

Ibogaine modifies GDNF, BDNF and NGF expression in brain regions involved in mesocorticolimbic and nigral dopaminergic circuits

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Keywords: ibogaine, neurotrophic factors, GDNF, BDNF, NGF

Abstract

Ibogaine is a psychedelic alkaloid which has been subject of intense scientific research due to its reported ability to attenuate drug-seeking behavior. Recent work suggested that ibogaine effects on alcohol self-administration in rats was related to the release of Glial Cell Derived Neurotrophic Factor (GDNF) in the Ventral Tegmental Area (VTA), a mesencephalic region which hosts soma of dopamine neurons. It is well known that neurotrophic factors (NFs) mediate the neuroadaptations induced in the mesocorticolimbic dopaminergic system by repeated exposure to drugs. Although previous reports have shown ibogaine's ability to induce GDNF expression in rat midbrain, there are no studies addressing its effect on the expression of GDNF, Brain Derived Neurotrophic Factor (BDNF) or Nerve Growth Factor (NGF) in distinct regions containing dopaminergic neurons. In this work, we examined the effect of ibogaine acute administration on the expression of these NFs in the VTA, Prefrontal Cortex (PFC), Nucleus Accumbens (NAcc) and the Substantia Nigra (SN). Thus, rats were i.p. treated with ibogaine 20 mg/kg (I₂₀), 40 mg/kg (I₄₀) or vehicle, and NFs expression was analyzed after 3 and 24 hours. Only at 24 h an increase of the expression for the three NFs were observed in a site and dose dependent manner. Results for GDNF showed that only I₄₀ selectively

upregulated its expression in the VTA and SN. Both doses of ibogaine elicited a large increase in the expression of BDNF in the NAcc, SN and PFC, while a significant effect was found in the VTA only for I₄₀. Finally, NGF was found to be upregulated in all regions after I₄₀, while a selective upregulation was found in PFC and VTA for the I₂₀ treatment. An increase in the content of mature GDNF was observed in the VTA but no significant increase in the mature BDNF protein content was found in all the studied areas. Interestingly, an increase in the content of proBDNF was detected in the NAcc for both treatments. Further research is needed to understand the neurochemical bases of these changes, and to confirm their contribution to the anti-addictive properties of ibogaine.

1. Introduction

Ibogaine is the main indole alkaloid isolated from the root bark of the African shrub *Tabernanthe iboga* (Lavaud and Massiot, 2017). Traditionally used in African religious ceremonies as a psychedelic, ibogaine became the subject of interest of the scientific community due to its reported ability to reduce craving and self-administration of several drugs of abuse in humans (Brown, 2013). These effects found mainly in uncontrolled clinical trials and observational studies, have been reported to be long-lasting enduring weeks to months after a single administration of large doses of ibogaine (Schenberg et al., 2014; Brown and Alper, 2017; Noller et al., 2017). In animal models for drug dependence, ibogaine also reduces the self-administration of morphine and heroin, (Glick et al., 1991; Glick et al., 1994; Dworkin et al., 1995) cocaine, (Cappendijk and Dzoljic, 1993; Glick et al., 1994) and alcohol, (He et al., 2005) with long-lasting effects that persists beyond pharmacokinetic elimination of the drug (Alper, 2001). In addition, ibogaine administration to animals also reduces naloxone or naltrexone precipitated-withdrawal signs (Dzoljic et al., 1988; Glick et al., 1992; Leal et al., 2003). Several ibogaine effects observed in humans, such as the dissociative or psychedelic effects, (Alper, 2001) tremor induction, (Glick et al., 1994) and prolongation of the QT_c interval in the EKG (which has been associated with sudden death cases after ibogaine intake), (Koenig and Hilber, 2015), have made it a controversial treatment option and resulted in reluctance to pursue rigorous controlled clinical trials (Brown, 2013). In this regard, unveiling the mechanism of action for ibogaine's anti-addictive property is critical for the development of safer derivatives for clinical use, and for a deeper understanding of the neurobiological basis of substance dependence and its possible treatment.

Although a vast amount of research has been done regarding the pharmacology of ibogaine, the mechanism of action of its anti-addictive effects remains unresolved (Alper,

2001; Maciulaitis et al., 2008; Brown, 2013). Ibogaine binds to numerous central nervous system (CNS) targets at the micromolar range such as: nicotinic acetylcholine receptors (nAChR $\alpha 3\beta 4$ and $\alpha 2\beta 4$), N-methyl-D-aspartate (NMDA), kappa and mu opioid, 5HT_{2A} and 5HT₃ receptors and the dopamine and serotonin transporters (Glick et al., 2001). However, these ibogaine-receptor interactions do not seem to account for the long-lasting effects of ibogaine found in rodents which are described to last for 48 to 72 hours after ibogaine administration (Glick et al., 1991; Cappendijk and Dzoljic, 1993; Glick et al., 1994). In rodents, ibogaine has a short half-life of 1-2 hours raising the hypothesis that its longer-lived active metabolite, noribogaine, should be responsible for the enduring effects elicited by ibogaine. However, no appreciable amounts of noribogaine have been found in rodents' brain tissue 19 hours after ibogaine intraperitoneal (i.p.) administration, (Pearl et al., 1997) and only approximately 5% of the noribogaine C_{max} was detected in serum 24 hours after the same treatment (Baumann et al., 2001).

A few years ago, a novel hypothesis linking ibogaine's attenuation of alcohol self-administration in rodents to its ability to modulate the expression of Glial Cell Derived Neurotrophic Factor (GDNF) in the brain was proposed. It was shown that a single ibogaine i.p. administration (40 mg/kg) increased the expression of GDNF in the midbrain of rats and mice for up to 24 hours (He et al., 2005). In addition, microinjection of ibogaine into the Ventral Tegmental Area (VTA), produced a long-lasting reduction of ethanol self-administration, a response that was attenuated by the intra-VTA delivery of anti-GDNF neutralizing antibodies. These results suggested that ibogaine mediates its effects against ethanol consumption by increasing GDNF content in the VTA (He et al., 2005). Accordingly, another study from the same research group showed that the intra-VTA infusion of noribogaine induced a long-lasting decrease in ethanol self-administration (Carnicella et al., 2010). Further, ibogaine-derived synthetic derivatives were recently shown to induce the release of GDNF *in vitro*, in established cell line systems (Gassaway et al., 2016). These observations formed the basis for a new rationale to explain the long-lasting effects of ibogaine; i.e., the induction of GDNF by ibogaine/noribogaine may activate an autocrine loop, leading a long-term synthesis and release of GDNF (that persists beyond elimination of both substances). This mechanism may reverse the biochemical adaptations to chronic exposure to drugs of abuse in the reward system (He and Ron, 2006).

Neurotrophic Factors (NFs), such as GDNF, BDNF (Brain Derived Neurotrophic Factor) or NGF (Nerve Growth Factor) are small proteins that promote the growth, differentiation, synaptogenesis, and survival of neurons. Their expression in the nervous tissue

is relatively high during the development of the CNS, where substantial growth, differentiation and remodeling of the nervous system occur (Barde, 1990; Lu and Figurov, 1997). More recently, it has been discovered that NFs play important roles in the adult brain where they modulate maintenance, protection, repair and plasticity of the nervous tissue (Reichardt, 2006; Schmidt and Duman, 2007). Furthermore, accumulating evidence has suggested that NFs mediate neuronal remodeling processes that occur during the development of substance use disorders (SUDs) (Bolaños and Nestler, 2004). Particularly, the role of GDNF and BDNF in the neuroadaptations in the mesocorticolimbic dopamine system (Prefrontal Cortex, PFC-VTA-Nucleus Accumbens, NAcc pathway) induced by repeated exposure to drugs of abuse has been extensively studied, including the impact of manipulating NFs levels on drug-seeking behavior in animal models (Russo et al., 2009; Ghitza et al., 2010; Koskela et al., 2017). It has been shown that the administration of BDNF or GDNF can either promote or inhibit drug-taking behaviors depending mainly on the brain site of administration, along with other several factors such as the drug type, the addiction phase (initiation, maintenance, abstinence or relapse), the time interval between site-specific NFs injections and the related behavioral assessments (Ghitza et al., 2010). For example, BDNF infusion into the NAcc increases cocaine-seeking behavior, (Graham et al., 2007) while BDNF infusion into the medial prefrontal cortex (mPFC) suppresses it (Berglind et al., 2007). The effect of administering other NFs (as NGF or Fibroblast Growth Factor, FGF) on drug-seeking behavior and modulation of neuroadaptations produced by chronic administration of drugs of abuse has been less studied. In the case of NGF, for example, it was found that NGF content decreases in the hippocampus and hypothalamus of alcohol-treated mice, (Aloe et al., 1993) and that its administration into the central nucleus of the amygdala mimicked the morphine reward sensitization (Bie et al., 2012). In contrast to BDNF, a single infusion of NGF into the VTA produced no changes in cocaine-seeking behavior (Lu et al., 2004).

Given the importance and the site-specificity of the elicited responses, we decided to analyze the effect of a single administration of ibogaine on the expression of GDNF, BDNF and NGF at two time points, 3 and 24 h after i.p. injection, in those brain areas which define the mesocorticolimbic dopamine system such as VTA, PFC and NAcc (Figure 1 and 2). As the Substantia Nigra (SN) is a major nucleus of dopaminergic neurons important in the basal ganglia functioning, the expression of these NFs in this region was also studied. In addition, locomotion of control and drug treated animals was recorded using an open field test.

