Converging action of alcohol consumption and cannabinoid receptor activation on adult hippocampal neurogenesis

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Abstract

Alcoholism is characterized by successive periods of abstinence and relapse, resulting from long-lasting changes in various circuits of the central nervous system. Accumulating evidence points to the endocannabinoid system as one of the most relevant biochemical systems mediating alcohol addiction. The endocannabinoid system regulates adult neurogenesis, a form of long-lasting adult plasticity that occurs in a few areas of the brain, including the dentate gyrus. Because exposure to psychotropic drugs regulates adult neurogenesis, it is possible that neurogenesis might be implicated in the pathophysiology, and hence treatment, of neurobiological illnesses related to drugs of abuse. Here, we investigated the sensitivity of adult hippocampal neurogenesis to alcohol and the cannabinoid receptor agonist WIN 55,212-2 (WIN). Specifically, we analysed the potential link between alcohol relapse, cannabinoid receptor activation, and adult neurogenesis. Adult rats were exposed to subchronic alcohol binge intoxication and received the cannabinoid receptor agonist WIN. Another group of rats were subjected to an alcohol operant self-administration task. Half of these latter animals had continuous access to alcohol, while the other half were subjected to alcohol deprivation, with or without WIN administration. WIN treatment, when administered during alcohol deprivation, resulted in the greatest increase in alcohol consumption during relapse. Together, forced alcohol binge intoxication and WIN administration dramatically reduced hippocampal neurogenesis. Furthermore, adult neurogenesis inversely correlated with voluntary consumption of alcohol. These findings suggest that adult hippocampal neurogenesis is a key factor involved in drug abuse and that it may provide a new strategy for the treatment of alcohol addiction and dependence.

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Introduction

Addiction is a multi-systemic disorder that follows a chronic course with cycles of abstinence followed by relapse (NIDA, 2008). These cycles have important consequences on the establishment of addictive behaviour (Koob & Le Moal, 2008). Vulnerability to relapse can persist even after years of abstinence (Robinson & Berridge, 2008), indicating that long-term, and perhaps permanent, neurobiological changes are responsible for addictive behaviour (Wolf, 2006).

Adult neurogenesis occurs in discrete regions of the mature brain, including the hippocampal formation and the olfactory system, and responds to environmental factors, such as learning and stress. Adult neurogenesis is also sensitive to psychotropic drugs (Abrous et al., 2005; Duman et al., 2001) and plays a key role in the neurobiological changes associated with the onset of addictive behaviour (Canales, 2007; Eisch
Given that hippocampal neurogenesis mediates several forms of learning (Santarelli et al. 2003; Trouche et al. 2009; Winocur et al. 2006), it is of particular interest in the investigation of addiction (Berke, 2001; Nestler, 2001).

Most drugs of abuse have deleterious effects on neurogenesis in a variety of circumstances, including chronic use (Eisch & Mandyam, 2006; Powrozek et al. 2004). Alcohol, for example, reduces neurogenesis (Nixon & Crews, 2002; Herrera et al. 2003; Nixon & Jang et al. 2002a; Rice et al. 2004). Similarly, cannabinoids have a detrimental effect on adult neurogenesis (Eisch & Mandyam, 2006; Rueda et al. 2002), although recent research has reported contrasting findings (Aguado et al. 2007; Jiang et al. 2005). For example, it has been demonstrated that there is an increase of hippocampal neurogenesis following kainic acid-induced excitotoxicity. Using transgenic mice in which CB1 cannabinoid receptors were ablated, it was shown that kainic acid-induced neurogenesis requires the activation of cannabinoid receptors (Aguado et al. 2007). Similarly, both embryonic and adult hippocampal neurogenesis were increased following administration of the potent synthetic cannabinoid receptor agonist HU-210 (10 nm to 1 μM, using in-vitro cultured embryonic hippocampal neural stem/progenitor cells, and 100 μg/kg, using in-vivo injection) (Jiang et al. 2005). Thus, the mechanism by which cannabinoids regulate adult neurogenesis is not yet clear.

