ARTICLE

Effects of chronic delta-opioid receptor agonist on the excitability of hippocampal glutamate and brainstem monoamine neurons, anxiety, locomotion, and habituation in rats

Eliyahu Dremencov[1,2] · Daniil Grinchii[1] · Zuzana Romanova[2,3] · Pavol Chomanic[2,3] · Lubica Lacinova[1] · Daniela Jezova[2]

Abstract

Background Short-term treatment with non-peptide agonists of delta-opioid receptors, such as agonist SNC80, induced behavioral effects in rodents, which could be modulated via changes in central neurotransmission. The present experiments aimed at testing the hypothesis that chronic treatment with SNC80 induces anxiolytic effects associated with changes in hippocampal glutamate and brainstem monoamine pathways.

Methods Adult male Wistar rats were used in experiments. Rats were treated with SNC80 (3 mg/kg/day) for fourteen days. Neuronal excitability was assessed using extracellular in vivo single-unit electrophysiology. The behavioral parameters were examined using the elevated plus maze and open field tests.

Results Chronic SNC80 treatment increased the excitability of hippocampal glutamate and ventral tegmental area dopamine neurons and had no effect on the firing activity of dorsal raphe nucleus serotonin cells. Chronic SNC80 treatment induced anxiolytic effects, which were, however, confounded by increased locomotor activity clearly confirmed in an open field test. The ability to cope with stressful situations and habituation processes in a novel environment was not influenced by chronic treatment with SNC80.

Conclusion Our study suggests that the psychoactive effects of SNC80 might be explained by its ability to stimulate hippocampal glutamate and mesolimbic dopamine transmission.

Abbreviations

SNC80	(+)-4-[(Alpha R)-alpha-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethyl-benzamide
DPDPE	(D-Pen2,5)-enkephalin
RM ANOVA	Analysis of variance for repeated measures
CA1/3	*Cornu Ammonis* 1/3
DOR	Delta-opioid receptor
DRN	Dorsal raphe nucleus
EPM	Elevated plus maze
HD	Head dipping
HCl	Hydrochloric acid
5-HT	Serotonin
NaCl	Sodium chloride
NaOH	Sodium hydroxide
SAP	Stretch attend postures
VTA	Ventral tegmental area

✉ Eliyahu Dremencov
eliyahu.dremencov@savba.sk

1   Institute of Molecular Physiology and Genetics, Center of Biosciences, Slovak Academy of Sciences, Dúbravská cesta 9, Bratislava, Slovakia

2   Institute of Experimental Endocrinology, Biomedical Research Center, Slovak Academy of Sciences, Bratislava, Slovakia

3   Department of Pharmacology and Toxicology, Faculty of Pharmacy, Comenius University Bratislava, Bratislava, Slovakia

Introduction

The endogenous opioid system, considering its dense interactions with limbic circuits and monoaminergic pathways [1], and its role in emotional and cognitive behavior [2], is

one of the possible targets for future psychotropic drugs. Out of opioid receptors, delta-opioid receptors (DORs) are of special interest, since they are responsible for the anxiolytic and antidepressant effects of opioids, but their involvement in the processes leading to addiction appears to be low [3].

SNC80, or (+)-4-[(alpha R)-alpha-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethyl-benzamide, is a selective non-peptide DOR agonist [4]. Several studies provide evidence of the anxiolytic effect of acute treatment with DOR agonists. Thus acute administration of SNC80 decreased signs of anxiety behavior reflected by several parameters measured in the elevated plus maze test which were antagonized by a selective DOR antagonist naltrindole [5]. Accordingly, the DOR antagonist naltrindole resulted in anxiogenic effects, which were antagonized by SNC80 [6]. Acute [5, 7] and sub-chronic (3 days) [8] treatment with SNC80 resulted in decreased immobility in the forced swim test, suggesting an antidepressant potential of SNC80. Repeated (7 days) SNC80 treatment induced an anxiolytic effect in the olfactory bulbectomized (an animal model of depression), but not in control rats [9]. However, the mentioned authors examined only the key characteristics of anxiety behavior in the elevated plus maze (EPM) test. To the author's best knowledge, the effect of chronic SNC80 on habituation to the changing environment has not yet been investigated.

In a recent study, we found that SNC80 potentiated the spontaneous firing activity of hippocampal neurons in primary culture [10]. Since a stimulatory effect on hippocampal markers of glutamatergic activity was also observed with the last-generation multimodal antidepressant drug vortioxetine [11], it is likely that behavioral effects induced by SNC80 administration are mediated, at least in part, via hippocampal glutamate mechanisms. It was indeed reported that intra-hippocampal microinjection of SNC80 increased local glutamate levels and stimulated glutamate-associated behaviors, such as tremor [12]. However, the effect of SNC80 on the excitability of the individual hippocampal neurons in in vivo conditions has not yet been studied.