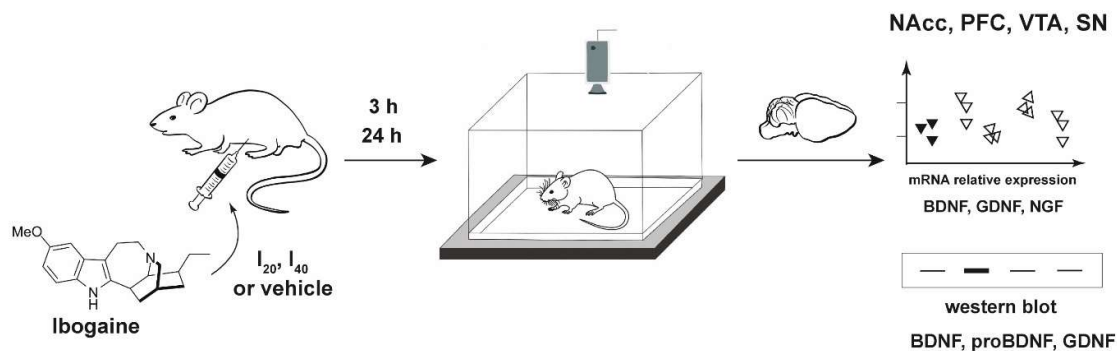


Figure 1. Schematic showing the experimental design of this work. Experimental groups of animals were i.p. treated with ibogaine 20 mg/kg (I₂₀), 40 mg/kg (I₄₀) or vehicle. After 3 and 24 hours, locomotion of control and treated animals was recorded using an open field test. Afterwards, animals were sacrificed, and selected brain regions were dissected. mRNA levels for BDNF, GDNF and NGF were determined by qPCR. Western Blot was used to determine BDNF, proBDNF and GDNF protein content. PFC = Prefrontal Cortex, NAcc = Nucleus Accumbens, VTA = Ventral Tegmental Area, and SN = Substantia Nigra, GDNF = Glial Cell Derived Neurotrophic Factor, BDNF = Brain Derived Neurotrophic Factor, NGF = Nerve Growth Factor.

2. Material and Methods

2.1 Ibogaine·HCl

The ibogaine used in this study was chemically synthesized using voacangine as starting material, which was extracted from the root bark of *Voacanga africana* using a modification of a previously described procedure (Jenks, 2002). Briefly, 100g of grounded root bark of *Voacanga africana* was extracted with a 1% aqueous solution of HCl (6 x 500 mL). The combined aqueous extracts were basified by adding concentrated NH₄OH until pH 10-11. A brown precipitate was separated by centrifugation and dried at 60°C for 24 h. This solid was taken in acetone and filtered to discard root impurities. The solvent was evaporated *in vacuo* to afford a total alkaloid extract of 3.5-4.0 g. Column chromatography (SiO₂, Hex:EtOAc:NH₄OH, 90:10:0.01) allowed to obtain 1g of pure voacangine which was analyzed by ¹H and ¹³C NMR (See supporting information). Voacangine was decarboxylated as follows. To a solution of voacangine in EtOH (0.45 M) in a double necked round bottomed flask, KOH in pellets (5 equivalents) was added. The solution was heated to reflux until consumption of the starting material was evident by thin layer chromatography (TLC) analysis. EtOH was removed under reduced pressure, and the residue was dissolved at 0 °C in a round bottomed flask using a 6% (v/v) aqueous solution of HCl (enough quantity to adjust pH to 1). The system was then heated to reflux for five minutes. Once the starting material consumption was evident by TLC analysis, the solution was carefully basified using 50% NaOH (pH 10-11). Precipitation of ibogaine as a white solid was observed. Ethyl acetate was added, and the resultant biphasic system was transferred into a separation funnel. The aqueous phase was extracted three times with EtOAc. The combined organic layers were dried under Na₂SO₄, and

the solvent was removed *in vacuo*. Ibogaine free base was obtained with an 86% yield and was analyzed by ^1H and ^{13}C NMR (see supporting information). Crystallization from EtOH afforded a crystalline solid which was converted to the corresponding hydrochloride by treatment with diethyl ether saturated with HCl(g). Purity of ibogaine·HCl was determined by GC-MS analysis as 98.3 % (see supporting information). Dissolution of ibogaine-HCl to prepare the samples for i.p. injection was carried out using warm saline that was previously degassed by nitrogen bubbling.

2.2 Experimental Animals

Thirty-six Wistar adult rats (270-300 g) were used in this study and assigned to one of the following groups: Vehicle group at 3 and 24 h (n= 6 per each group); Ibogaine 20- (I_{20}) treated group at 3 h and 24 h (n= 6 per each group) and Ibogaine 40- (I_{40}) treated group at 3 and 24 h (n= 6 per each group). Animals were housed four to five per cage and maintained on a 12-h light/dark cycle (lights on at 07.00 h) with food and water freely available before and after i.p. injection of vehicle or ibogaine until behavioral testing and sacrifice. All experimental procedures were conducted in agreement with the National Animal Care Law (#18611) and with the "Guide to the care and use of laboratory animals" (8th edition, National Academy Press, Washington D. C., 2010). Furthermore, the Institutional Animal Care Committee approved the experimental procedures. Adequate measures were taken to minimize pain, discomfort or stress of the animals, and all efforts were made to use the minimal number of animals necessary to obtain reliable scientific data.

2.2.1 Behavioral analysis

Animals were brought to the experimental room in their home cages, identified and weighed prior to the behavioral test. An open field (OF) apparatus consisting of a square area (45 cm wide×45 cm long×40 cm high) with transparent plastic walls indirectly illuminated (35 luxes) to avoid reflection and shadows were employed. The OF was placed in a quiet experimental room with controlled temperature (22 ± 2 °C). As rats were not habituated to the OF before drug or vehicle administration, novelty-induced motor activity was automatically recorded by a camera connected to a computer equipped with the Ethovision XT 12.0 software (Noldus, Netherlands) located above the OF. Using this video tracking software, we specifically measured the total distance traveled in meters (m) during 30 min, starting 3 and 24 h after ibogaine or vehicle administration. Animals were randomly assigned to different experimental groups and were used only once. Taking into account that immediately after i.p. administration

ibogaine can produce a dose-dependent unusual motor profile and some prototypical serotonergic syndrome-related behaviors (e.g., tremor, flat body posture, forepaw treading) (Gonzalez et al. 2018), these specific behaviors were assessed by a trained investigator every 5 min (for a total of 30 min) starting 3 and 24 h after ibogaine administration. During all experiments, the OF was cleaned with 30% alcohol before placing the following rat. All experiments were done between 9 AM and 3 PM.

2.3 Ex Vivo Studies

2.3.1 Brain dissection

Three or twenty-four hours after I_{20} , I_{40} or vehicle (i.p.) injection, animals were sacrificed by decapitation and the brains were carefully removed and chilled in ice cold saline. According to Paxinos and Watson (Paxinos and Watson, 2005), the whole NAcc (shell and core), PFC (including mPFC), Substantia Nigra (SN, pars compacta-SNpc and pars reticulata-SNpr) and VTA were dissected out on ice and the tissue obtained was immediately frozen and rapidly stored at -80°C until the processing day (Scorza et al., 1997; Meikle et al., 2013). Representative examples of coronal section at the level of each dissected brain area are shown in Figure 2.

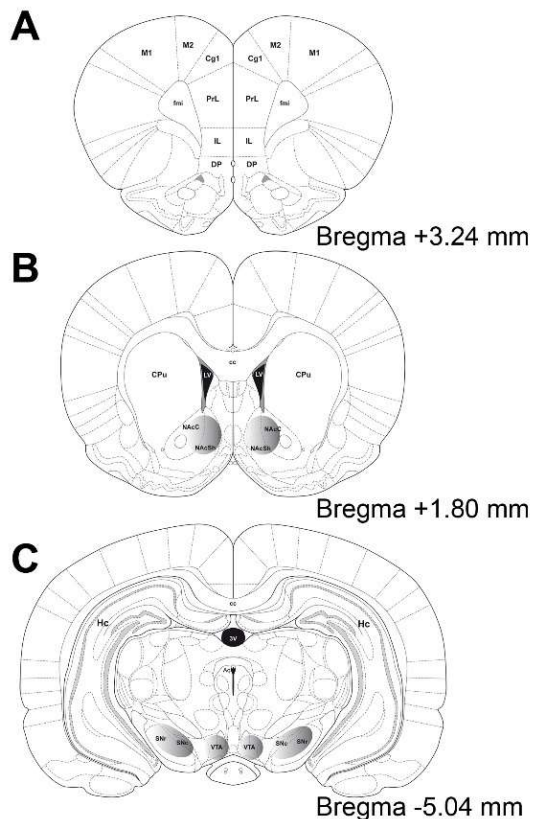


Figure 2. Dissected brain areas. Figure shows a schematic diagram of coronal sections at three different levels. A: +3.24 for PFC, B: + 1.80 for NAcc, and C: -5.04 for VTA and SN, from Bregma) according to Paxinos and Watson (2005) atlas. In A, a complete slice (2 mm) was taken for PFC (including the mPFC). In B and C, gray areas indicate an approximately extension of the brain tissue dissected for NAcc ((this region was punched by using a sample corer with an inner diameter of 2 mm), VTA and SN, respectively. Cg, cingulate cortex; PrL, prelimbic; IL, infralimbic; M1 and M2, motor cortex; CPu, corpus striatum; NAcC and NAcSh, nucleus accumbens core and shell; Hc, hippocampus; CC, corpus callosum; 3V, third ventricle, SNc and SNr, compact and reticular substantia nigra; VTA, ventral tegmental area.

2.3.2 Semiquantitative qPCR

For RT-PCR analysis total RNA was extracted from the different brain regions using Trizol reagent (ThermoFisher Scientific) followed by chloroform extraction and isopropanol precipitation. Possible DNA contaminations were eliminated with DNase treatment using DNase free Kit (Thermo Fisher Scientific). RNA quality was evaluated by agarose gel electrophoresis followed by ethidium bromide staining and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). 500 ng of this total RNA was reverse-transcribed using 200 U M-MLV-reverse transcriptase (Thermo Fisher Scientific) following manufacturer instructions. 25 ng of the resulting cDNA was diluted in Biotools Quantimix Easy master mix (Biotools) in 10 µl volume. All reactions were performed in triplicates in strip tubes (Axygen® Brand Products), using specific forward and reverse primers. The sequences of the quantitative PCR primers (IDT, Integrated DNA Technologies) used are as follows: for GAPDH F: 5'- CAC TGA GCA TCT CCC TCA CAA - 3' and R: 5'- TGG TAT TCG AGA GAA GGG AGG - 3', for BDNF F: 5'- GAG GGG TAG ATT TCT GTT TGT T -3' and R: 5'- TTG CCT TAA TTT TTA TTC GTT T -3', for GDNF F: 5'- AAA TCG GGG GTG CGT CTT AAC T -3' and R: 5'- AAC ATG GGC CTA CCT TGT C -3', for NGF F: 5'- AAG TTA TCC CAG CCA AAC TA -3' and R: 5'- ATG TCA GTG TTG GGA GTA GG -3'. PCR amplification was done over 40 cycles using a Rotor-Gene 6000 System (Corbett Life Science) and data were analyzed using Rotor Gene 6000 software (Corbett Life Science). Quantification was performed with $\Delta\Delta C_t$ method using rats treated with vehicle as a negative control, and GAPDH mRNA as reference.

