

# **Role for Neuronal Nitric Oxide Synthase in Cannabinoid-induced Neurogenesis**

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tetrahydrocannabinol (THC), *N*-methyl-D-aspartate (NMDA), nitric oxide (NO), (+)-[2, 3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone (R(+)-Win 55212-2), nitric oxide synthase (NOS), neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), 7-nitroindazole (7-NI), dentate gyrus (DG), subventricular zone (SVZ), subgranular zone (SGZ), bromodeoxyuridine (BrdU), doublecortin (Dcx)

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## Abstract

Cannabinoids, acting through the CB1 cannabinoid receptor (CB1R), protect the brain against ischemia and related forms of injury. This may involve inhibiting the neurotoxicity of endogenous excitatory amino acids and downstream effectors, such as nitric oxide (NO). Cannabinoids also stimulate neurogenesis in the adult brain through activation of CB1R. Because NO has been implicated in neurogenesis, we investigated whether cannabinoid-induced neurogenesis, like cannabinoid neuroprotection, might be mediated through alterations in NO production. Accordingly, we measured neurogenesis in dentate gyrus (DG) and subventricular zone (SVZ) of CB1R-knockout (CB1R-KO) and wild-type mice, some of whom were treated with the cannabinoid agonist (+)-[2, 3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone (R(+)-Win 55212-2) or the NO synthase (NOS) inhibitor 7-nitroindazole. NOS activity was increased by ~25%, whereas bromodeoxyuridine (BrdU) labeling of newborn cells in DG and SVZ was reduced by ~50%, in CB1R-KO compared to wild-type mice. 7-NI increased BrdU labeling in both DG and SVZ, and to a greater extent in CB1R-KO than in wild-type mice. In addition, R(+)-Win 55212-2 and 7-NI enhanced BrdU incorporation into neuron-enriched cerebral cortical cultures to a similar maximal extent, and in non-additive fashion, consistent with a shared mechanism of action. Double-label confocal microscopy showed coexpression of BrdU and the neuronal lineage marker doublecortin (Dcx) in DG and SVZ of untreated and 7-NI-treated CB1R-KO mice, and 7-NI increased the number of Dcx- and BrdU/Dcx-immunoreactive cells in SVZ and DG. Thus, cannabinoids appear to stimulate adult neurogenesis by opposing the anti-neurogenic effect of NO.

## Introduction

Cannabinoids, which include naturally occurring plant-derived compounds (e.g.,  $\Delta^9$ -tetrahydrocannabinol, or THC), endogenous signaling molecules found in animal brains (e.g., anandamide and 2-arachidonoylglycerol), and synthetic drugs (e.g., R(+)-Win 55212-2)), act on receptors in the brain to modify neuronal function. In addition to their effects on normal physiological functions such as blood pressure, immunity, pain perception, appetite and cognition, cannabinoids can also regulate the severity of brain injury.

We reported previously that administration of cannabinoids acting on the CB1 cannabinoid receptor (CB1R) reduces neuronal death from cerebral ischemia (Nagayama et al., 1999). Consistent with this finding, stroke size is increased in mice with genetic deletion of CB1R (Parmentier-Batteur et al., 2002). Although the molecular mechanisms that underlie cannabinoid-induced protection from brain ischemia are not fully understood, one possibility is that cannabinoids act by suppressing excitotoxic signaling cascades, which have been implicated in ischemic brain injury. Supporting evidence includes the ability of cannabinoids to inhibit excitatory neurotransmission (Shen et al., 1996) and to block the neurotoxicity of excitatory amino acids like *N*-methyl-D-aspartate (NMDA) (Shen and Thayer, 1998). A principal signaling module through which NMDA operates to produce its toxic effects is thought to involve the calcium-dependent activation of neuronal nitric oxide synthase (nNOS) and the resulting production of NO and metabolites such as peroxynitrite (ONOO<sup>-</sup>) (Dawson et al., 1991). It is, therefore, notable that cannabinoids reduce NMDA- and NO-mediated neurotoxicity in retina (El-Remessy et al., 2003) and brain (Kim et al., 2005).