Effects of SNC80 on behavior might also be mediated, in addition to the hippocampal glutamatergic system, by central monoamine mechanisms. The excitability of hippocampal neuronal circuits is under a modulatory influence of monoamines, namely serotonin (5-HT) and dopamine [13]. The cell bodies of 5-HT, noradrenergic, and dopaminergic neurons projecting to the hippocampus are located in the dorsal raphe nucleus (DRN) and ventral tegmental area (VTA), respectively [14]. These brain nuclei express DORs [15] and thus a role of monoamines in the modulation of hippocampal excitability by the DORs is possible.

The modulation of the central monoaminergic pathways by DORs was indeed reported in previous studies. In olfactory bulbectomized rats, an animal model of depression,

SNC80 increased extracellular serotonin (5-HT) concentrations in the prefrontal cortex, hippocampus, and amygdala, to the levels observed in control animals [9]. Behavioral effects of SNC80 and another non-peptide DOR agonist BW373U86 were abolished by pre-treatment with naltrindole and reduced by pre-treatment with D_2/D_3 receptor antagonist raclopride. Amphetamine-mediated efflux of dopamine from rat striatum was found to be enhanced by SNC80 [16]. These studies suggest that DORs influence central 5-HT and dopamine transmission, which may play a role in the anxiolytic and antidepressant effects of SNC80. The effect of DOR ligands on the excitability of central monoamine neurons is, however, unknown.

The present experiments aimed at testing the hypothesis that chronic treatment with SNC80 induces anxiolytic effects associated with changes in hippocampal glutamate and brainstem monoamine pathways. To approach this goal, we examined the effects of chronic SNC80 treatment on the excitability of hippocampal glutamate and midbrain 5-HT and dopamine neurons. With the aim to obtain behavioural characteristics induced by chronic activation of DORs, the behaviours in the elevated plus maze and open field tests were evaluated in rats treated chronically with SNC80 or vehicle, including anxiety behaviour, locomotor activity and habituation process.

Methods

Animals

Adult male Wistar rats (250–300 g) were ordered from the Animal Breeding facility of the Institute of Experimental Pharmacology and Toxicology, Centre of Experimental Medicine, Slovak Academy of Sciences (Dobra Voda, Slovakia). The animals were housed 2–3 per cage under standard laboratory conditions (temperature: 22 ± 2 °C, humidity: $55 \pm 10\%$) with a 12 h light/12 h dark cycle (lights on at 7 a.m.). Pelleted food and tap water were available ad libitum. In this type of investigation, the use of animals could not be avoided [17]. All experimental procedures were approved by the Animal Health and Animal Welfare Division of the State Veterinary and Food Administration of the Slovak Republic (Permit number Ro 3592/15-221) and conformed to the Directive 2010/63/EU of the European Parliament and the Council on the Protection of Animals Used for Scientific Purposes.

Chemicals

SNC80 was ordered from Bio-Techne Ltd., Abingdon, UK. Other chemicals were purchased from Merck Life Science s.r.o, Bratislava, Slovakia. Chloral hydrate and urethane were

dissolved in saline (0.9% sodium chloride: NaCl in water). SNC80 was dissolved in 1 M hydrochloric acid (HCl). The SNC80 solution in HCl was subsequently diluted with saline (1:100) and titrated with sodium hydroxide (NaOH) to pH ≈ 7.

Chronic SNC80 treatment

Animals were treated with SNC80 (1.5 mg/kg subcutaneously, twice a day at 09:00 and 17:00 h) or its vehicle (1 M HCl, adjusted to pH ≈ 7 with NaOH) for 14 consecutive days. Behavioral tests were performed on days 13th and 14th of the treatment, one hour after the morning SNC80 or vehicle injection. The last vehicle or SNC80 injection was performed on day 15th, and the electrophysiological assessments were performed one hour thereafter (Fig. 1).

Electrophysiology in vivo

In vivo electrophysiological experiments were performed as previously described [18–22]. Animals were anesthetized by urethane (1.25 g/kg, intraperitoneally: i.p., for the assessment of excitability of hippocampal glutamate neurons) or chloral hydrate (0.4 g/kg, i.p., for the assessment of excitability of brainstem monoamine neurons) and mounted in the stereotaxic frame (David Kopf Instruments, Tujunga, CA). Body temperature was maintained between 36 and 37 °C with a heating pad (Gaymor Instruments, Orchard Park, NY, USA).