2.3.3 Western Blot analysis

The selected brain regions were sonicated in a lysis buffer containing 50 mM NaCl, 50 mM HEPES, 2 mM sodium orthovanadate, 1% Triton X-100, and SigmaFAST Protease inhibitor cocktail (Sigma-Aldrich). After quantification and denaturation, the samples were loaded and separated by 12% SDS-PAGE gels and then transferred into a nitrocellulose membrane. The membranes were incubated for 1 h in blocking solution (BS: 5% Bovine serum albumin, 1% Tween 20 in PBS), and incubated overnight at 4°C with primary antibodies to GDNF (1:500 in BS; Abcam ab119473), BDNF (1:400 in BS; Promega G1641), or proBDNF (1:500 in BS; Invitrogen PA1-18360), together with anti-alpha-tubulin (1:3000 in BS; Abcam ab184613) as loading control. Afterwards, the membranes were washed and incubated for 1 hour at room temperature with IRDye 680RD/ IRDye 800CW-Conjugated Goat Anti-Mouse IgG/ Goat Anti-Rabbit IgG/ Donkey Anti-Chicken IgG secondary antibodies (1:15000 in PBS each, LI-

COR Biosciences #926-68070, #926-32210, #926-68071, #926-32211, and #925-32218). The Odyssey system (LI-COR Biosciences) was used to detect the bands. Quantification of band intensity was performed using Image Studio software version 5.2.5.

2.4 Data analysis

GraphPad Prism software 5 was used to design figure graphs and data analysis. Data are presented as mean \pm S.E.M values. Six animals per group were assessed for comportamental and PCR studies. In some cases, some data was excluded from the analysis due to insufficient sample or high deviation from the mean of the group, rendering a lower n , but never smaller than 4. The total sample size (N) is given in figure legends and the sample for each treatment (n) can be observed in the scatter plot graphs in each figure. For western blot analysis, samples from 4 animals per group were assessed. Data from qPCR and western blot were analyzed and compared by one-way ANOVA followed by post hoc Tukey's Multiple Comparison Test. In all cases, statistical significance was set at $p < 0.05$. General P and F values from ANOVA, and p values from Tukey's multiple comparison test are provided in figure legends for each data set when significance is reached. Data from motor activity were analyzed by two-way (treatment, time, and interaction between factors) ANOVA for repeated measures followed by Newman-Keuls multiple comparison post hoc test and Unpaired- t -test. In all cases, statistical significance was set at $p < 0.05$.

3.0 Results

In a previous study, we reported a very high impact of the I_{40} treatment on novelty-induced locomotion after two hours of ibogaine administration and the concomitant induction of some of the behavioral signs related to the serotonergic syndrome (Gonzalez et al., 2018). Thus, we decided to analyze the behavioral effect of ibogaine treatment in the time points used in the present study. The behavioral response induced by ibogaine is shown in Figure 3. Compared to the control group, novelty-induced locomotion was not altered by I_{20} at any evaluated time (data not shown). Whereas I_{40} was not effective to induce any behavioral alterations 3 h after i.p. administration, it elicited a significantly reduction of the animal locomotion 24 h after injection (Fig. 3 A and B, respectively). No abnormal behaviors were present for both time points and animals were qualitatively indistinguishable from the vehicle group animals (data not shown). Immediately after each behavioral test, animals were sacrificed to pursue brain dissection for the qPCR and Western Blot studies.

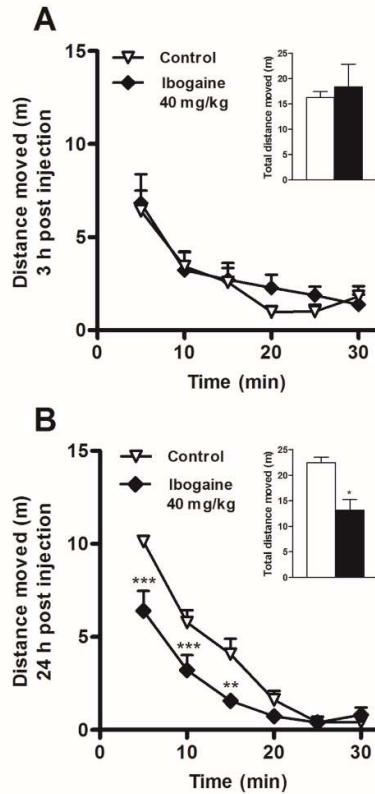


Figure 3. Effects of ibogaine administration on locomotor activity.

Locomotor activity of rats was recorded in the OF test during 30 min, at 3 h (A) and 24 h (B) after ibogaine (40 mg/kg) i.p. administration. The inset graphs represent the total locomotor activity (30 min). Data are expressed as mean + SEM. Data were analyzed by the two-way ANOVA of repeated measured followed by Newman-Keuls test and Unpaired *t*-test (insets). For 24 h after treatment, two-way ANOVA revealed a significant effect of the treatment $F_{(1,8)} = 11.14$, $P < .01$, time $F_{(5,40)} = 66.56$, $P < .001$, and treatment x time interaction $F_{(5,40)} = 4.85$, $P < .01$. *, respective to saline group. *** $p < .001$; ** $p < .01$; * $p < .05$. $N = 18$, $n = 6$ per group.

3.1 qPCR quantification of NFs mRNA

qPCR results for the GDNF (Figure 4) showed that ibogaine acute administration differentially regulated GDNF mRNA expression levels in the selected brain regions in a dose and time-dependent manner. At 3 hours, no changes in the GDNF mRNA expression was found for both doses of ibogaine in all the studied areas. In contrast, after 24 hours of treatment, changes in the expression of GDNF were found in a dose and site-specific manner. While the I_{20} dose did not affect the GDNF expression in any of the studied areas, the I_{40} dose selectively increased GDNF mRNA content in the midbrain regions: VTA (12-fold increase compared to the control group) and SN (6-fold increase vs the control group) with no appreciable effects in the PFC and NAcc.

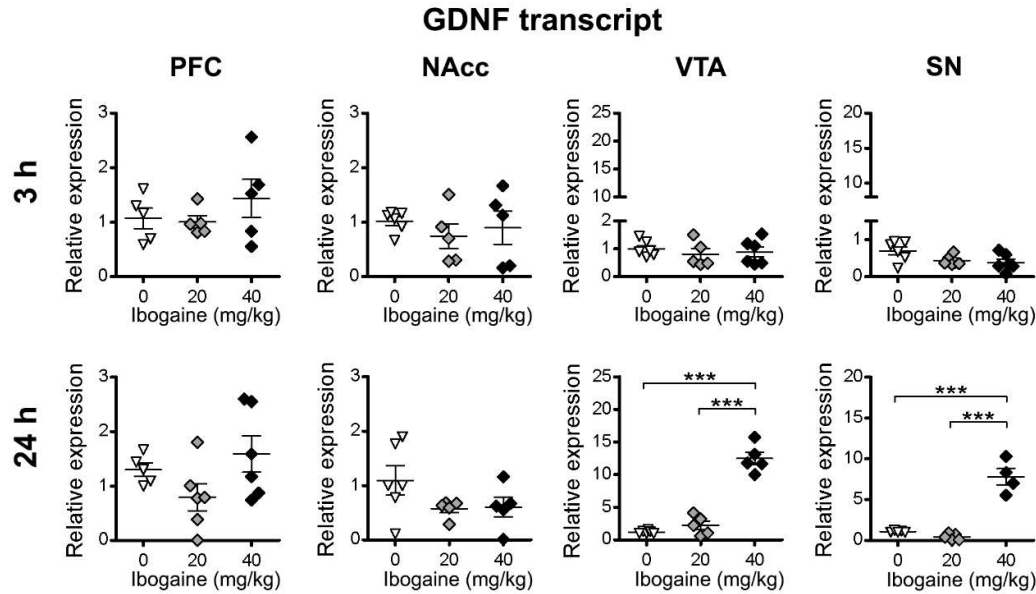


Figure 4. Effects of ibogaine administration on GDNF expression in specific brain areas. Quantitative analysis of GDNF transcript levels in the indicated brain areas after 3 hours (upper panels) or 24 hours (lower panels) of vehicle (0), 20 or 40 mg/kg ibogaine administration. For 24 h after treatment VTA, $N = 16$, $P < .0001$, $F_{2,13} = 96.11$; For 24 h after treatment SN, $N = 14$, $P < .0001$, $F_{2,11} = 60.75$; *** $p < .001$ between indicated groups.

For BDNF, ibogaine treatment produced an appreciable downregulation of its expression in the PFC at 3 hours after injection (1.7 and 2-fold decrease for I_{20} and I_{40} respectively, compared to control), while no response was seen for the other brain areas at this time point (Figure 5). At 24 hours, ibogaine administration upregulated the mRNA expression of BDNF in all the brain regions studied in a dose-dependent manner (Figure 5). A large effect was found in the NAcc for both doses of ibogaine (220-fold increase compared to the control for I_{20} , and 340-fold increase for I_{40}). The I_{20} dose increased BDNF expression in PFC (55-fold increase compared to the control) but not in the VTA or SN. On the other hand, in addition to the NAcc, the I_{40} dose also upregulated BDNF expression in PFC (107-fold increase compared to the control), VTA (43-fold increase compared to the control) and SN (21-fold increase compared to the control).