Cannabinoids also stimulate adult neurogenesis, or the generation of new neurons from endogenous precursors in the dentate gyrus (DG) and subventricular zone (SVZ) of adult brain (Jiang et al., 2005; Jin et al., 2004c; Rueda et al., 2002). This effect, like protection from cerebral ischemia, appears to arise through activation of CB1R, because it is diminished in CB1R-knockout (CB1R-KO) mice (Jin et al., 2004c). Of interest, signaling by NO has been implicated in neurogenesis as well. Studies with NO-donor or NOS-inhibitor drugs have shown variable effects (Cheng et al., 2003; Matarredona et al., 2004; Moreno-Lopez et al., 2004; Packer et al., 2003; Zhang et al., 2001; Zhu et al., 2003), probably because they differ in their specificities for NOS isoforms, and since nNOS, endothelial NOS (eNOS) and inducible NOS (iNOS) affect neurogenesis differently. This is evident from studies that employ isoform-specific NOS-knockout mice, which show that nNOS activity inhibits (Packer et al., 2003; Sun et al., 2005), whereas iNOS (Zhu et al., 2003) and eNOS (Reif et al., 2004) promote neurogenesis.

In view of these findings, we asked whether in neurogenesis as in stroke, the biological effect of cannabinoids might be mediated through their antagonism of nNOS-derived NO. To address this question, we measured neurogenesis in DG and SVZ of CB1R-KO (Ledent et al., 1999) and wild-type mice, some of whom were given a cannabinoid agonist (R(+)-Win 55212-2) or NOS inhibitor (7-nitroindazole) drug. We report that cannabinoids stimulate adult neurogenesis in DG and SVZ, at least partly by relieving the neurogenesis-inhibiting effect of nNOS.

## Methods

**Mice.** CB1R-KO and wild-type littermates were bred in-house from mice originally obtained from Dr. Catherine Ledent (Ledent et al., 1999). Mice weighing 30-35 g were

used for experiments and were euthanized by decapitation. Genotyping was performed as described in a previous publication (Parmentier-Batteur et al., 2002). Experiments were approved by local committee review and conducted according to NIH guidelines.

**NOS activity assay.** NOS activity was measured using a commercial nitrate/nitrite colorimetric assay kit (Catalog No. 78001; Cayman Chemical, Ann Arbor, MI), according to the manufacturer's instructions. Briefly, the cerebral cortex was dissected on ice, homogenized in PBS and centrifuged at  $10,000 \times g$  for 20 min. The supernatant was centrifuged at  $100,000 \times g$  for 30 min, then filtered through a 10-kDa molecular weight cutoff filter. Nitrate/nitrite was measured in triplicate in 40- $\mu$ l aliquots of the final filtrate on 96-well plastic microtiter plates. Absorbance was read at 570/620 nm using a Cytofluor series 4000 multiwell plate reader (Applied Biosystems, Foster City, CA).

**Western Blot analysis.** Cerebral cortex was homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 150 mM NaCl, 0.5% deoxycholic acid, 0.1% SDS, 100  $\mu$ g/ml PMSF, and 1  $\mu$ g/ml aprotinin. Cell lysates were centrifuged at  $12,000 \times g$  for 15 min, and 10  $\mu$ g of protein was electrophoresed on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were incubated overnight at 4°C with rabbit polyclonal anti-CB1 (1:1,000, Calbiochem, San Diego, CA) or mouse monoclonal anti- $\beta$ -actin (1:5,000, Sigma, St. Louis, MO), then washed with PBS containing 0.1% Tween-20 and reacted with anti-rabbit or anti-mouse secondary antibodies (1:2000, Santa Cruz Biotechnology). Immunoreactivity was detected with luminol reagent (PerkinElmer Life Sciences Inc., Boston, MA).