The scalp was opened, and a 3 mm hole was drilled in the skull for the insertion of electrodes. Glass-pipettes were pulled with a DMZ-Universal Puller (Zeitz-Instruments GmbH, Martinsried, Germany) to a fine tip approximately 1 μm in diameter and filled with 2 M sodium chloride (NaCl) solution. Electrode impedance ranged from 4 to 6 MΩ. The pipettes were inserted into the Cornu Ammonis 1/3 (CA1/3) area of the hippocampus (3.9–4.2 mm posterior to bregma, 2.2–2.8 mm lateral to the midline, and 1.9–3.5 mm ventral to brain surface), dorsal raphe nucleus (DRN; 7.8–8.3 mm posterior to bregma and 4.5–7.0 mm ventral to brain surface), or ventral tegmental area (VTA; 4.5–5.5 mm posterior to bregma, 0.6–0.8 mm lateral to the midline, and 7.0–8.5 mm ventral to the brain surface) [23] by hydraulic micro-positioner (David Kopf Instruments, Tujunga, CA).

Electrophysiological assessments were performed one hour thereafter (Fig. 1). The action potentials generated by monoamine-secreting neurons were recorded using the AD Instruments Extracellular Recording System (Dunedin, New Zealand). Pyramidal neurons were identified based on the following criteria: large amplitude (0.5–1.2 mV), long-duration (0.8–1.2 ms) simple action potentials alternating with complex spike discharges [24, 25]. The 5-HT neurons were identified by bi- or tri-phasic action potentials with a rising phase of long duration (0.8–1.2 ms) and regular firing rate of 0.5–5.0 Hz [18, 26]. Dopamine neurons were recognized by tri-phasic action potentials lasting between 3 and 5 ms with a rising phase lasting over 1.1 ms, inflection or "notch" during the rising phase, marked negative deflection, irregular firing rate of 0.5–10 Hz, mixed single-spike and burst firing with a characteristic decrease of the action potentials amplitude within the bursts [27].

The same number of electrode descents per brain structure (four for the CA1/3, and DRN and five for the VTA) were made in vehicle- and SNC80-treated rats. All spontaneously active neurons were recorded for two minutes. Action potentials (spikes) of 5-HT, norepinephrine, and dopamine neurons were detected using the spike sorting algorithm, with version 6.02 of Spike2 software (Cambridge Electronic Design, Cambridge, UK). The neuronal firing rate and burst activity characteristics were calculated using the burstiDAtor software (www.github.com/nno/burstidator). The onset of a burst was signified by the occurrence of two spikes with an interspike interval (ISI) < 0.16 s for hippocampal glutamate, ISI < 0.01 s for DRN 5-HT, and ISI < 0.08 s for VTA dopamine neurons. The termination of a burst was defined as an ISI > 0.16 s for hippocampal glutamate [28], ISI > 0.01 s for DRN 5-HT neurons [29], and ISI > 0.16 s for VTA dopamine neurons [30, 31].

Assessment of anxiety behavior

The anxiety behavior was evaluated by spatiotemporal and ethological measures in the elevated plus maze (EPM) test as well as by the number of entries and the time spent in the central zone of the open field. All behavioral data were performed on 16 SNC80-treated and 14 vehicle-treated rats.

The EPM apparatus (Ekoplast, Telc, Czech Republic) consisted of two opposite open (50 × 15 cm) and two encosed (50 × 15 × 40 cm) arms made of black PVC that radiated from a central platform (15 × 15 cm) to form a plus sign. The maze was elevated to a height of 60 cm above the floor. The apparatus was illuminated by dim light with an intensity of 10–20 lx in the closed arms, and 35–50 lx in the open arms. Each rat was placed on the central platform of the maze facing an enclosed arm. The tests were performed during the light phase of the cycle, between 9 and 11 am. The rats were allowed to habituate in the test room 1 h before

14 days, 2x daily 1.5 mg/kg s.c. SNC80 (n = 16) / vehicle (n = 14)

OFT EPM electrophysiology
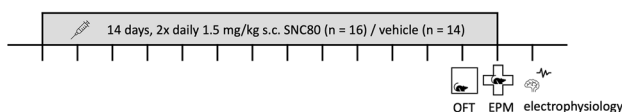
Fig. 1 Schematic study design of chronic treatment with SNC80. *OFT* open field test, *EPM* elevated plus maze

testing. The experimenter was not in the room during the test. Each test was recorded by a video camera positioned above the apparatus and behavior was analyzed after the test.