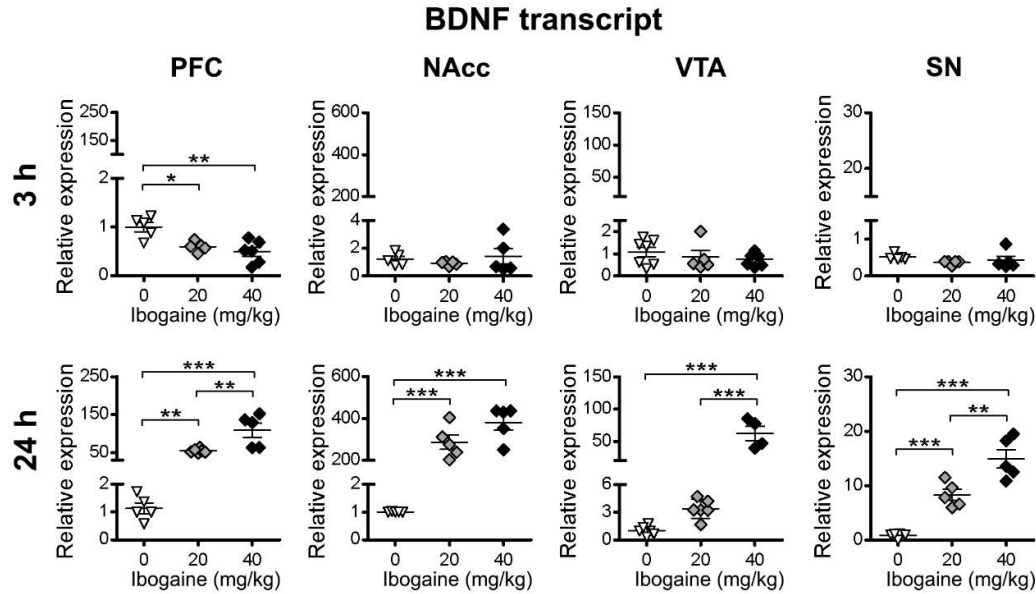


Figure 5. Effects of ibogaine administration on BDNF expression in specific brain areas. Quantitative analysis of BDNF transcript levels in the indicated brain areas after 3 hours (upper panels) or 24 hours (lower panels) of vehicle (0), 20 or 40 mg/kg ibogaine administration. For 3 h after treatment PFC, $N = 16$, $P < .0001$, $F_{2,13} = 9.80$; For 24 h after treatment PFC, $N = 16$, $P < .0001$, $F_{2,13} = 25.26$; For 24 h after treatment NAcc, $N = 15$, $P < .0001$, $F_{2,12} = 46.62$; For 24 h after treatment VTA, $N = 14$, $P < .0001$, $F_{2,11} = 46.46$; For 24 h after treatment SN, $N = 16$, $P < .0001$, $F_{2,13} = 45.50$; * $p < .05$, ** $p < .01$ and *** $p < .001$ between indicated groups.

For NGF (Figure 6), no difference in the content of mRNA was found 3 hours after ibogaine treatments. At 24 hours, an upregulation of NGF mRNA content was found in: PFC (14-fold increase compared to the control), NAcc, (15-fold increase compared to the control), VTA (11-fold increase compared to the control), and SN (4-fold increase compared to the control). For the I_{20} dose a significant effect was only found in the PFC (7-fold increase compared to the control) and VTA (5-fold increase compared to the control). However, the levels of increase in the NGF mRNA were not as high as those for BDNF.

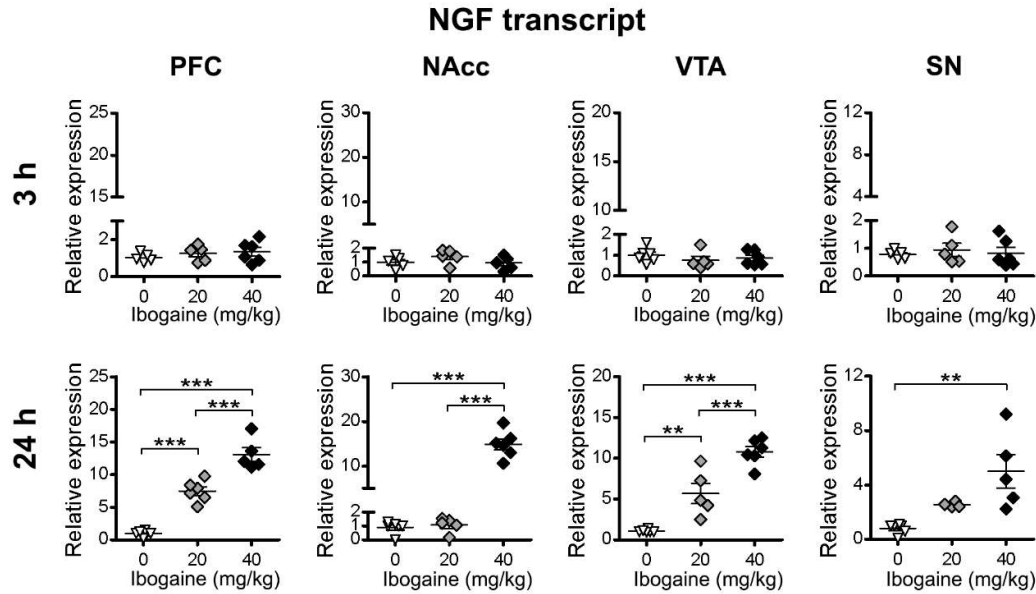


Figure 6. Effects of ibogaine administration on NGF expression in specific brain areas. Quantitative analysis of NGF transcript levels in the indicated brain areas after 3 hours (upper panels) or 24 hours (lower panels) of vehicle (0), 20 or 40 mg/kg ibogaine administration. For 24 h after treatment PFC, $N = 17$, $P < .0001$, $F_{2,14} = 76.40$; For 24 h after treatment NAcc, $N = 17$, $P < .0001$, $F_{2,14} = 107.1$; For 24 h after treatment VTA, $N = 17$, $P < .0001$, $F_{2,14} = 44.88$; For 24 h after treatment SN, $N = 16$, $P = .0050$, $F_{2,13} = 8.16$; ** $p < .01$ and *** $p < .001$ between indicated groups.

3.2 GDNF, BDNF and proBDNF protein content by Western Blot

Considering the changes found for the expression of NFs after 24 hours of ibogaine administration, we decided to analyze the content of mature proteins BDNF and GDNF for all the studied brain regions, because of their involvement in the addictive behavior. Precursor of BDNF, proBDNF was also considered since it is well described that it shows opposite effects to the mature protein because of a higher affinity to the p75 receptor (Woo et al., 2005; Xu et al., 2011; Sun et al., 2012). For GDNF, a single dose of ibogaine affected mature protein content in a region- and dose-dependent manner (Figure 7). While no changes in GDNF content were observed for I_{20} in any of the studied regions, GDNF content was increased in VTA for the I_{40} dose (2-fold increase compared to the control group). No effect was observed in the GDNF content at the NAcc, SN and PFC in comparison to the control group. For BDNF no significant change in the mature protein content was detected for all the studied regions for both doses of ibogaine. Nevertheless, in the case of proBDNF we found a selective increase in the protein content for I_{20} and I_{40} in the NAcc (2.7 and 2.8-fold increase for I_{20} and I_{40} doses respectively, compared to control), while no significant change was detected in the other brain areas.