**Injection of BrdU and 7-NI.** Bromodeoxyuridine (BrdU, 50 mg/kg in saline), and 7-nitroindazole (7-NI, 50 mg/kg) or vehicle (DMSO:1,2-propanediol:distilled water::1:3:6), were administered i.p. twice daily at 8-h intervals, for 3 consecutive days. After 24 h, 50- $\mu$ m coronal brain sections were stained with mouse monoclonal anti-BrdU (2  $\mu$ g/ml;

Roche, Indianapolis, IN) and BrdU-positive cells in DG subgranular zone (SGZ) and SVZ were counted blindly in 5 sections per animal, spaced 200  $\mu\text{m}$  apart, under high power on a Nikon E800 microscope with a Magnifier digital camera.

**Cell Culture.** Cerebral cortical cultures enriched in cells of neuronal lineage were prepared from 16-day Charles River CD1 mouse embryos in Neurobasal medium containing 2% B27 supplement, 2 mM glutamate, and 1% each of penicillin and streptomycin. After 4 days, one-half of the medium was replaced with Neurobasal medium containing 2% B27, and experiments were conducted after 5 days *in vitro*. At this time, cultures were maintained for 24 hr in the presence of 50  $\mu\text{g}/\text{ml}$  of BrdU, and in the absence or presence of 100 nM R(+)-Win 55212-2, 100  $\mu\text{M}$  7-NI, or both drugs in combination.

**Immunohistochemistry.** Immunohistochemistry was performed on 50- $\mu\text{m}$  frozen coronal brain sections, corresponding to coronal coordinates interaural 8.7 to 10.2 mm, bregma -0.30 to bregma -1.2 mm (for SVZ), and interaural 4.48 to 5.86 mm, bregma -4.52 to bregma -3.14 (for DG SGZ). Sections were cut with a cryostat, fixed with 100% methanol for 10 min, incubated in 2 M HCl at 25°C for 1 h, and rinsed in 0.1 M boric acid (pH 8.5) at room temperature for 10 min. Sections were placed in 1%  $\text{H}_2\text{O}_2$  for 15 min, then in blocking solution (2% goat serum, 0.3% Triton X-100 and 0.1% BSA in PBS) for 2 h at room temperature, and incubated with 2  $\mu\text{g}/\text{mL}$  of mouse monoclonal anti-BrdU (Roche Applied Science, Indianapolis, IN; 1:200) and goat polyclonal anti-doublecortin (Dcx) (Santa Cruz Biotechnology; Santa Cruz, CA; 1:100) at 4°C overnight. Sections were washed with PBS, incubated for 2 h at 25°C with biotinylated goat-anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA; 1 :200), washed, and placed in avidin-peroxidase conjugate solution (Vector Laboratories) for 1 h. The horseradish peroxidase reaction was detected with 0.05% diaminobenzidine and 0.03%  $\text{H}_2\text{O}_2$ .

Processing was stopped with H<sub>2</sub>O and sections were dehydrated through graded alcohols, cleared in xylene, and coverslipped in permanent mounting medium (Vector Laboratories). For confocal microscopy, a Nikon PCM-2000 laser-scanning confocal microscope and Simple PCI imaging software (Compix, Cranberry Township, PA) were used.

**Statistics.** Data were analyzed using Student's t-test (single comparisons) or ANOVA with post hoc Student-Newman-Keuls tests (multiple comparisons). Differences having  $p < 0.05$  were considered significant.

## Results

If CB1R-mediated signaling provides an important constraint on nNOS, then NOS activity might be increased in CB1R-KO mice. Accordingly, we measured cerebral NOS activity (which includes the nitrate- and nitrite-generating capacity of all NOS isoforms) in CB1R-KO and wild-type mice. **Figure 1** shows that NOS activity was about 25% higher in knockouts, although expression of nNOS, which is not inducible, was not increased. These results are consistent with an inhibitory effect of CB1Rs on NOS activation.

As noted previously, nNOS inhibits (Packer et al., 2003; Sun et al., 2005), whereas iNOS (Zhu et al., 2003) and eNOS (Reif et al., 2004) promote, adult neurogenesis. Because deletion of the CB1R gene enhances nNOS activity, CB1R-KO mice might be expected to show reduced levels of basal neurogenesis. In addition, basal neurogenesis in these mice might be more readily stimulated by NOS inhibitors. We compared BrdU incorporation, an index of cell proliferation, in DG and SVZ of wild-type versus CB1R-KO mice (**Figure 2**). In line with our prior findings (Jin et al., 2004c), BrdU labeling in DG and SVZ of CB1R-KO mice was only about 50% of that observed in wild-type mice.