Spatiotemporal measures of behavior (number of open and closed arm entries, total arm entries, and the time spent in each section of the maze) were recorded in a 5 min EPM test as described previously [32]. Rats were considered to have entered an open arm when all four paws were in the arm.

Moreover, the ethologically derived parameters related to exploration and risk assessment behavior were also considered [32, 33]. Ethological measures included the frequency of stretch attend postures, and head dipping (exploratory movement in which the animal head is protruding over the side of the open arm and down towards the floor). Stretch attend postures (SAP) and head dipping (HD) were differentiated as protected (occurring in the closed arms or central platform) or unprotected (occurring in the open arms).

Next, the number of entries and time spent in the central zone of an open field were used as measures of the anxiety level. A central zone entry was defined as four paws entering. The testing was performed in the open field apparatus (Ekoplast, Telc, Czech Republic) which consisted of a rubber square area of 100×100 cm surrounded by plastic 52 cm high walls made of black PVC. The apparatus was illuminated by dim light with an intensity of 40–45 lx in the central area, and 15–25 lx in the peripheral area. At the beginning of the test, the animal was placed in the corner of the open field. The duration of the open field test was 15 min [34, 35].

To evaluate anxiety behavior, the movement of the rat was continuously tracked and recorded using a video camera. The records were analyzed using the H77 program (Institute of Experimental Medicine, Budapest, Hungary).

Assessment of habituation

The total locomotor activity and locomotor activity in selected time intervals throughout a 15 min open field test were recorded to evaluate the habituation of the animal in a new environment. The video records were analyzed using the Noldus EthoVision XT program. The parameters of locomotor activity included total distance, distance traveled in the central zone, and a ratio of central to the total distance traveled.

For the assessment of habituation processes, the video recording arena was divided into 36 squares grid. The number of crossed squares was recorded. Average values of squares crossed in the first, second, and third 5-min periods of the test were calculated according to the approach published in our previous studies [34, 35].

The rate of habituation was evaluated by using linear regression. The exponential function $Y_{(t)} = Y_0 e^{-kt}$ ($Y =$ amount of activity in individual minutes of session habituation assessment, $k =$ individual rate of habituation, $t =$ number of sessions/time of session) was used as a model of the habituation course of locomotor activity. The individual rate of habituation (k-value) expresses the rapidity of habituation [34, 35].

Statistical analyses

All values were checked for normal distribution (as revealed by the Shapiro–Wilk's test). The firing characteristics of the neurons in rats chronically treated with SNC80, such as spikes and bursts frequency and an average number of spikes per burst, as well as behavioral parameters of SNC80-treated rats, were compared to the corresponding parameters in vehicle-treated control animals, using two-tailed Student's t-test for parametric values and Mann–Whitney U test for nonparametric values. For the examination of the habituation, the locomotor activity in the three-time period during the 15 min open field test reflecting the habituation was analyzed by the analysis of variance for repeated measures (RM ANOVA) followed by Bonferroni post hoc test. The probability of $p \leq 0.05$ was considered significant.
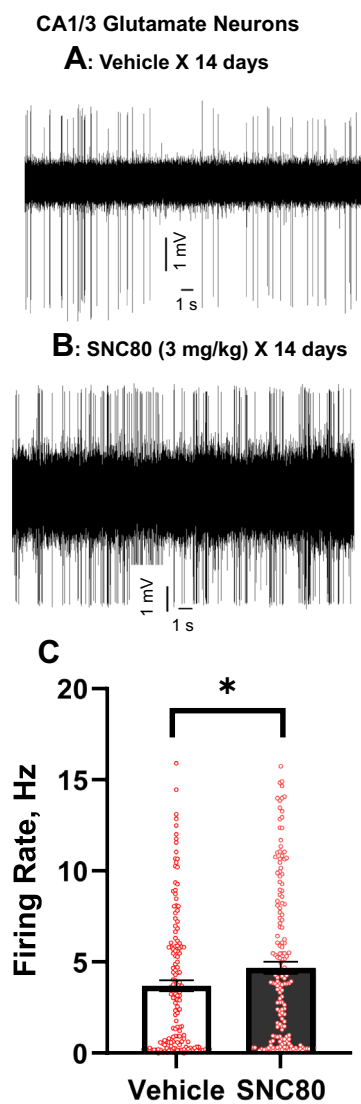
Results

Chronic SNC80 treatment alters the excitability of hippocampal glutamate and brainstem dopamine, but not 5-HT neurons

Chronic treatment with SNC80 led to a significant increase in the mean frequency of action potentials ($U = 8023.50$, $p < 0.05$) generated by hippocampal glutamate neurons (data from 151 neurons from 5 vehicle and 168 neurons from 5 SNC80-treated rats, Fig. 2). Other characteristics of glutamate neuronal firing activity were not affected by SNC80 in a statistically significant way.