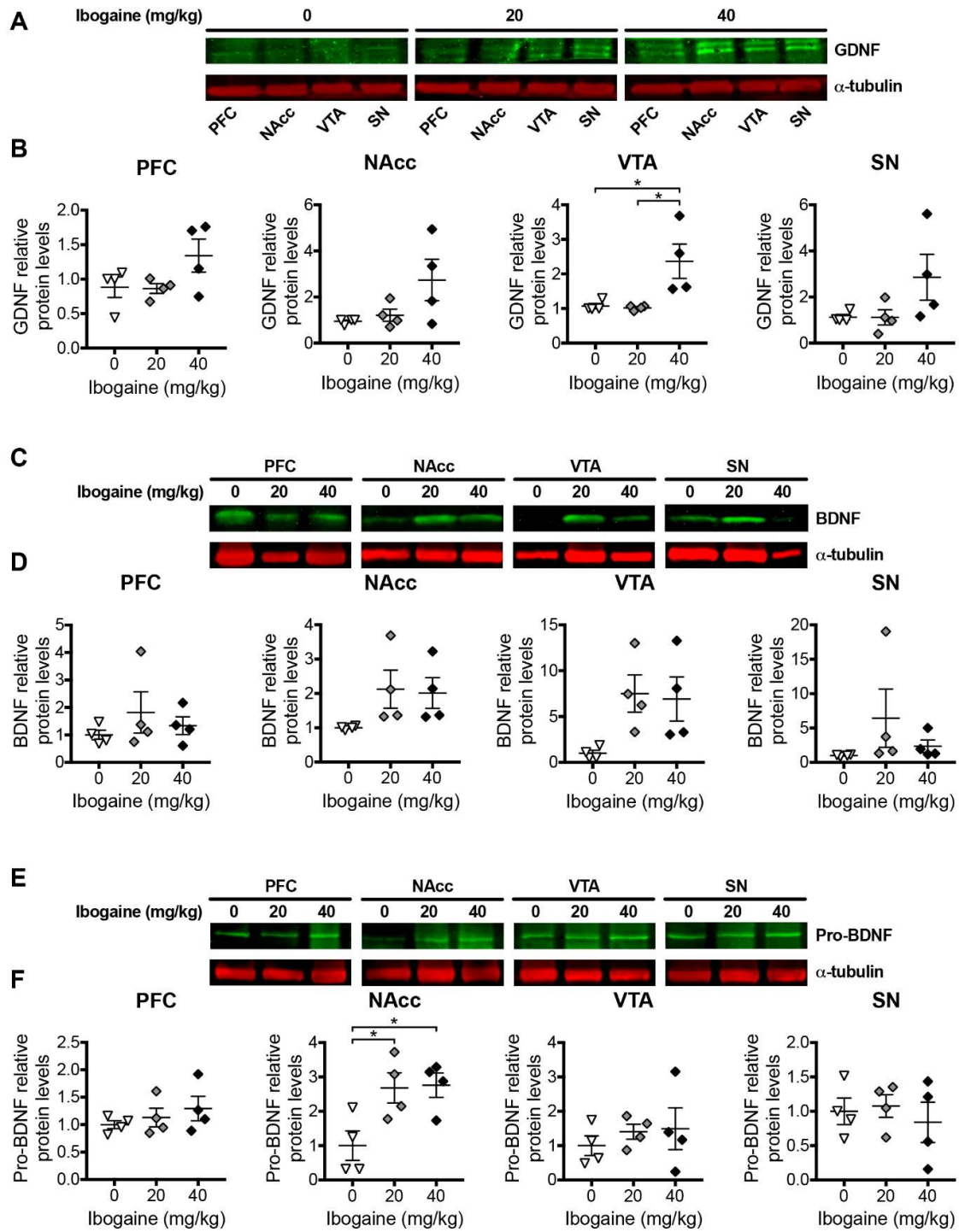


Figure 7. Effects of ibogaine administration on GDNF, BDNF and proBDNF protein levels in specific brain areas. Western blot analysis of GDNF (A-B), BDNF (C-D) and proBDNF (E-F) protein levels in the indicated brain areas after 24 hours of vehicle (0), 20, or 40 mg/kg ibogaine administration. A representative image from immunostained membrane of each condition is shown (A, C, E) with the corresponding quantification below (B, D, F). Data represent mean \pm SEM of $n = 4$ biological replicates assayed in triplicate. For GDNF/VTA, $N = 12$, $P < .05$, $F_{2,9} = 6.86$; For proBDNF/NAcc, $N = 12$, $P < .05$, $F_{2,9} = 5.87$; * $p < .05$ between indicated groups.

4.0 Discussion

In the present study, we have demonstrated that ibogaine administration simultaneously alters the expression of the two main trophic factors involved in addictive behavior: GDNF and BDNF, but also NGF in rat brain regions related to the dopamine neurotransmission in a dose and time dependent manner. In addition, we showed that after 24 h of treatment, I₄₀ selectively increased the content of mature GDNF in the VTA, while proBDNF content was increased selectively in NAcc by both doses. Considering that dopamine neurotransmission, specifically in the mesocorticolimbic pathway, is related to rewarding/reinforcing and motivational actions of most drugs of abuse (Di Chiara and Imperato, 1988; Koob and Bloom, 1988; Kalivas and Volkow, 2005) our findings contribute to shed light on a mechanism underlying the anti-addictive action of ibogaine. According to previous pharmacokinetics reports in rats using i.p. administration (Baumann et al., 2001), ibogaine concentration in blood rapidly decreases in the first hour while noribogaine concentration is at maximum at 2.4 h and lasts up to 24 h. Regarding concentrations in the brain, no appreciable amounts of noribogaine have been found in the brain tissue of rodents 19 h after ibogaine i.p. administration (Pearl et al., 1997). Given these previous reports, we chose to study NFs expression/content in the selected brain areas at 3 h, where the parent drug and its metabolite are present in relevant concentrations, and at 24 h where both drugs are no longer detectable in the brain. In this manner, the observed effects found at 24 h involving NFs expression and the outcome of the locomotion study, would be due to signaling mechanisms elicited by the drug which remain after it has been cleared from the brain, and not from the acute effects of ibogaine/noribogaine. These results may provide the rationale for the previously reported long-lasting anti-addictive effects of ibogaine (Brown, 2013).

Regarding the motor function, a decrease in the novelty-related motor activity was observed 24 h after I₄₀ (while 3 h after the same treatment, animals displayed a similar activity than the control). There is no evidence at this point to establish a potential connection between this intriguing behavior and the observed changes in NFs expression. In this regard, considering the changes in the expression of NFs at 24 h in the SN, it is plausible that a neurochemical imbalance in the basal ganglia output may underlie the changes in the motor activity (Day et al., 2008; Calabresi et al., 2014). Also, since the induced increase of GDNF content in the VTA by ibogaine has been proposed previously as a putative mechanism to reduce motivational behaviors in alcohol self-administration paradigms (Carnicella et al. 2009; 2010; He et al. 2005), we cannot rule out that this acute motor impairment is related to this neurochemical effect eliciting a decrease in the animal overall motivation. In order to address this hypothesis,

another experimental design would be needed, including specific behavioral paradigms. Further studies should also include the relationship between changes in the expression of NFs and other factors which may be altered in ibogaine-treated animals at this time point.

At 3 h after I₂₀ and I₄₀ treatments, no alteration of GDNF expression was found in all the studied brain areas. This is in contrast with previous work reported by He et al. (2005), where a significant GDNF upregulation was found 3 h after I₄₀ treatment in the midbrain of rats. Differences between both reports may rely in the analyzed brain regions. We studied the effect of ibogaine administration on GDNF expression in specific brain areas (PFC, VTA, NAcc and SN), while the whole midbrain was used in the study by He et al. On the other hand, after 24 h, we found that the I₄₀ dose increases GDNF expression and mature protein content specifically in the rat VTA, which was also found in the whole midbrain at this time point in the mentioned previous report. In this manner our study identifies the VTA as the key brain region of the mesocorticolimbic system where GDNF is upregulated after 24 h of ibogaine administration. This finding is important since the ability of ibogaine to attenuate ethanol self-administration had previously been proposed to be mediated, at least in part, by the increase in GDNF content in the VTA. (He et al., 2005; He and Ron, 2006) Furthermore, we show that I₂₀ administration does not increase GDNF expression in any of the studied brain areas, which is in accordance with the observation that this dose was not effective in reducing drug self-administration in the majority of previous studies in rodents (Glick et al., 1991; Cappendijk and Dzoljic, 1993; Glick et al., 1994; Dworkin et al., 1995). In addition, our results are in line with the reports indicating that GDNF infusions into the VTA has been effective in reducing drug self-administration or conditioned place preference in rodents (for cocaine and alcohol) (Messer et al., 2000; He and Ron, 2006; Carnicella et al., 2008; Carnicella et al., 2009), and with the proposal that upregulation of the GDNF pathway represents a potential strategy to treating SUDs (Carnicella and Ron, 2009). Lastly, I₄₀ administration increases GDNF expression in the SN, which was not accompanied with a significant increase of the GDNF protein content at this time point.

Since in this study the mRNA content was analyzed in whole tissue from the different regions, the precise cell source of GDNF was not identified. Literature indicates that GDNF may be produced either by neuronal or glial cells (Schaar et al., 1993; Moretto et al., 1996; Pochon et al., 1997). Ibogaine treatment upregulated GDNF secretion in dopaminergic neuron-like SHSY5Y cells in culture, (He and Ron, 2006) however since astrocytes are a major source of NFs (Moretto et al., 1996) the glial origin cannot be excluded. This observation deserves further attention, since an increase in GDNF in different cell types in regions containing

dopaminergic neurons by ibogaine/noribogaine administration could be important for future development of therapeutics for neurodegenerative disorders.

With regard to BDNF, a selective downregulation of its expression in the PFC for both doses of ibogaine was found after 3 h of administration, while no changes in other areas were observed. Ibogaine and noribogaine administration in rats stimulate the secretion of corticosterone, being ibogaine a more potent releaser (Baumann et al., 2001). Since corticosterone decreases BDNF expression in the frontal cortex (Dwivedi et al., 2006; Huang et al., 2011), ibogaine induced corticosterone secretion during the first hours after treatment (where ibogaine concentrations in blood are high), could be the reason behind this result. In contrast, at 24 h, an impressive upregulation of BDNF expression was found, which was much more pronounced compared to the effect on GDNF and NGF expression in all the studied brain areas at this time point. Nevertheless, this high effect on BDNF expression was not reflected on an increase in the content of BDNF mature protein, since no significant differences were found between both treatments and the control group at this time point, although trending toward increased BDNF protein levels in NAcc and VTA for both doses (Figure 7). Since BDNF is synthesized in a precursor form, we included proBDNF in our experimental design. A selective increase in the proBDNF content was selectively found for NAcc for both ibogaine doses. It is known that the mature BDNF protein and its precursor proBDNF have opposite effects on neuronal protection, axonal growth, maturation of dendrites and synaptic plasticity, owing to different affinities of each form to the TrkB and p75 receptors.(Xu et al., 2011; Benarroch, 2015; Borodinova and Salozhin, 2016). These opposite effects have been hypothesized specifically in the NAcc in the context of animal models of depression as learned helplessness and social defeat stress, where BDNF content is increased while proBDNF content is decreased compared to control animals in this brain area (Shirayama et al., 2015; Yang et al., 2016; Montagud-Romero et al., 2017). In this regard, since it is well-documented that an increase in BDNF content in the NAcc increases cocaine-seeking behavior (Graham et al., 2007) and vulnerability to substance abuse (Krishnan et al., 2007; Burke and Miczek, 2015), an increase in proBDNF in this brain area could have an opposite impact. In this line of reasoning, the increase in proBDNF content in NAcc generated by ibogaine after 24 h of administration in rats, could also be implicated in ibogaine's effect in drug self-administration paradigms. Further experiments are required to address this hypothesis.

Despite implicit assumption that differentially expressed mRNAs are reflected in protein content, numerous previous studies comparing mRNA and protein levels concluded that the correlation is poor (de Sousa Abreu et al., 2009; Maier et al., 2009). While the increase

in GDNF mRNA expression was linked to augmented mature protein content, our data showing an impressive increase in BDNF mRNA expression and no changes in mature protein are intriguing. The possibility exists that the time frame of protein synthesis is different for both NFs, however many other factors should be considered to explain this incongruousness. These include post-transcriptional regulation, for example miRNA-based translation repression or alternative splicing, or translational and post-translational modifications. Indeed, it has been previously described that sortilin, an intracellular chaperon, acts as a regulatory switch for delivery of BDNF to the regulatory secretory pathway or to degradation in the lysosome, modulating in this way the neurotrophic factor availability (Evans et al., 2011). Interestingly, BDNF levels have been shown to be modified in PFC after chronic ethanol exposure (Yang et al., 2017).