Cannabinoids and alcohol are two of the most commonly co-abused drugs in the Western world (EMCDDA, 2008). Both drugs alter motor activity and emotional behaviour (Kotlinska & Bochenski, 2008; Sharma et al. 2007; Viveros et al. 2005), and currently there are numerous results in various experimental paradigms that demonstrate how the manipulation of the endocannabinoid system can modulate alcohol-related behaviours. First, pharmacological studies reveal that whereas cannabinoid receptor agonists can stimulate the intake, preference, reinstatement, relapse, and craving for alcohol in rats (Colombo et al. 2005; De Vries & Schoffelmeer, 2005), cannabinoid receptor antagonists reduce or fully block these effects (Economidou et al. 2006; Lallemand & De Witte, 2006). Second, biochemical studies demonstrate that alcohol administration causes alterations in the endocannabinoid system in the whole brain (Basavarajappa et al. 1998, 2003) as well as in various brain structures. For example, alcohol administration increases the levels of endogenous cannabinoid ligands in the rat prefrontal cortex and striatum (Malinen et al. 2009). Third, genetic studies show that deletion of the CB1 cannabinoid receptor reduces alcohol preference (Vinod et al. 2008) and alcohol self-administration (Thanos et al. 2005) in different strains of mice. Finally, our group has demonstrated previously, using operant task paradigms, that cannabinoid receptor activation during periods of alcohol abstinence can promote a dose-dependent alcohol relapse (Alen et al. 2008; López-Moreno et al. 2004). Together, these results highlight the synergistic action of cannabinoids and alcohol in the context of an alcohol operant self-administration paradigm. Indeed, an operant behavioural paradigm gives a more realistic prediction of the response in humans (O’Dell & Khroyan, 2009) than studies in which animals are forcibly or passively exposed to alcohol.

In the first series of experiments presented here, we investigated the regulation of adult hippocampal neurogenesis following alcohol consumption, cannabinoid receptor activation, and the combination of these two conditions. In the second series of experiments, we explored the relationships between operant alcohol self-administration, alcohol deprivation with relapse-like drinking periods, cannabinoid receptor activation, and their correlation with adult hippocampal neurogenesis. During these experiments, we assessed behaviours that indicate emotional aspects, such as anxiety-like behaviour, and the stimulant-depressant locomotor effects of alcohol and cannabinoid receptor activation. We found that cannabis and alcohol consumption reduced neurogenesis in the adult dentate gyrus.

**Methods**

**Subjects**

Adolescent male Wistar rats (aged 5–6 wk upon arrival at the animal facilities) (Harlan, Spain), weighing 150–175 g, were kept under an inverted light/dark photoperiod regimen (lights on 20:00 hours). Subjects were pair-housed during the experiments, with one exception (at the end of the second experimental series, half of the animals that had been subjected to alcohol operant sessions were sacrificed 10 d before the others). All experiments were conducted under dim red light between 09:00 and 19:00 hours. Procedures were conducted in accordance with the European Community Council Directive (86/609/EEC) and were approved by our institutional animal care and utilization committees.

**Drugs**

WIN 55,212-2 [WIN; (R)-(+)\[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethylone mesylate] (Sigma...
Chemical Co., Spain) was dissolved in sterile physiological saline containing 0.1% Tween-80 and administered by subcutaneous (s.c.) injection between the shoulder blades. WIN has affinity for CB1 and CB2 in the low nanomolar range and exhibits relatively high efficacy at both of these receptor types (Pertwee & Ross, 2002). WIN (2.0 mg/kg) was administered once per day for 5 consecutive days in all experiments. Alcohol solutions, 10% alcohol w/v, were prepared daily from 99% alcohol. The dose of WIN (2.0 mg/kg) was chosen following the dose–response curve established by López-Moreno & colleagues (2004) in an alcohol operant self-administration context.

Forced alcohol/saccharin administration

In the first series of experiments, we used four groups of animals. All four groups were submitted to intragastric administration of a solution using a gavage needle. Two of these groups were treated with an alcohol solution, and the other two groups, functioning as controls for alcohol groups, were treated with a saccharin solution. One group from the two alcohol groups was treated with the cannabinoid receptor agonist WIN (2.0 mg/kg), and the other alcohol group was treated with vehicle. Identically, one group from the two saccharin groups was treated with WIN and the other group with vehicle. The injections of WIN and vehicle were subcutaneous. Rats were given a dose of alcohol (5 g/kg) using a 35% alcohol solution once a day for 5 d. The saccharin groups received the same volume but of a 0.02% saccharin solution instead of alcohol. The treatments were given at the beginning of the dark cycle. Injection of WIN and alcohol/saccharin intragastric administration occurred within 1 min of each other.