Next we examined the effect of 7-NI, a relatively selective nNOS inhibitor (Southan and Szabo, 1996). 7-NI (50 mg/kg i.p., given twice daily for 3 days) increased BrdU labeling in both DG and SVZ, and in both wild-type and CB1R-KO mice. However, the extent of this effect was greater in knockouts. Thus, 7-NI raised BrdU labeling in DG by only about 20% in wild-type, but by almost 80% in CB1R-KO, mice. In SVZ, the observed increases were about 40% and about 85%, respectively. Hence, the disinhibition of NOS activity in CB1R-KO mice is accompanied by enhanced sensitivity to the neurogenesis-promoting action of a NOS inhibitor.

We next examined the relative effects of a cannabinoid agonist and 7-NI on BrdU incorporation into cortical cultures enriched in cells of neuronal lineage (**Figure 3**). As reported previously (Jin et al., 2002), antibodies against the neuronal lineage markers,  $\beta$ III tubulin and Hu, each stain  $\geq 75\%$  of cells in these cultures. Cultures were treated for 24 h with concentrations of R(+)-Win 55212-2 (100 nM) or 7-NI (100  $\mu$ M) that produced maximal enhancement of BrdU incorporation. The extent of increase in BrdU labeling was similar for R(+)-Win 55212-2 and 7-NI, and the combination both drugs produced no further increase. These findings would be expected if R(+)-Win 55212-2 and 7-NI acted through the same pathway.

Although our in vivo studies focused on the two principal neuroproliferative zones in the adult brain, and our in vitro studies employed cultures consisting mostly of cells of neuronal lineage, BrdU labels dividing cells irrespective of cell type. Consequently, it was important to determine directly whether the BrdU-immunoreactive cells that we analyzed were neuronal. To address this question, brain sections through DG or SVZ of BrdU-treated mice were stained with antibodies against both BrdU and the early

neuronal marker, Dcx ( Francis et al., 1999; Gleeson et al., 199). Sections examined by double-label confocal fluorescence immunohistochemistry showed extensive coexpression of nuclear BrdU and cytoplasmic Dcx in DG and SVZ, in both untreated and 7-NI-treated CB1R-KO mice (**Figure 4a**). In addition, 7-NI treatment increased the number of Dcx-immunoreactive (**Figure 4b**) and of BrdU- and Dcx- doubly-immunoreactive (**Figure 4c**) cells in SVZ and DG, confirming that the number of new neurons was increased.

## Discussion

Understanding how adult neurogenesis is regulated is important because of its proposed role in brain repair and recovery from injury. Neurogenesis in DG, SVZ or both is enhanced in response to diverse cerebral insults, including animal models of stroke (Jin et al., 2001), Alzheimer's disease (Jin et al., 2004a) and Huntington's disease (Jin et al., 2005), and Alzheimer's disease (Jin et al., 2004b) and Huntington's disease (Curtis et al., 2003) in patients. These findings have led to the hypothesis that an increase in neurogenesis may be one of the adaptive mechanisms the brain uses to limit injury and resulting functional impairment. If this is the case, then even greater benefit might be achieved by therapeutic enhancement of neurogenesis,

A surprisingly broad range of drugs have been found to stimulate neurogenesis in vitro or in the brains of experimental animals in vivo. These appear to include cannabinoids, although some studies have yielded discrepant results, at least partly because some of the drugs employed were not entirely specific for cannabinoid receptors (discussed in (Jin et al., 2004c)). We reported previously (Jin et al., 2004c) that CB1R are expressed on neuronal precursor cells in the adult mouse brain, and that the number of BrdU-