How does ibogaine administration produce this long-term upregulation of GDNF and BDNF? It is well established that an increase in serotonin transmission leads to an increase in BDNF expression/signaling both *in vitro* and *in vivo* (Rantamaki et al., 2007; Popova et al., 2017). In addition, serotonin and SSRIs (Selective Serotonin Re-uptake Inhibitors) induce GDNF expression *in vitro* (Hisaoka et al., 2001; Mercier et al., 2004; Tsuchioka et al., 2008; Golan et al., 2011), and recently it has been shown that chronic treatment in mice using SSRIs induce GDNF content in SN and Striatum (Shadfar et al., 2018). It is well-established that ibogaine and noribogaine increase serotonin transmission (Wei et al., 1998; Wells et al., 1999; Baumann et al., 2001). Both substances are serotonin-reuptake inhibitors (Jacobs et al., 2007; Bulling et al., 2012), and noribogaine is more potent at increasing serotonin levels in the NAcc than ibogaine, which correlates with the ability of both compounds to inhibit SERT *in vitro* (IC₅₀ of 3.85 and 0.18 μ M for ibogaine and noribogaine, respectively) (Baumann et al., 2001). In this manner, a sustained enhancement on serotonin transmission due to ibogaine and its long-lasting metabolite noribogaine could account, at least in part, for the observed effect on BDNF and GDNF expression after 24 h of ibogaine administration.

Finally, in addition to GDNF and BDNF, ibogaine also modulated the expression of NGF 24 h after treatment, while no changes were found at 3 h. The effect of NGF administration in specific brain areas on drug-seeking behavior has been less studied in comparison to GDNF and BDNF, and scarce data is available on the effects of NGF in brain regions related to the dopaminergic mesocorticolimbic circuitry. Nevertheless, NGF (as other neurotrophins) is likely involved in mediating important responses related to chronic intake of drugs of abuse, as illustrated by a previous study that points to a role NGF plays in the central amygdala in the development of increased sensitivity to opioid reward (Bie et al., 2012).

The modifications in NFs levels induced by ibogaine/noribogaine, may underlie neuroplasticity processes in the discrete brain regions analyzed as has been described by several drugs used in clinical practice including drugs of abuse (Castren and Antila, 2017). Most of these drugs regulate the expression of NFs, reactivating a process defined as induced plasticity (iPlasticity), which allows networks reorganization in the adult brain (Castren and Antila, 2017).

5.0 Conclusions and future perspectives

This study demonstrates for the first time that ibogaine administration simultaneously alters the expression of GDNF, BDNF and NGF in rat brain regions related to the dopamine neurotransmission in a dose and time dependent manner. Our results add relevant information concerning specific brain areas involved in the increment of GDNF levels (VTA) as a putative mechanism of action underlying the anti-addictive effect of ibogaine. In addition, we showed that only I₄₀ promoted this increase in GDNF content, which is in accordance with previous reports where the I₂₀ treatment was not effective in reducing drug self-administration in rodents (Glick et al., 1991; Cappendijk and Dzoljic, 1993; Glick et al., 1994; Dworkin et al., 1995). Also, we found that both doses of ibogaine produced an increase in the proBDNF content in NAcc after 24 h of treatment, which could be an important factor mediating ibogaine anti-addictive long-lasting effects, in addition to the already highlighted increase in GDNF. Future experiments are needed in order to clarify these important implications.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest.

Author Contributions

SM, BG and SR did the qPCR experiments. EM and SR performed the Western Blot experiments. SM, BG, LMP and MP contributed in the experiments and in the analysis of the data. BG prepared the ibogaine HCl used in this study. JPP, PR and CS, did the experiments with animals and the brain dissection. IC, PC, GS and CS provided the funding for the experiments. MP, PC, GS, CS, DS and IC planned the experiments, and wrote the manuscript. All the authors participated in critical revision the manuscript, added important intellectual content, and approved the definitive version.

Funding

Agencia Nacional de Investigación e Innovación (ANII, Montevideo - Uruguay) Project Fondo María Viñas 103488, Comisión Sectorial de Investigación Científica (UdelaR) - Projects Grupos I+D 981 and 1104, and Programa de Desarrollo de Ciencia Básicas (PEDECIBA).

Acknowledgments

We would like to thank Agencia Nacional de Investigación e Innovación (ANII) and Comisión Sectorial de Investigación Científica (CSIC-UdelaR) for financial support. We thank Inés Carrera, Analía Richeri and Juan Pablo Rodríguez for their help in designing the manuscript figures. We also would like to thank Dr. Kenneth Alper for important discussions regarding ibogaine and noribogaine pharmacology.

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Supplementary Material

Ibogaine modifies GDNF, BDNF and NGF expression in brain regions involved in mesocorticolimbic and nigral dopaminergic circuits

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Supplementary Data

Ibogaine-HCl Preparation. Nuclear Magnetic Resonance spectra were obtained on a Bruker Avance DPX-400 instrument.

Voacangine (12-methoxy-16-carbomethoxyibogamine) isolated from *Voacanga Africana* root bark (see Material and Methods section in the manuscript)

¹H NMR (400 MHz, CDCl₃) δ 7.81 (s, 1H), 7.13 (d, *J* = 8.7 Hz, 1H), 6.93 (d, *J* = 2.3 Hz, 1H), 6.80 (dd, *J* = 8.7, 2.4 Hz, 1H), 3.84 (s, 3H), 3.70 (s, 3H), 3.55 (s, 1H), 3.44 – 3.33 (m, 1H), 3.26 – 3.08 (m, 2H), 3.03 – 2.86 (m, 2H), 2.81 (d, *J* = 8.5 Hz, 1H), 2.58 (dd, *J* = 11.8, 2.1 Hz, 1H), 1.94 – 1.81 (m, 2H), 1.73 (t, *J* = 11.1 Hz, 1H), 1.56 (dt, *J* = 22.0, 7.4 Hz, 1H), 1.49 – 1.39 (m, 1H), 1.37 – 1.28 (m, 2H), 1.15 – 1.08 (m, 1H), 0.90 (t, *J* = 7.3 Hz, 3H). **¹³C NMR** (100 MHz, CDCl₃) δ 175.71, 153.98, 137.51, 130.53, 129.19, 116.62, 111.81, 111.07, 110.12, 100.77, 99.99, 77.34, 77.03, 76.71, 57.52, 56.04, 55.14, 53.13, 52.59, 51.52, 39.15, 36.56, 32.03, 27.35, 26.76, 22.22, 11.68.

Ibogaine·HCl prepared by decarboxylation of voacangine (see Material and Methods section in the manuscript)

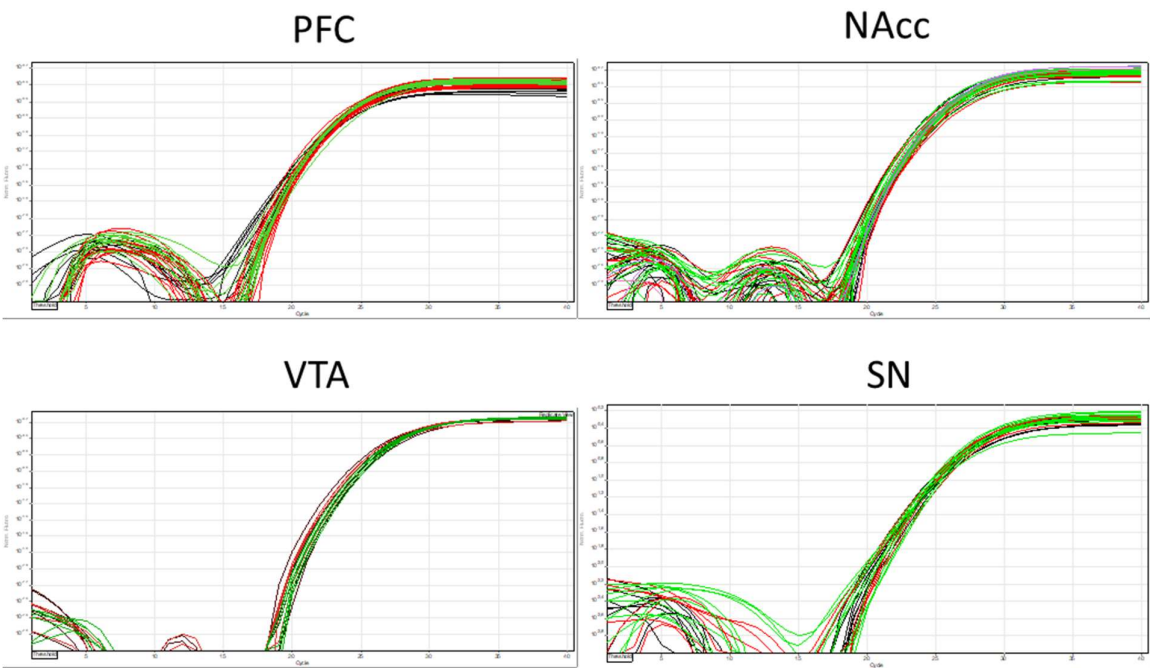
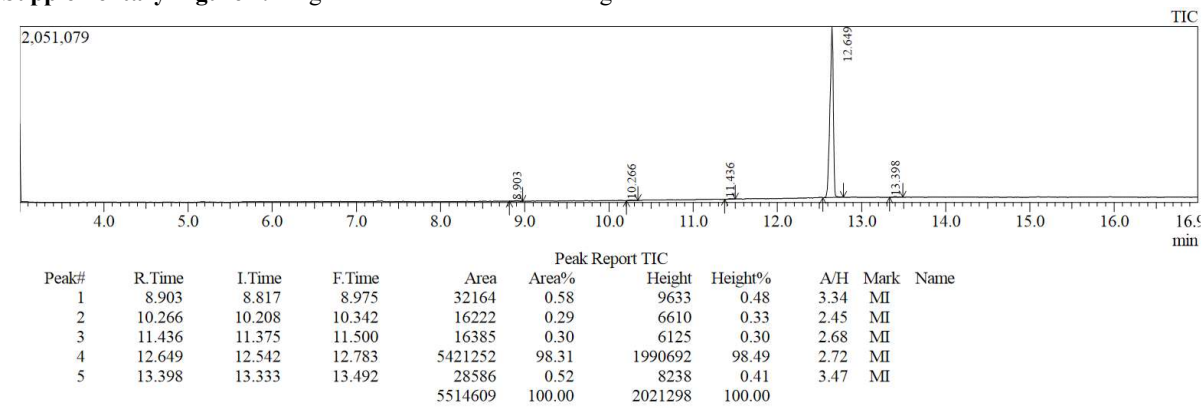
¹H NMR (400 MHz, CD₃OD) δ (ppm) = 7.19 (d, *J* = 8.9 Hz, 1H), 6.97 (d, *J* = 2.4 Hz, 1H), 6.76 (dd, *J* = 8.8, 2.5 Hz, 1H), 3.87 (s, 3H), 3.70 (dt, *J* = 13.4, 4.2 Hz, 1H), 3.63 – 3.53 (m, 2H), 3.45 – 3.34 (m, 3H), 3.31 – 3.14 (m, 2H), 2.32 (ddt, 13.5, 12.1, 2.7 Hz, 1H), 2.19 – 2.09 (m, 2H), 2.06 (hept, *J* = 7.5 Hz, 1H), 1.74 – 1.65 (m, 3H), 1.46 – 1.34 (m, 1H), 1.03 (t, *J* = 7.3 Hz, 3H) **¹³C NMR** (100 MHz, CD₃OD) δ(ppm) = 153.0, 139.1, 130.4, 128.5, 111.2, 111.1, 106.0, 99.5, 60.1, 56.0, 54.9, 50.5, 39.0, 35.1, 31.2, 28.8, 26.0, 23.9, 18.0, 10.5