Alcohol operant self-administration and relapse model

Animals were trained to self-administer alcohol in operant chambers equipped with two retractable levers (an active lever, and an inactive lever) located on either side of a drinking dipper (0.1 ml). A modification of the method, which has been described by López-Moreno & colleagues (2004), was used for training. We decided not to use sucrose (2.0%) but saccharin (0.02%), as smaller amounts of this solution can be used and because it is less sticky than sucrose for all the operant chamber machinery. Briefly, the rats were subjected to a restricted water schedule for 2–4 d to facilitate training in lever pressing. For the first 3 d, the animals received a 2% saccharin solution in the dipper. Thereafter, the following sequence was used on a fixed ratio-1 (FR-1) schedule (i.e. one lever press results in one alcohol solution delivery): 0.2% saccharin for four sessions, 0.16% saccharin and 2% alcohol for three sessions, 0.12% saccharin and 4% alcohol for three sessions, 0.08% saccharin and 6% alcohol for four sessions, 0.04% saccharin and 8% alcohol for four sessions, 0.02% saccharin and 10% alcohol for four sessions, and 10% alcohol for the remainder of the sessions. The experiments began once an alcohol concentration of 10% w/v was reached without saccharin. Each of the alcohol operant sessions lasted 30 min/d under a FR-1 schedule for the entire study. All of the animals had access to a 10% alcohol solution for 45 d; however, half of these animals were subjected to two periods of 5 d of abstinence from alcohol, and half of the abstinence group was treated with WIN, while the other half was injected with the appropriate control vehicle. A schematic representation of the protocol is shown in Fig. 1.

Several animal models mimic aspects of human alcoholism. The alcohol-deprivation effect (ADE) is one of the most robust effects mimicked by these animal models (Spanagel & Holter, 2000). The ADE shows that alcohol consumption by animals is greater after, than before a period of alcohol deprivation. We used a slightly modified version of the ADE test. According to our approach, termed ‘Drug During Deprivation’, animals are treated with a drug (WIN) during the period of alcohol deprivation and the long-lasting behavioural and neurochemical changes induced by the drug (see López-Moreno et al. 2008a) are subsequently evaluated.

Saccharin operant self-administration

Each alcohol operant self-administration group described above had a control group treated with a 0.02% saccharin solution. The reduction sequence (described above) included only the saccharin solution.

BrdU injection protocol and brain fixation

Newborn cells were labelled with 5-bromo-2-deoxyuridine (BrdU, Sigma), a DNA synthesis marker and hence of cell division. Animals were given two BrdU injections of 75 mg/kg at an interval of 2 h, following a modified version of the protocol used by Reif & colleagues (2006). The first injection was given 4 h after the alcohol/saccharin operant self-administration session or the forced alcohol/saccharin administration. BrdU solution and reagents for brain fixation in 4% paraformaldehyde were prepared as described by Wojtowicz & Kee (2006). The rats were sacrificed 20–24 h after the last BrdU injection. They were first
anaesthetized with chloral hydrate and then perfused with 0.1 M PBS (pH 7.4). The brains were extracted and immersed in the fixative (4% paraformaldehyde in 0.1 M PBS) for 48 h. Finally, the brains were cryopreserved with sucrose and stored at -80 °C.

**Immunohistochemistry**

See Supplementary Methods (available online).

**Complementary behavioural tests: hole-board and elevated plus-maze tests**

See Supplementary Methods.

**Analysis of BrdU- and DCX-immunopositive cells**

Light microscopy was used for DCX staining, in which a 400 × total magnification was used to reconstruct images of each section (Compix Imaging; Hamamatsu Photonics, France). Fluorescence images were acquired for BrdU staining, in which a fluorescence microscope with a 400 × magnification (Olympus, USA) was used. Counting was restricted to the granular and subgranular layers of the dentate gyrus of the hippocampal formation (Crews et al. 2006; He et al. 2005). For each rat, exhaustive counts of BrdU- or DCX-immunopositive cells were collected from every tenth 40 μm coronal section along the entire rostro-caudal axis of both dentate gyri (1.80–7.64 mm from bregma). Sections were analysed blind until the completion of all data analysis.

**Data analyses**

BrdU- and DCX-immunopositive cell count data from the first series of experiments were analysed using Student’s t tests (Fig. 2) and one-way ANOVAs, with treatment as the between-group factor (Fig. 3). Data shown are percentages of DCX-expressing cells compared to the control group. Data from the operant self-administration of alcohol or saccharin experiments were analysed using a one-way ANOVA with treatment as the between-group factor (Fig. 4). Data from the neurogenesis experiments were analysed with a two-way ANOVA (Fig. 5). Data from the elevated plus-maze and hole-board experiments were analysed using a three-way ANOVA (Figs 5 and 6). Significant effects (p<0.05) revealed by ANOVA analyses were subjected to Tukey’s honestly significant difference test (between-groups factor) using SPSS software (version 17.0 for Windows; SPSS Inc. USA).
Results