labeled cells in DG and SVZ was reduced by about 50% in CB1R-KO compared to wild-type mice; as in the present study, most BrdU-labeled cells in these regions expressed *Dcx*, indicative of neuronal lineage. These data argue that CB1R-mediated activity normally exerts a pro-neurogenic influence. However, we did not examine the signaling mechanisms involved downstream of CB1R. In another study, Jiang and colleagues also found colocalization of CB1R with nestin, a marker for neuroepithelial precursors, some of which will undergo neuronal differentiation, in adult rat brain (Jiang et al., 2005). They showed that the synthetic cannabinoid HU210 stimulated proliferation of neuronal precursors in vitro, and that this effect was mediated through CB1R, guanine nucleotide binding ( $G_{i/o}$ ) proteins, and extracellular signal-regulated kinase. Further, long-term administration of HU210 to rats increased neurogenesis, measured by colocalization of BrdU with the mature neuronal marker protein NeuN, in DG. Cannabinoids appear to regulate the production of oligodendrocytes as well, as CB1R activation promotes the survival of oligodendrocyte precursors from rat brain, via  $G_{i/o}$ , phosphatidylinositol 3-kinase and Akt (Molina-Holgado et al., 2002).

In the present study, we found evidence implicating an interaction between cannabinoid and NO signaling in the regulation of adult neurogenesis. Thus, NOS activity was increased in CB1R-KO mice, and the associated reduction in neurogenesis was reversed by the nNOS-preferring NOS inhibitor, 7-NI. Moreover, the cannabinoid agonist R(+)-Win 55212-2 and 7-NI increased BrdU labeling in mouse brain cultures to a similar extent, and their effects were not additive. Finally, BrdU was localized to cells of neuronal lineage, defined by *Dcx* expression, in DG and SVZ.

Cannabinoid- and NO-signaling pathways have been linked in the past, although not in the setting of neurogenesis. For example, several studies have reported that

cannabinoids inhibit iNOS in macrophages or glia (Esposito et al., 2001; Jeon et al., 1996; Molina-Holgado et al., 2003; Waksman et al., 1999). Inhibition of iNOS could not account for cannabinoid-induced enhancement of neurogenesis, however, because iNOS is itself pro-neurogenic, and neurogenesis is defective in iNOS-KO mice (Zhu et al., 2003). Cannabinoids also inhibit nNOS activity. In cultured cerebellar granule cells, CB1R agonists blocked the stimulation of nNOS by K<sup>+</sup>-induced depolarization (Hillard et al., 1999), by reducing voltage-gated Ca<sup>2+</sup> influx, upon which nNOS activation depends.  $\Delta^9$ -THC protected rat retinal neurons from NMDA-induced, NO- and ONOO<sup>-</sup>-mediated toxicity in vivo, partly through interaction with CB1R (EI-Remessy et al., 2003). However, cannabidiol, which does not bind to CB1R, was also protective, and the mechanism employed by  $\Delta^9$ -THC was not further defined. Cannabinoids and NO also appear to interact in the regulation of body temperature. The hypothermic effect of  $\Delta^9$ -THC was abolished in nNOS-KO mice (Azad et al., 2001), and the combination of a cannabinoid agonist (R(+)-Win 55212-2) and a NOS inhibitor (*N*<sup>o</sup>-nitro-L-arginine methyl ester) reduced temperature in a synergistic fashion (Rawls et al., 2004).

These studies, and especially that of Hillard and colleagues (Hillard et al., 1999), suggest a plausible mechanism for the co-regulation of adult neurogenesis by cannabinoids and NO. CB1R activation inhibits Ca<sup>2+</sup> influx through neuronal (N- and P/Q-type) voltage-gated Ca<sup>2+</sup> channels (Twitchell et al., 1997), which uncouples membrane depolarization from nNOS activation. Ca<sup>2+</sup> signaling appears to be an important determinant of neuronal maturation (Owens and Kriegstein, 1998). However, the route of Ca<sup>2+</sup> influx exerts a strong influence on its downstream effects (Bading et al., 1993). Thus, although reduced Ca<sup>2+</sup> influx through N- and P/Q-type channels may explain how cannabinoids promote neurogenesis, proliferation of neuronal precursors in

the adult DG is also enhanced by increased  $\text{Ca}^{2+}$  influx through L-type voltage-gated or NMDA receptor-gated channels (Deisseroth et al., 2004). Further studies will be required to achieve a fuller understanding of the relationships between cannabinoids, signal transduction and neurogenesis.