With regards to the VTA dopamine neurons, chronic administration of SNC80 led to a significant increase in their firing rate ($U = 22,413.50$, $p < 0.05$; Fig. 3). Other characteristics of firing activity of the VTA dopamine neurons, such as the density of spontaneously active neurons, and the percentage of spikes occurring in the bursts, were not affected by chronic SNC80 treatment (data from 97 neurons from 5 vehicle- and 77 neurons from 5 SNC80-treated rats).

The mean firing rate of 5-HT neurons in rats chronically treated with SNC80 and the frequency of burst firing were not statistically different compared to the same measures in vehicle-treated controls. Other characteristics of their excitability, such as the density of spontaneously active neurons, frequency of burst firing, percentage of neurons exhibiting burst firing, percentage of spikes occurring in the bursts and

CA1/3 Glutamate Neurons

A: Vehicle X 14 days

B: SNC80 (3 mg/kg) X 14 days

C

VTA Dopamine Neurons

A: Vehicle X 14 days

B: SNC80 (3 mg/kg) X 14 days

C

Fig. 2 Effect of chronic treatment with SNC80 ($n = 168$ neurons from 5 rats, 1.5 mg/kg subcutaneously, twice a day at 09:00 and 17:00 h) on the excitability of glutamate neurons of the hippocampus. **A** and **B** Representative recordings from a vehicle- and an SNC80-treated animal, respectively. **C** Summary effect on the firing rate; *$p < 0.05$ in comparison with vehicle controls ($n = 151$ neurons from 5 rats), Mann–Whitney U test

the mean number of spikes in a burst, were not statistically different, either (data not shown).

## The effects of chronic SNC80 treatment on anxiety behavior

Chronic treatment with SNC80 failed to significantly modify classical spatiotemporal parameters of anxiety (frequency of open arm entries, time spent in open arms) measured in the EPM test. No differences between the treatment groups were observed in the ratio of open to
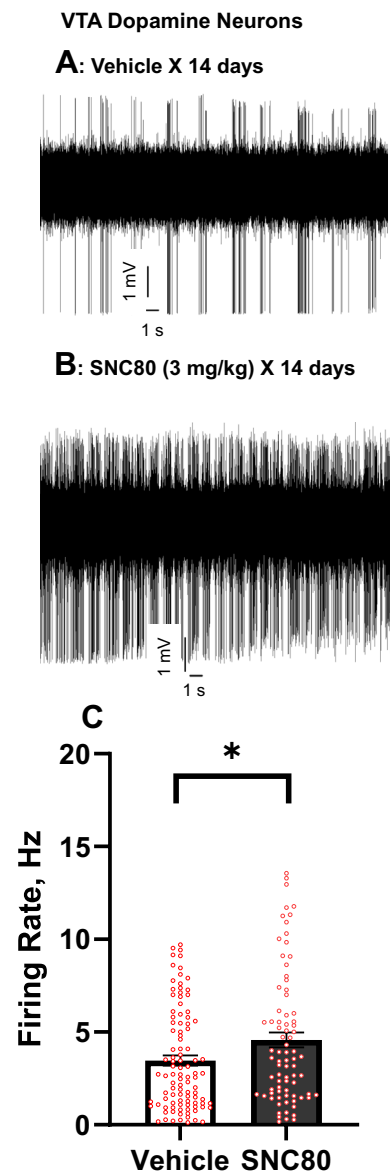
Fig. 3 Effect of chronic treatment with SNC80 ($n = 77$ neurons from 5 rats, 1.5 mg/kg subcutaneously, twice a day at 09:00 and 17:00 h) on the excitability of dopamine neurons of the ventral tegmental area (VTA). **A** and **B** Representative recordings from a vehicle- and an SNC80-treated animal, respectively. **C** Summary effect on the firing rate; *$p < 0.05$ in comparison with vehicle controls ($n = 97$ neurons from 5 rats), Mann–Whitney U test