Reduced neurogenesis after subchronic alcohol binge intoxication

We assessed neural cell proliferation after a 5-d treatment of 5 g/kg alcohol per day (Fig. 2) to test the effect of alcohol on adult neurogenesis. This treatment significantly reduced BrdU staining in the dentate gyrus of the alcohol-treated animals (a reduction of 88 ± 6%, p < 0.01 with a Student’s t test; Fig. 2a). DCX immunoreactivity was quantified to confirm the neural identity of the newborn cells. DCX is a microtubule-associated protein produced by neuronal progenitors and immature neurons (Rao & Shetty, 2004). Thus, it serves as a reliable marker for quantification of adult neurogenesis (Brown et al., 2003). The number of DCX-immunopositive cells showed a similar reduction after binge alcohol intoxication (59 ± 9%, p < 0.05 with a Student’s t test; Fig. 2b), indicating that subchronic alcohol intoxication significantly reduced adult neurogenesis in the hippocampal formation.

Reduction of adult neurogenesis following activation of cannabinoid receptors

We compared BrdU-immunopositive cell counts in four groups that drank either alcohol or saccharin and received either a cannabinoid receptor agonist (WIN) or vehicle for five consecutive days (Fig. 3) in order to investigate the effects of alcohol consumption and the simultaneous activation of cannabinoid receptors on neurogenesis. Two-way ANOVA [between solutions: F(1, 16) = 66.77, p < 0.001; between drug-treatment: F(1, 6) = 7.84, p < 0.05; interaction: F(1, 16) = 5.65, p < 0.05] revealed significant treatment effects that were further analysed with Tukey’s post-hoc test. Hippocampal neurogenesis was reduced with 5-d WIN treatment alone relative to the control group (p < 0.05) (Fig. 3). The alcohol-induced reduction of neurogenesis (86% reduction, n = 4, p < 0.01) (Fig. 2) was more pronounced than that induced by WIN alone (43% reduction, n = 4, p < 0.05) (Fig. 3). In alcohol-treated animals, the WIN effect was lost (p = 0.98), suggesting total occlusion of the WIN effect by forced alcohol administration.

Activation of cannabinoid receptors increases operant alcohol self-administration in animals subjected to repetitive alcohol deprivations

To further investigate the functional interaction between activation of the endocannabinoid system and
alcohol deprivation, we performed additional experiments, similar to those described above, on four different groups of rats (Fig. 1) and assessed the level of hippocampal neurogenesis. No significant differences were found between the WIN-treated group with continuous access to alcohol and the alcohol control group. However, when two alcohol-deprivation periods were introduced, the response to alcohol self-administration increased significantly (150%, \( p < 0.001 \)). Furthermore, animals treated with WIN during the alcohol-deprivation period displayed the highest levels of alcohol operant self-administration of the four groups (Fig. 4a). Two-way ANOVA showed a significant effect of the treatments [between deprivations: \( F(1, 36) = 44.03, p < 0.0001 \); between drug treatment: \( F(1, 36) = 8.77, p < 0.01 \); interaction: \( F(1, 36) = 0.45, \text{n.s.} \)]. Four other groups of animals that worked to obtain a non-drug reward (saccharin) were analysed.

Although we used a very low concentration of saccharin (0.02%), the levels of saccharin responses were significantly higher than alcohol responses at every time-point. These rats were sensitive to repetitive deprivation phases and WIN treatment (Fig. 4b). Saccharin administration responses were significantly

Fig. 4. Cannabinoid receptor activation leads to a long-lasting increase in operant alcohol self-administration only for animals subjected to repetitive alcohol deprivation. Data represent the last 10 d of operant alcohol/saccharin self-administration after WIN/control vehicle treatment. (a) Animals treated with WIN 30 min prior to the alcohol self-administration sessions and not exposed to alcohol deprivation (hatched bar), do not show a significant increase in their alcohol responses in the following 10 d compared to the group treated with vehicle and non-deprived group (□). Note that repetitive alcohol-deprivation periods increase the response for alcohol (■), and this increase is the highest in the group exposed to the combination of alcohol deprivation and WIN treatment (■) \((n=10)\); \( * p < 0.05 \), \( ** p < 0.01 \), \( *** p < 0.005 \). (b) Only the combination of saccharin-deprivation periods with WIN treatment (■) induces a greater response for saccharin compared to the group treated with vehicle and non-deprived group (□) \((n=8)\); \( * p < 0.05 \). Values are mean ± S.E.M.