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## **Footnotes**

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## Legends for Figures

**Fig. 1.** NOS activity (a) and nNOS expression (b) in wild-type (WT) and CB1R-KO mice. \*,  $p < 0.05$  compared to WT.

**Fig. 2.** Effect of in vivo inhibition of NOS with 7-NI on BrdU labelling in SVZ (a) and DG (b) of WT and CB1R-KO mice. 7-NI (50 mg/kg i.p.) or vehicle was administered together with BrdU, twice daily at 8 hr intervals for 3 consecutive days. \*,  $p < 0.05$  compared to same-genotype control.

**Fig. 3.** Effects of the CB1R agonist R(+)-Win 55212-2 (100 nM), the NOS inhibitor 7-NI (100  $\mu$ M), and both drugs in combination, on BrdU incorporation into wild-type cerebral cortical cultures at day 5 in vitro. (a) DAPI, blue; BrdU, green. (b) \*,  $p < 0.05$  compared to control.

**Fig. 4.** Effects of in vivo inhibition of NOS with 7-NI on Dcx expression in SVZ and DG of CB1R-KO mice. 7-NI (50 mg/kg i.p.) or vehicle was administered together with BrdU, twice daily at 8 hr intervals for 3 consecutive days. (a) xyz-plane confocal images of sections stained with antibodies against BrdU (red nuclei) and Dcx (green cytoplasm). (b) Dcx-immunopositive cell counts \*,  $p < 0.05$  compared to controls not given 7-NI. (c) Dcx- and BrdU- doubly-immunopositive cell counts. \*,  $p < 0.05$  compared to controls not given 7-NI.

Fig. 1

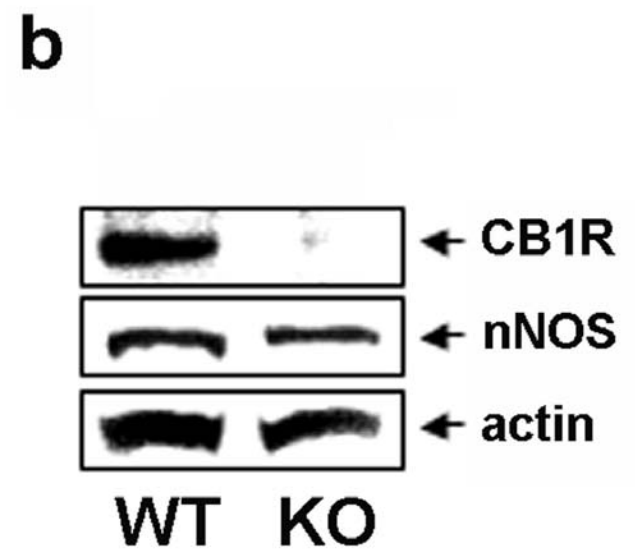
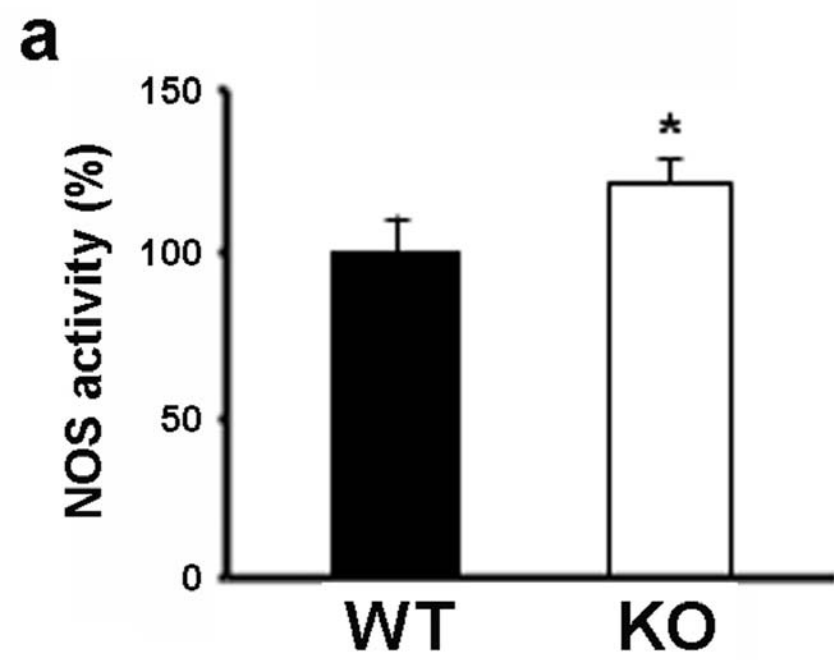