total arm entries; the time spent and the number of entries to the central zone within the first 5 min was not different between the groups (data not shown). SNC80 treatment resulted in a mild modulation of ethological parameters in the EPM test (Fig. 4). SNC80-treated rats showed a significantly increased frequency of the protected head dipping ($U = 165.00$, $p < 0.05$). Other ethological parameters were not significantly affected by chronic SNC80 treatment.
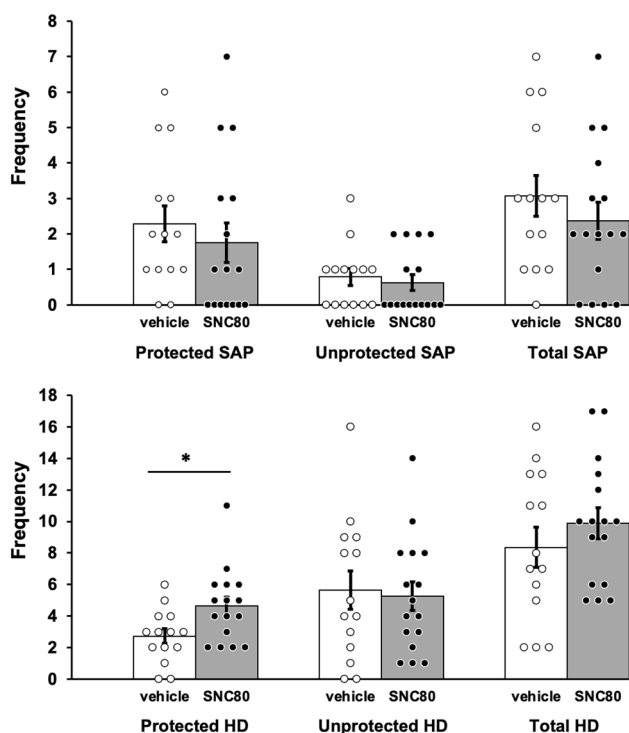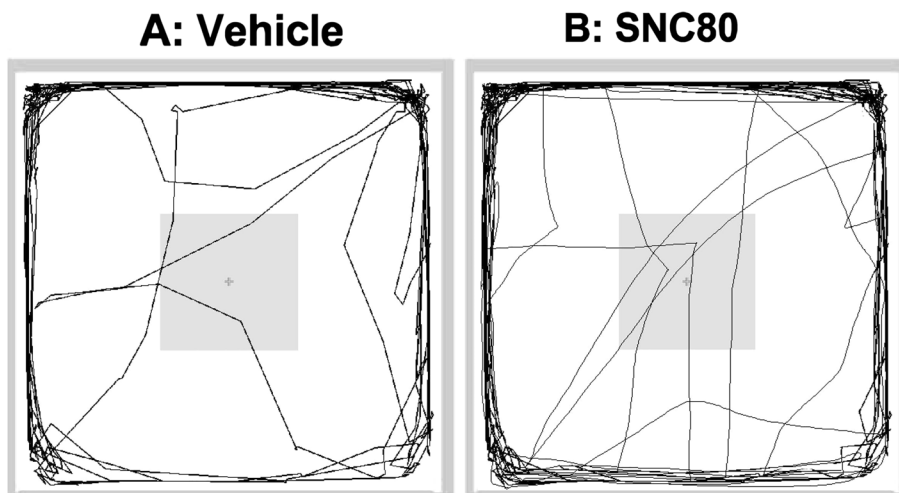
Fig. 4 Effect of chronic treatment with SNC80 ($n = 16$, 1.5 mg/kg subcutaneously, twice a day at 09:00 and 17:00 h) on ethologically derived parameters of anxiety behaviour measured in the elevated plus maze test. SAP: Stretch attend postures. *HD* head dipping; *$p < 0.05$ in comparison with vehicle controls ($n = 14$), two-tailed Student's *t*-test or Mann–Whitney *U* test, as appropriate

The trajectories of the SNC80 (A)- and vehicle (B)-treated rats within the open-filed apparatus are shown in Fig. 5. The rats treated with SNC80 entered more often and spent more time in the central zone of the open field compared to vehicle-treated animals (Fig. 6A). Mann–Whitney *U* test revealed a significant effect of treatment on

the frequency of entries ($U = 166.50$, $p < 0.05$) and the percentage of time spent in the central area ($U = 168.00$, $p < 0.05$).