Fig. 5. Hippocampal neurogenesis is inversely correlated with the responses for alcohol/saccharin. (a) Alcohol deprivation and WIN treatment cause a similar reduction in the number of DCX-immunopositive cells. This reduction is more significant in the group exposed to the combination of alcohol deprivation and WIN treatment than in the control group \((n=5–8)\); \( * p < 0.05 \), \( ** p < 0.01 \). Values are mean ± S.E.M. (b) Only the combination of saccharin-deprivation periods and WIN treatment induces a significant decrease in the number of DCX-immunopositive cells compared to the control group \((n=5–8)\); \( * p < 0.05 \).
higher in animals treated with WIN during the saccharin-deprivation period than responses in the saccharin control group [two-way ANOVA, between deprivations: $F(1, 28) = 7.05, p < 0.05$; between drug treatment: $F(1, 28) = 6.56, p < 0.05$; interaction: $F(1, 28) = 0.53, n.s.$].

Before any experimental treatment, the means of saccharin alcohol responses were between 57.72 and 63.72 (S.E.M.’s 4.58 and 4.49, respectively), whereas alcohol response means were between 29.98 and 32.55 (S.E.M.’s 1.78 and 1.80, respectively). It is also important to note that whereas the increase of saccharin responses was about 65.5%, alcohol increase is about 154.3% when they were compared to their respective control groups (non-deprivations and non-WIN treatment). This demonstrates the synergistic action of alcohol consumption on the WIN-induced effect.

The average alcohol intake and saccharin intake per 30-min session in the operant self-administration tasks over 10 d after WIN treatment are shown in Tables 1 and 2. These tables are related to the number of responses necessary to obtain the alcohol/saccharin solutions shown in Fig. 4a, b, respectively. These results highlight that when WIN treatment is not given during an alcohol-deprivation period, there is no effect on the alcohol/saccharin response.

**Hippocampal neurogenesis inversely correlates with the response to alcohol/saccharin consumption**

We attempted to correlate the behavioural response to alcohol/saccharin with changes in adult hippocampal neurogenesis. We found significant differences in the level of neurogenesis between experimental groups [two-way ANOVA, between deprivations: $F(1, 28) = 6.65, p < 0.05$; between drug treatment: $F(1, 28) = 5.86, p < 0.05$; interaction: $F(1, 28) = 0.22, n.s.$] (Fig. 5a). Both WIN administration and alcohol-deprivation periods reduced DCX staining compared to that for animals with continuous alcohol self-administration sessions ($p < 0.05$). This reduction was even greater in the group that underwent both alcohol deprivation and WIN treatment ($p < 0.01$). The tendency for operant alcohol self-administration inversely correlated with the level of neurogenesis ($r = −0.43, p < 0.01$ with a Pearson correlation test). For saccharin self-administration groups, decrease in neurogenesis was in some way similar to that of the alcohol self-administration groups (Fig. 5b). Yet, only animals subjected to the
combination of saccharin deprivation and WIN treatment showed a significant reduction in DCX staining when compared to animals with continuous saccharin self-administration and vehicle treatment [two-way ANOVA, between deprivations: \( F(1,28) = 0.01, \) n.s.; between drug treatment: \( F(1,28) = 1.13, \) \( p < 0.05 \); interaction: \( F(1,28) = 5.21, \) \( p < 0.05 \)].

The combination of operant alcohol self-administration and WIN causes long-lasting behavioural effects

Behaviour and spontaneous locomotor activity were investigated during the period of alcohol self-administration and WIN treatment because cannabinoid receptors affect motor-related behaviour and locomotor activity is altered during drug sensitization (see Supplementary methods). Only animals that self-administered alcohol and were treated with WIN showed a significant decrease in their rearing activity relative to animals that self-administered alcohol and were not treated with WIN (Fig. 6a). This effect was independent of alcohol-deprivation periods and was specific to rearing behaviour given that external ambulation was not affected (Fig. 6b) and that the number of lever presses necessary to obtain alcohol was elevated in these animals (Fig. 4a). Animals were assessed 4–5 d after the last WIN injection, suggesting that the WIN-induced decrease in rearing was long-lasting. A three-way ANOVA showed that the decrease in rearing in animals that self-administered alcohol and were treated with WIN was significant \( [F(1,63) = 4.72, \) \( p < 0.05 \)] (Fig. 6b). No significant differences were found in frequency or duration of head-dipping responses in the hole-board test (data not shown).