Fig. 2

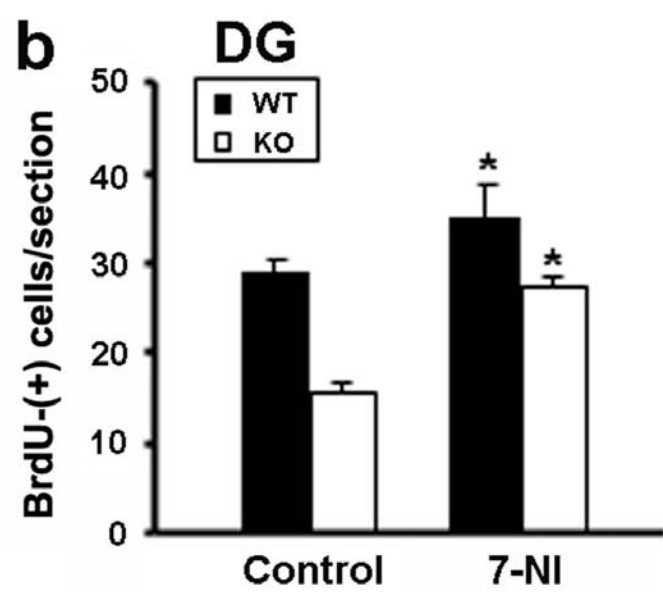
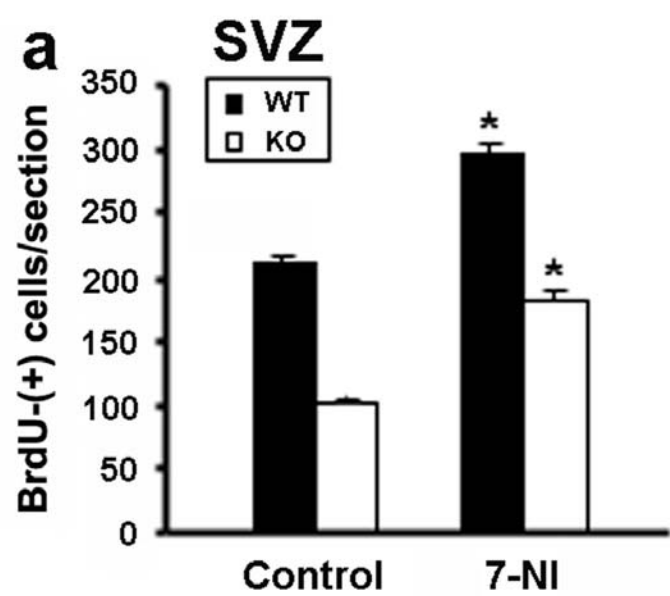