To determine ibogaine-HCl purity (after crystallization and purification procedures described in the Materials and Methods section of the manuscript) Gas Chromatography analysis was carried out in a GC-MS Shimadzu QP 1100 EX instrument using the electron impact mode, 70 eV. For analysis sample was previously dissolved in aqueous NaOH 10% and extracted with Ethyl Acetate. Conditions: Column HP-5MS (30m x 0.25mm x 0.25um) Temperature Program 200 °C (Hold time, 2 minutes) to 300 °C (Hold time, 5 minutes) with a rate of 10 °C/min. Ibogaine purity was determined as 98.3% (See chromatogram below)

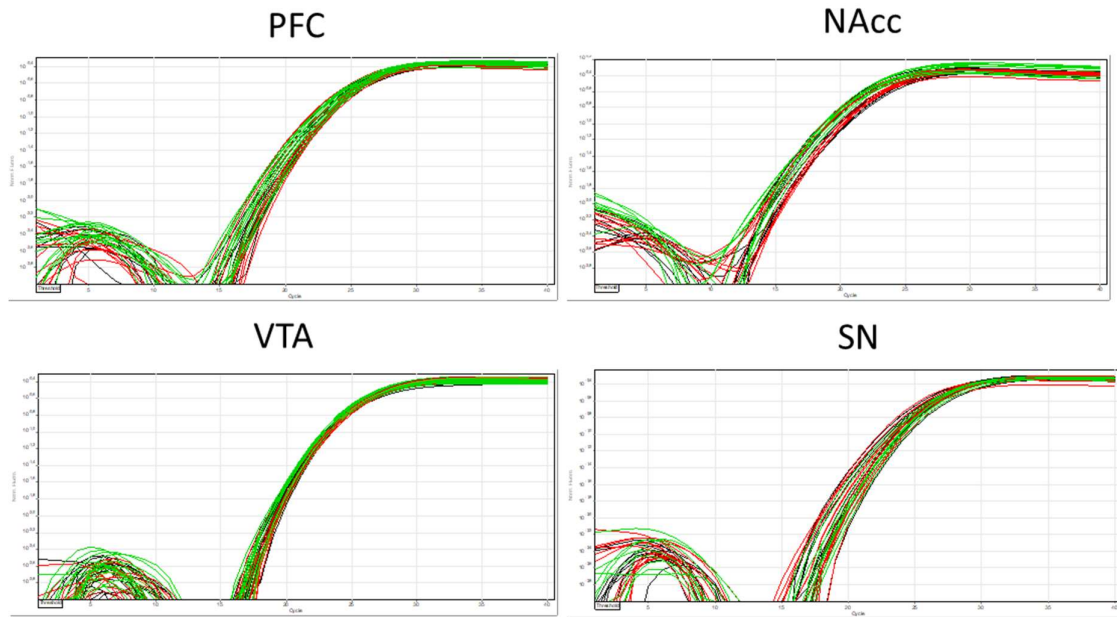
Supplementary Figures and Tables

Supplementary Figures

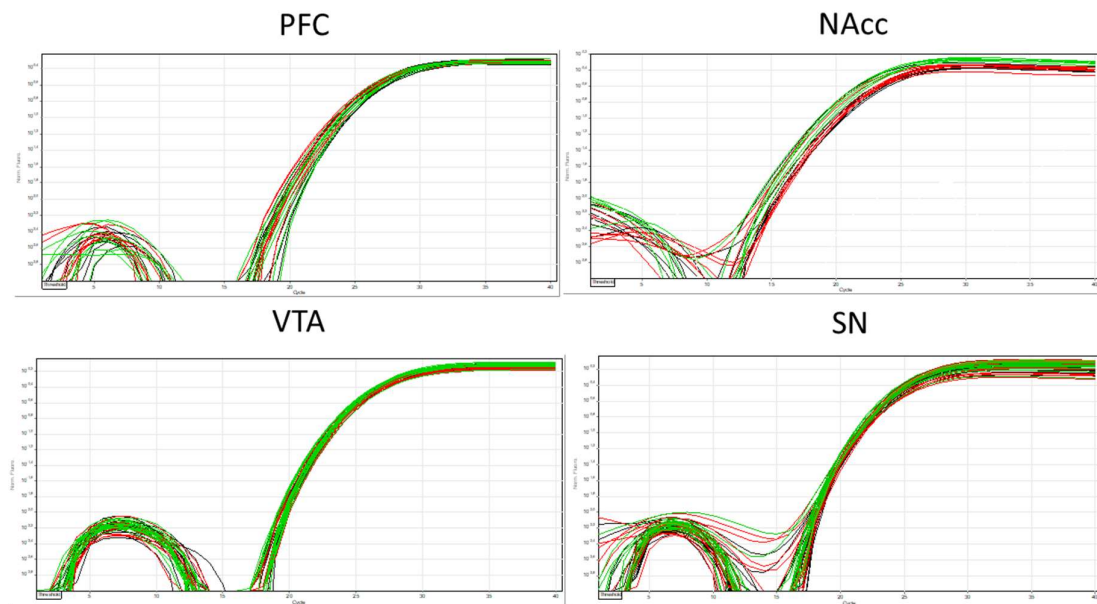
Supplementary Figure 1. Ibogaine-HCl GC-MS chromatogram.



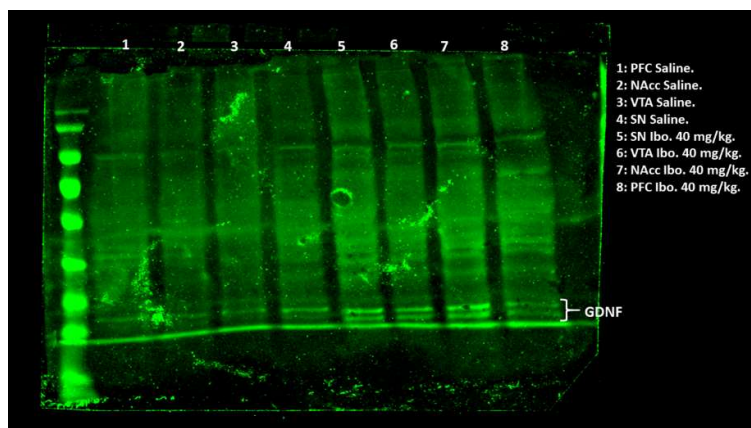
Supplementary Figure 2. PCR amplification curves showing the raw data of BDNF mRNAs at 3 hours after ibogaine administration. Norm fluorescence vs amplification cycle. Black vehicle, red 20 mg Ibogaine, green 40 mg Ibogaine.



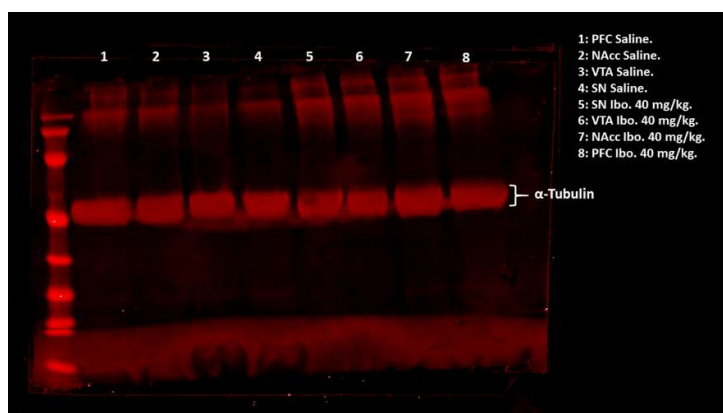
Supplementary Figure 3. PCR amplification curves showing the raw data of GDNF mRNAs at 3 hours after ibogaine administration. Norm fluorescence vs amplification cycle. Black vehicle, red 20 mg Ibogaine, green 40 mg Ibogaine.