Moderate anxiety-like behaviours after operant alcohol self-administration

Anxiety-related behaviour is usually studied by measuring avoidance responses to potentially threatening situations that expose rodents to predators, such as open and brightly lit environments (see Supplementary methods). Anxiety-like behaviour was tested using the elevated plus-maze test (Fig. 7). This test consists of two closed arms and two open arms, the latter constituting the threatening areas. We found that rats made fewer open-arm entries at 4–5 h after the alcohol self-administration session, but these results were not significant [initial three-way ANOVA: \( F(1,59) = 3.59, \) \( p = 0.06 \)] (Fig. 7a). Tests between groups showed that open-arm entries were fewer for animals self-administering alcohol than for those with continuous access to saccharin solution \( [F(1,27) = 4.22, \) \( p < 0.05 \)] . The proportion of time spent by the animals in the open arms is shown in Fig. 7b. Differences in the percent of time spent in the open arms between the animals with continuous access to saccharin and those subjected to alcohol deprivation were significant and independent of WIN treatment \( [F(1,43) = 4.44, \) \( p < 0.05 \)] . This effect did not depend on non-specific modification of locomotor activity, suggesting that operant alcohol self-administration increases anxiety-related behaviour.

Discussion

Our results show that activation of cannabinoid receptors interacts with alcohol intake and that both of...
these components individually reduce adult hippocampal neurogenesis. First, we found that subchronic binge alcohol intoxication reduced adult neurogenesis, and that this decrease was mimicked by activation of cannabinoid receptors. Second, using animals that had learned how to gain an alcohol reward, we demonstrated that two successive alcohol-deprivation periods greatly increased relapse drinking, and that this increase was enhanced by alcohol deprivation in combination with WIN treatment. We found that these alcohol-consumption patterns inversely correlated with the degree of hippocampal neurogenesis.

**Neurogenesis-dependent action of alcohol and drug abuse**

Structural plasticity is a potential mechanism involved in neurodegenerative and psychiatric diseases, such as drug abuse and depression. Chronic alcoholism is a progressive neurodegenerative disease during alcohol abuse that can cause severe to extreme diseases such as Wernicke–Korsakoff syndrome (Harper, 2009). The neuronal plasticity observed in chronic alcoholism coupled with conflicting reports on alcohol-induced hippocampal neuropathology indicates that the potential link between adult neurogenesis and alcoholism should be reconsidered. Only recently has the first evidence that binge alcohol exposure causes inhibition of neural cell proliferation and survival in adult rat hippocampus been provided (Nixon & Crews, 2002). Here, we describe the effect of alcohol intoxication and abstinence with simultaneous activation of cannabinoid receptors on neurogenic processes.

Our first set of data supports previous studies showing the deleterious effects of alcohol and other drugs of abuse on adult neurogenesis (Crews et al. 2006; Eisch & Mandyam, 2004; He et al. 2005; Herrera et al. 2003; Jang et al. 2002b; Nixon & Crews, 2002; Rice et al. 2004). Neurogenesis is regulated by drugs of abuse (Abrous et al. 2002; Eisch et al. 2000; Nixon & Crews, 2002; Noonan et al. 2008), and the hippocampus influences both drug-taking and drug-seeking behaviour via its projections into limbic regions involved in reward (Floresco et al. 2001; Lodge & Grace, 2006; Taepavarapruk et al. 2000). Using subchronic, forced administration of alcohol, our results confirm and extend these observations. We also demonstrate that subchronic WIN administration causes a similar reduction in adult neurogenesis, which contrasts with the findings of previous studies (Aguado et al. 2005, 2007; Jiang et al. 2005). For example, it has been reported that acute and chronic administration of Δ⁹-THC did not significantly alter cell proliferation in the adult mouse dentate gyrus (Kochman, 2006). Yet, others have reported decreases in adult neurogenesis similar to that observed in the present study (Galver-Roperh et al. 2008; Rueda et al. 2002). These discrepancies might result from the use of different cannabinoid agonists, the participation of endogenous vs. exogenous cannabinoid receptor ligands, the use of in-vivo vs. in-vitro experiments, the type of administration (s.c. vs. i.p.), or the different periods of WIN administration. It could also be argued that the reduction of neurogenesis observed in the second series of experiments, 11 d after treatment withdrawal (Fig. 1), is not directly linked to cannabinoid receptor activation. This hypothesis cannot be discarded since other non-cannabinoid receptors could be recruited (like vaniloid receptors; e.g. Marchalant et al. 2009). Nevertheless, it is unlikely since we provide here clear evidences showing that adult neurogenesis was reduced the day following withdrawal of WIN treatment (Fig. 3).

Our study shows that adult neurogenesis mediates several aspects of addictive behaviour, such as the desire for and memory of alcohol consumption, as well as the sensitivity to and reward received from alcohol ingestion. These findings are consistent with both basic research and clinical observations, including the altered proliferation and neurogenesis associated with abuse of various drugs (nicotine, alcohol, opiates, cannabinoids) and the strong propensity to relapse to drug-taking in a paired-drug context (Canales, 2007; Gould, 2006). Our study demonstrates the implication of adult neurogenesis in drug-reward and alcohol-context memory, suggesting that study of the role of contextual cues in alcoholism and drug addiction would be valuable. Such studies may have implications for the development of treatments.