The effects of chronic SNC80 treatment on habituation

Concerning the locomotor activity, the rats treated with SNC80 traveled a greater distance in the central zone of the open field compared to animals treated with a vehicle (Fig. 6B). Mann–Whitney *U* test revealed a significantly greater distance traveled by SNC80-treated rats in the central zone ($U = 167.00$, $p < 0.05$) compared to vehicle-treated controls. Similarly, chronic SNC80 treatment resulted in a greater total distance traveled ($t = 2.89$, $p < 0.01$) in the open field. The ratio of the two above-mentioned parameters (Fig. 6B) showed higher values in SNC80-treated rats ($U = 163.00$, $p < 0.05$). The rapidity of habituation (*k*-values) in the open field tended to be lower in SNC80-treated rats compared to controls ($U = 75.50$, $p = 0.13$). However, this difference failed to be statistically significant (data not shown). By evaluating the number of squares crossed in the consecutive three 5-min time periods in the open field, RM ANOVA (Fig. 7) revealed a significant main effect of time ($F_{2,56} = 46.55$, $p < 0.001$) and treatment ($F_{1,28} = 9.17$, $p < 0.01$). In both groups, the number of squares crossed decreased in time, while the rats chronically treated with SNC80 crossed significantly more squares in comparison with vehicle-treated controls. No effect of treatment × time interaction was detected ($F_{2,56} = 2.01$, $p = 0.14$). Bonferroni post hoc test showed that the number of crossed squares at the third 5 min was significantly lower compared to the numbers at the first 5 min in both SNC80- ($p < 0.001$) and vehicle-treated ($p < 0.001$) animals.

Fig. 5 Open-field trajectories of the rats treated with SNC80 ($n = 16$, 1.5 mg/kg subcutaneously, twice a day at 09:00 and 17:00 h) or vehicle ($n = 14$)
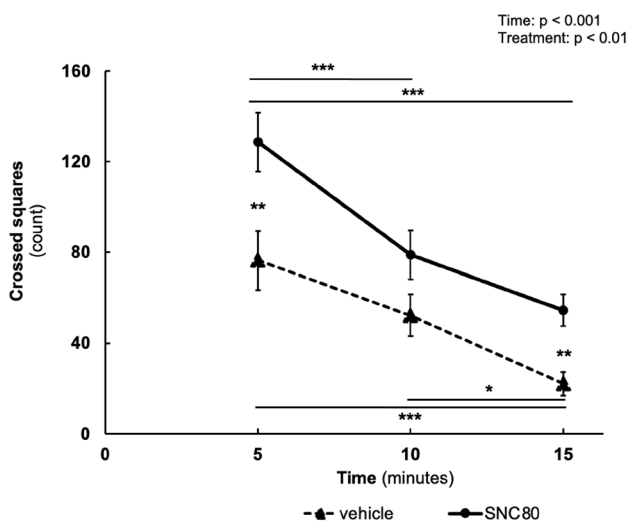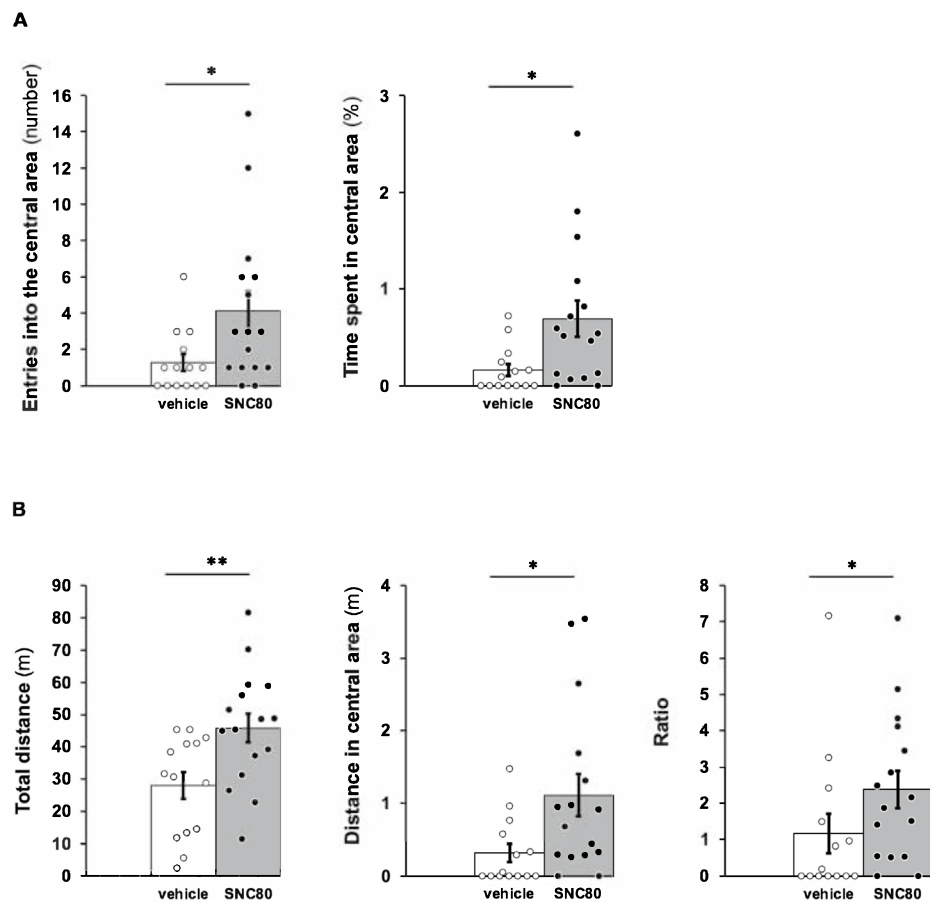
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Fig. 6 Effect of chronic treatment with SNC80 ($n=16$, 1.5 mg/kg subcutaneously, twice a day at 09:00 and 17:00 h) on anxiety behavior (**A**) and locomotor activity (**B**) measured in the open field test; *$p<0.05$ and **$p<0.01$ in comparison with vehicle controls ($n=14$), two-tailed Student's t-test or Mann–Whitney U test, as appropriate