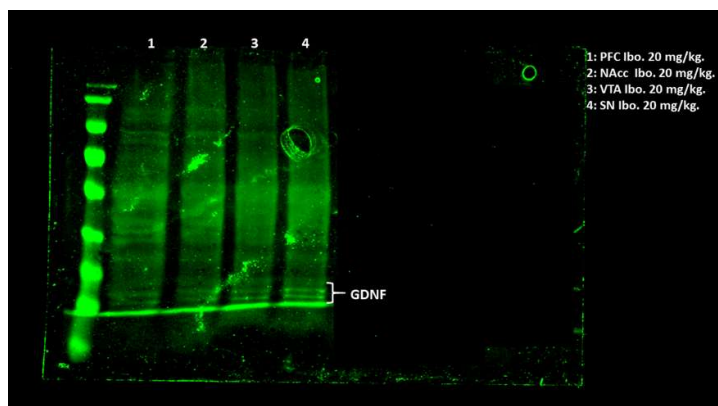
Supplementary Figure 4. PCR amplification curves showing the raw data of NGF mRNAs at 3 hours after ibogaine administration. Norm fluorescence vs amplification cycle. Black vehicle, red 20 mg Ibogaine, green 40 mg Ibogaine.



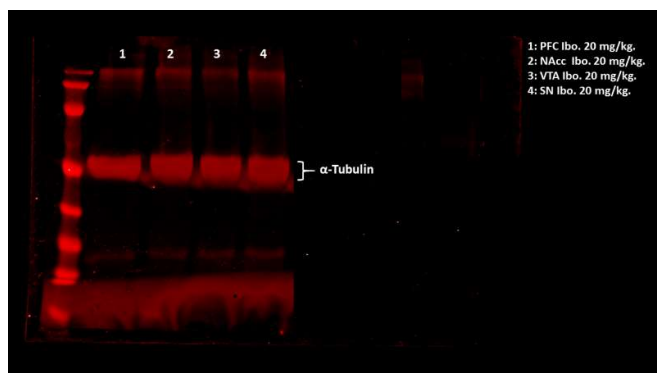
Supplementary Figure 5. Nitrocellulose membrane incubated with GDNF antibody, for Saline and Ibogaine 40 mg/kg samples.



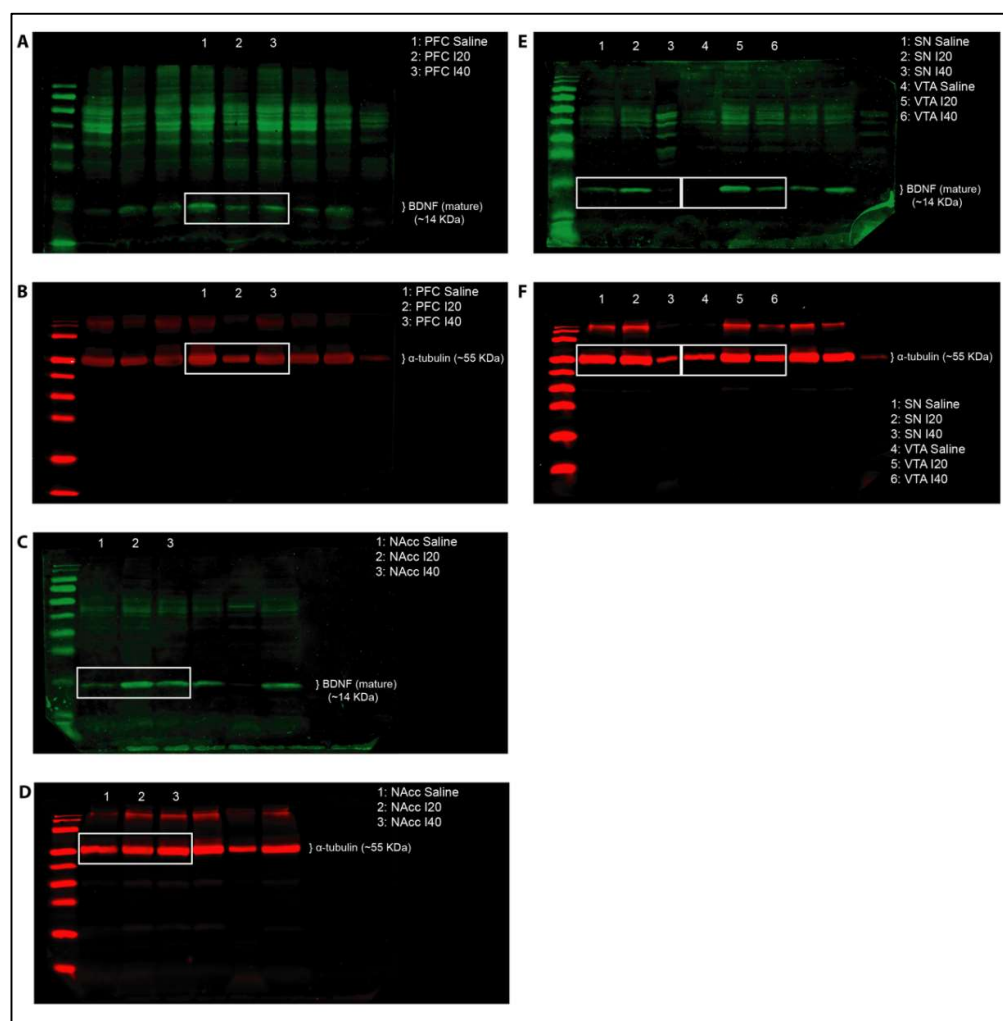
Supplementary Figure 6. Nitrocellulose membrane incubated with alpha-tubulin antibody, for Saline and Ibogaine 40 mg/kg samples.



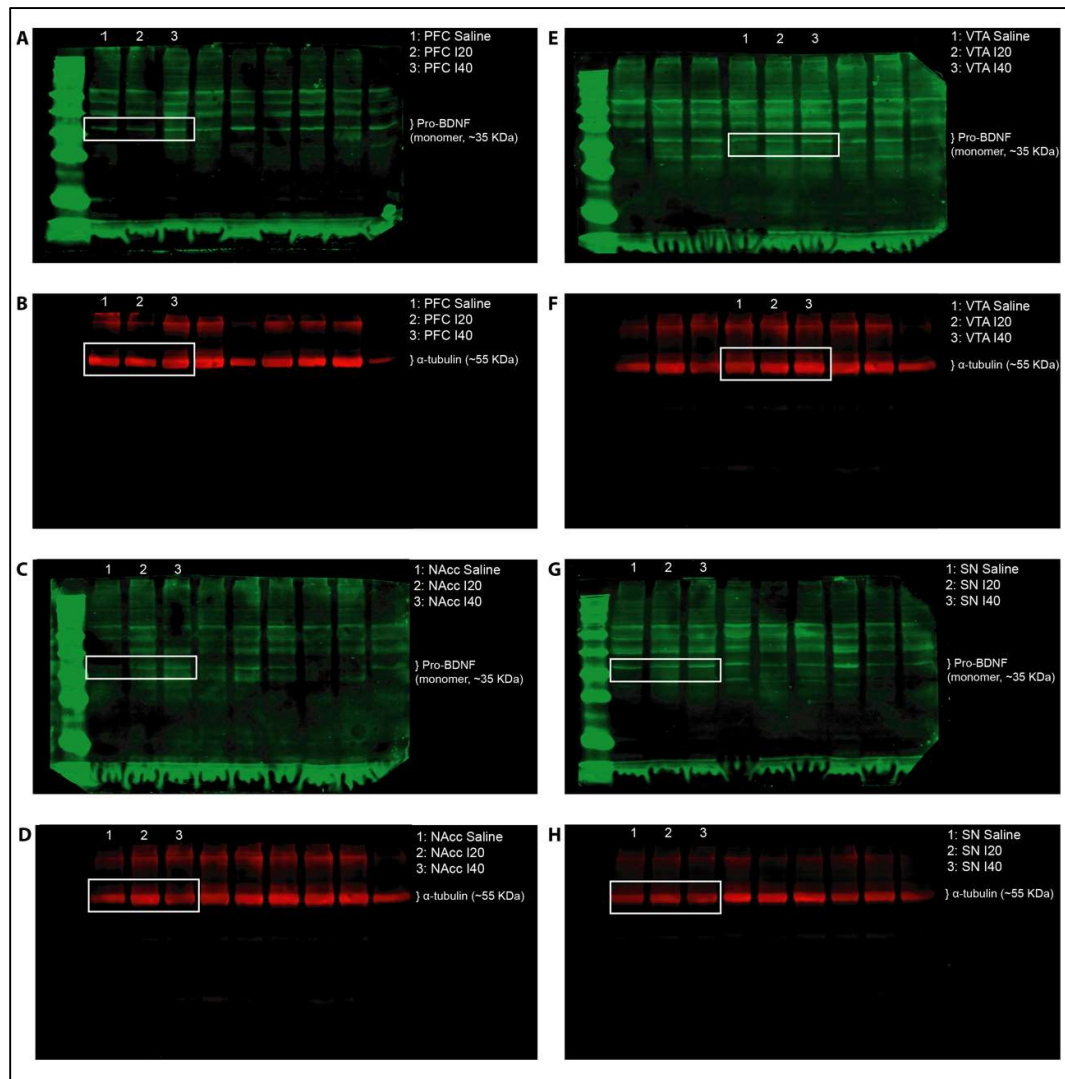
Supplementary Figure 7. Nitrocellulose membrane incubated with GDNF antibody, for Ibogaine 20 mg/kg samples.



Supplementary Figure 8. Nitrocellulose membrane incubated with alpha-tubulin antibody, for Ibogaine 20 mg/kg samples.



Supplementary Figure 9. Full-scanned images of western blots in main Figure 7-C. Nitrocellulose membranes corresponding to samples of PFC (A-B), NAcc (C-D), VTA and SN (E-F) from saline, I20 and I40-treated rats incubated with antibodies to BDNF (A, C, E) and α -tubulin (B, D, F).



Supplementary Figure 10. Full-scanned images of western blots in main [Figure 7-E](#). Nitrocellulose membranes corresponding to samples of PFC (A-B), NAcc (C-D), VTA (E-F) and SN (G-H) from saline, I20 and I40-treated rats incubated with antibodies to ProBDNF (A, C, E, G) and α-tubulin (B, D, F, H).