**Functional interactions between operant alcohol self-administration and cannabinoid receptor activation**

The second series of experiments showed that the rate of operant alcohol self-administration was greatly increased in animals subjected to alcohol-deprivation periods combined with WIN administration. This group also showed the lowest level of hippocampal neurogenesis. We conclude that cannabinoid receptor activation reduced adult neurogenesis in the dentate gyrus. It remains to be determined whether this effect is specific to the hippocampal formation or whether it can be extended to the second neurogenic area, the so-called subventricular zone of the lateral ventricle.
WIN treatment did not alter alcohol relapse when administered outside of the alcohol-deprivation period. The finding that the same dose of WIN could have distinct effects on alcohol consumption, and subsequently on adult neurogenesis, demonstrates the importance of timing and availability of other drugs that mediate the neurogenic effects of cannabinoids. Again, this adds more complexity to the role of the endocannabinoid system in alcohol-related behaviours. Although a clear relationship has been found between the activation of cannabinoid receptors and the increase in intake, preference, reinstatement, relapse, and craving for alcohol, our results demonstrate that such effects depend on the context of this activation (i.e. during abstinence or just prior to alcohol exposure). Interestingly, we also found that adult neurogenesis was implicated in both cannabinoid-induced intake and alcohol relapse. In addition, the reduction in adult neurogenesis during saccharin self-administration after concurrent WIN treatment and saccharin deprivation would suggest that these alterations could take place in a more subtle way with a natural reward. There are many experimental pharmacological, biochemical, and behavioural evidences that link the cannabinoid system to alcohol addiction (see Introduction). At a molecular level, alcohol exposure causes a significant decrease in CB1 receptor expression (Basavarajappa et al. 1998, 2006; Ortiz et al. 2004), CB1 receptor–G-protein activation (Vinod & Hungund, 2006), as well as the levels of the fatty acid amidohydrolase (Vinod & Hungund, 2006) and the reuptake of anandamide (Basavarajappa et al. 2003). At a behavioural level, cannabinoid receptor agonists/antagonists are known to modulate most parts of alcohol-addiction processes (Colombo et al. 2007; Hungund & Basavarajappa, 2004), although the detailed mechanisms still remain elusive. In line with these observations, several authors have demonstrated that cannabinoid receptor activation alters the normal activity of the ventral tegmental area and nucleus accumbens, key brain regions of the reward circuitry (Lupica et al. 2004). It has been shown, particularly in the nucleus accumbens, that WIN inhibits GABAergic and glutamatergic synaptic transmission (Manzoni & Bockaert, 2001; Robbe et al. 2003) and that a single in-vivo exposure to Δ⁹-THC blocks the endocannabinoid-mediated synaptic plasticity (Mato et al. 2004). Together, WIN produces an increase in the spontaneous electrical activity of meso-accumbens dopaminergic neurons (Gessa et al. 1998), whereas this electrical activity is reduced in alcohol-dependent rats (Diana et al. 1992). Here, we provide evidences that adult hippocampal neurogenesis could also contribute to these complex interactions. WIN seems to have a synergistic effect with alcohol operant self-administration and alcohol-deprivation periods, which causes a significant increase in the number of responses for obtaining alcohol and a significant neurogenesis reduction.

The dose range of WIN should also be taken into account. The effect of cannabinoids on anxiety is biphase and depends on the drug dose (Viveros et al. 2005). Adult neurogenesis in the dentate gyrus reflects stress and anxiety (Brene, 2007; Duman et al. 2001; Santarelli et al. 2003). It has been suggested that the influence of adult neurogenesis on addictive behaviour could be achieved through regulation of anxiety and stress because these factors are both known to influence drug consumption (Canales, 2007; Goeders, 2003; Koob, 2008). A critical role of the cannabinoid system in stress-stimulated alcohol drinking has been proposed (Racz et al. 2003). Results from the elevated plus-maze showed greater anxiety-like behaviour for animals with access to alcohol self-administration than for animals with continuous access to saccharin. Importantly, the elevated plus-maze test was performed 4–5 h after alcohol withdrawal from the operant alcohol self-administration cages. Thus, this anxiogenic-like response may be attributable to an alcohol-withdrawal effect (Rubio et al. 2008). Moreover, in the hole-board test, cannabinoid agonist treatment reduced rearing behaviour in the animals with a history of alcohol use. Rearing behaviour is particularly sensitive to acute cannabinoid administration (Borcel et al. 2004; Marco et al. 2004; Marín et al. 2003). In this study, rearing was markedly reduced at 5 d after the last WIN injection, suggesting that previous alcohol administration facilitated, or prolonged, the effect of the cannabinoid receptor agonist.