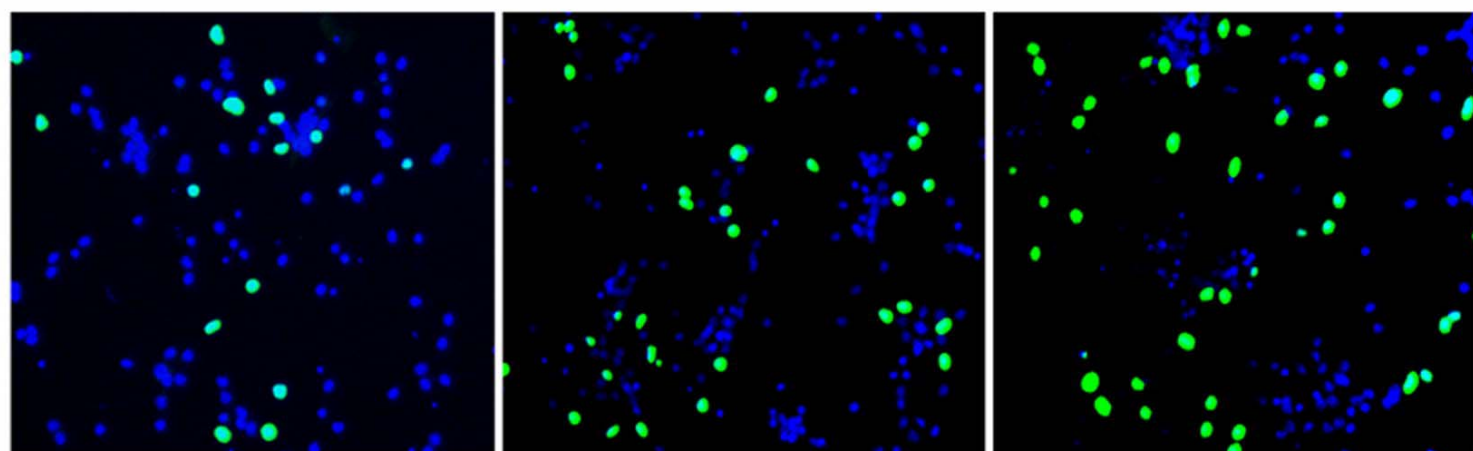
Fig. 3

**a**

Control

Win 55212-2

7-NI



**b**

BrdU-(+) cells (% DAPI)

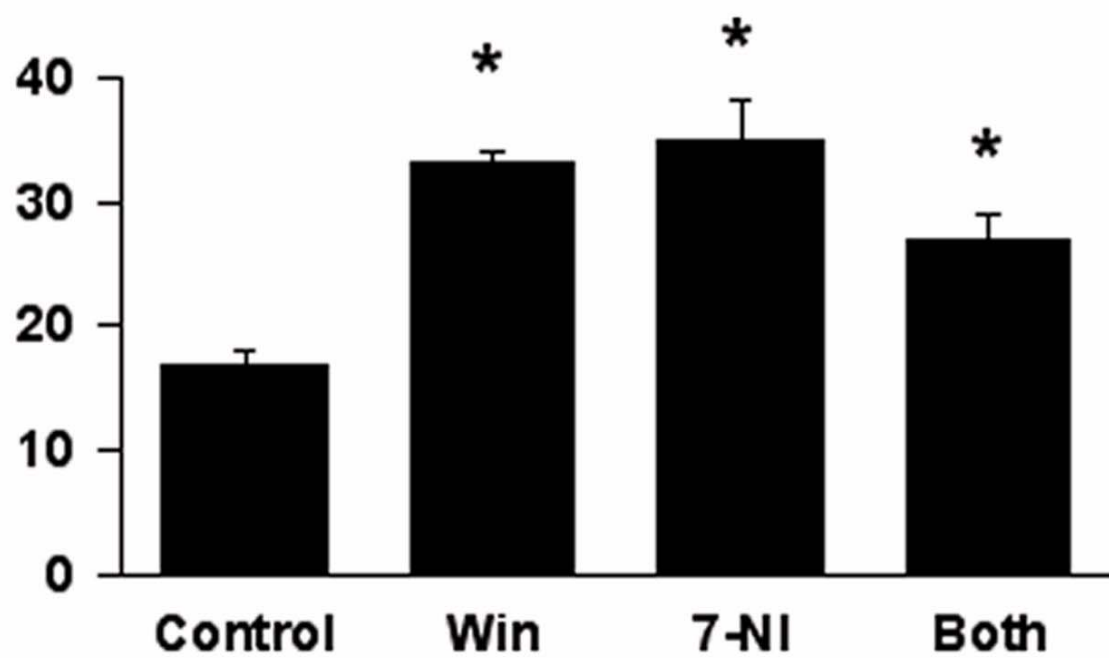




Fig. 4

