) in 100 mL of dry methanol and the reaction mixture was then heated to 60 °C for 12 h. The resultant solution was then concentrated under reduced pressure and the precipitated beige solid was resuspended in 100 mL of water. The resulting slurry was stirred at room temperature for 1 h before the product was collected by filtration through a sintered glass fritted funnel to yield compound **2** as a beige solid in quantitative yield. $^{1}$H NMR (300 MHz, $CDCl_3$): $\delta$ 7.56 (dd, $J$ = 3.8, 1.2 Hz, 1H), 7.50 (dd, $J$ = 5.0, 1.2 Hz, 1H), 7.14 (dd, $J$ = 5.1, 3.7 Hz, 1H), 6.78 (s, 1H), 3.99 (s, 3H); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 167.0 (C), 160.4 (C), 156.8 (C), 129.0 (CH), 128.4 (CH), 128.3 (C), 128.0 (CH), 99.7 (CH), 53.1 ($CH_3$); IR (KBr, $cm^{-1}$) 3134 (s), 3116 (m), 3083 (w), 2954 (m), 1731 (s), 1593 (s), 1476 (s), 1452 (s), 1275 (s), 1250 (s), 1138 (s), 1004 (s), 930 (s), 853 (s), 810 (s), 779 (s), 703 (s).

*5-(Thiophen-2-yl)isoxazole-3-carboxylic acid (**3**).* An aqueous solution of LiOH (1.0 M, 140 mL) was added to a stirred solution of ester **2** (16.9 g, 80.6 mmol) in 90 mL of THF and reacted at 50 °C for 3 h. The reaction mixture was cooled to room temperature and the aqueous layer was acidified to pH 1 (with 3 M HCl) and then extracted with ethyl acetate (2 × 100 mL). The combined organic extracts were washed with brine, dried over anhydrous $Na_2SO_4$, filtered and concentrated *in vacuo* to afford compound **3** as a yellow crystalline solid in 71% yield. $^{1}$H NMR (300 MHz, $(CD_3)_2CO$): $\delta$ 7.79 (dd, $J$ = 5.1, 1.0 Hz, 1H), 7.76 (dd, $J$ = 3.5, 1.2 Hz, 1H), 7.26 (dd, $J$ = 5.1, 3.7 Hz, 1H), 7.06 (s, 1H);