Clinical studies revealed no difference between a group of patients with a alcohol history and a placebo group, with respect to relapse rates after administration of rimonabant (a cannabinoid receptor antagonist) (Soyka, 2008). Interestingly, since rimonabant has been retired from the market because of a non-negligible risk of depression and suicide, numerous preclinical studies support the notion that endocannabinoid remains a potential target to treat drug addiction (Le Foll, 2009).

**Adult hippocampal neurogenesis and alcoholism**

The hippocampus, together with the ventral tegmental area, nucleus accumbens, amygdala, and prefrontal cortex, is associated with drug addiction and alcoholism (Koob, 2009; López-Moreno et al. 2008b;
Maldonado et al. 2006). The hippocampus has recently attracted attention in relation to addiction (Abrous et al. 2005; Eisch & Harburg, 2006; Vorel et al. 2001). Cannabinoidergic activity modulates hippocampal long-term potentiation and depression (Viveros et al. 2007; Zhu & Lovinger, 2007), two forms of neural plasticity that underlie addictive processes (Carlson, 2002; Wolf, 2003). However, the relationship between drug-related behaviour, the formation of new neurons and their correct integration into the hippocampus, both functionally and electrophysiologically, remains unknown. Studies assessing the direct effects of alcohol self-administration on adult neurogenesis are required, as articulated by some authors (Eisch & Mandyam, 2004, 2006; Rodd et al. 2008). This report is the first to show the effects of operant alcohol self-administration on adult neurogenesis. Our results complement those of a previous study, which found gene expression modifications related to adult neurogenesis using operant self-administration procedures (Rodd et al. 2008). However, the previous study did not include an index of actual neurogenesis rates, as reported here.

There are two main lines of study in the postnatal neurogenesis field. One line seeks to understand the functional meaning of adult neurogenesis and the contribution of newborn neurons to brain circuits (Lledo et al. 2006). The second line seeks to manipulate these cells for the purposes of brain repair and regeneration (Abrous et al. 2005; Zhao et al. 2008). Research on cellular mechanisms of alcoholism should benefit from both lines of work. First, understanding the contribution of newborn neurons to hippocampal function may reveal mechanisms that underlie hippocampal dysfunction. In the case of alcoholism, hippocampal dysfunction and neurodegeneration often result from the neurotoxic effects of alcohol. Moreover, functions related to hippocampal neurogenesis, such as learning, memory and mood, are deregulated in chronic alcoholism (reviewed in Nixon, 2006). Second, understanding the relationship between neural stem cells and neural regeneration in the adult brain may help explain how the brain recovers during abstinence from alcohol. Our results indicate that stimulating hippocampal neurogenesis during abstinence might help to reconstruct hippocampal circuits during the period of recovery from the neurodegenerative effects associated with chronic alcoholism. Further studies are needed to understand how alcohol affects neural stem cells. However, our results from the alcohol binge exposure model are consistent with evidence of cell death in the dentate gyrus following binge alcohol exposure (Obernier et al. 2002) and dentate gyrus granule cell loss following chronic alcohol exposure (Walker et al. 1980). Thus, alcohol inhibition of adult neurogenesis is important in alcohol-induced neurodegeneration.

Our results demonstrate a clear relationship between binge alcohol intoxication, alcohol-deprivation periods, relapse-like drinking periods, cannabinoid receptor activation, and adult hippocampal neurogenesis. We have shown that alcohol and WIN treatment reduce neurogenesis and that an increase in alcohol craving inversely correlates with the rate of adult neurogenesis. Changes in motor activity and anxiety-related behaviour followed a slightly different pattern. Operant alcohol self-administration procedures are appropriate for addressing sophisticated questions about alcohol addiction and relapse and provide better predictions of human responses than other procedures (O’Dell & Khroyan, 2009). Therefore, we conclude that there is a harmful link between drug addiction and adult neurogenesis. Mounting evidence indicates that use of cannabis is often associated with alcohol consumption in Western societies. This study suggests that pharmacological agents designed to treat both addictions should take into account their common anti-neurogenic action on the adult hippocampus.

Note
Supplementary material accompanies this paper on the Journal’s website (http://journals.cambridge.org/pnp).

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Statement of Interest
None.
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