Fig. 7 Effect of chronic treatment with SNC80 ($n=16$, 1.5 mg/kg subcutaneously, twice a day at 09:00 and 17:00 h) on habituation process in the open field test lasting 15 min; *$p<0.05$, **$p<0.01$ and ***$p<0.001$ in comparison with vehicle controls ($n=14$), ANOVA for repeated measures, Bonferroni post-hoc test

Discussion

Chronic treatment with SNC80 resulted in the stimulation of hippocampal glutamate and VTA dopamine neurons and did not affect the firing activity of DRN 5-HT neurons. Chronic SNC80 treatment induced anxiolytic effects, which were, however, confounded by increased locomotor activity confirmed in an open field test. Chronic treatment with SNC80 did not influence the habituation processes in a new environment.

We found that chronic SNC80 treatment failed to alter the excitability of 5-HT neurons of the DRN in a statistically significant way. This finding is startling since the administration of another DOR agonist (D-Pen2,5)-enkephalin (DPDPE) led to an enhancement of DRN 5-HT concentrations [36]. It should be, however, noted that Tao and Auerbach [36] tested the effect of an acute local (intra-DRN) administration of DPDPE, while we examined the effect of chronic peripheral treatment with a more selective agonist SNC80.

In the present study, we observed a statistically significant stimulatory effect of chronic SNC80 treatment on the firing rate and burst firing activity of glutamate neurons

of the CA1/3 area of the hippocampus. The burst firing of glutamate neurons enhances the nerve terminal transmitter release, in comparison with the same amount of action potentials fired in a single-spike mode [37]. It is thus likely that chronic activation of DORs stimulates central glutamate and dopamine neurotransmission via an increase of the neuronal firing rate, as well as via the triggering of their burst activity.

The stimulatory effect of chronic SNC80 treatment on the excitability of CA1/3 glutamate neurons in in vivo conditions, observed in the present study, is consistent with the stimulatory effect of this DOR agonist on the hippocampal neurons in primary culture, observed in our previous experiments [10]. It is also coherent with the stimulatory effects of SNC80 [38] and DPDPE [39] on the population spike amplitude observed in CA1 neurons in brain slices. SNC80-induced increase in hippocampal glutamate concentrations, reported in a previous study [12], might be, therefore, mediated, at least in part, via the stimulation of firing activity of the individual hippocampal neurons.

Since a stimulatory effect on hippocampal markers of glutamatergic activity was previously observed with the antidepressant drug vortioxetine [11], it is possible that antidepressant-like effects of SNC80 [5, 7, 8] are mediated, at least in part, via the stimulation of hippocampal glutamate neurons. On the other hand, SNC80-induced increase of in vivo excitability of hippocampal glutamate neurons, observed in the present study, might be also responsible for the seizure- [8, 40, 41] and tremor-inducing [12] effects of this DOR ligand.

With regards to dopamine neurons of the VTA, chronic treatment with SNC80 increased their mean spontaneous firing rate. It is thus possible that the stimulatory effect of DOR agonists on mesolimbic dopamine transmission, reported in a previous study [42] might be explained, at least in part, via the DOR-mediated increase in the firing rate of dopamine neurons. Since activation of dopamine transmission might be beneficial in the treatment of depression [14], antidepressant-like effects of SNC80 [5, 7, 8] might be mediated, at least in part, via the activation of mesolimbic dopamine neurons.

DOR agonists were considered agents with promising beneficial effects in the context of emotional responses and mood disorders [3]. Anxiolytic effects were described following single peripheral or central administration of DOR agonists. Thus, peripheral administration of nonpeptide DOR agonists SNC80 [5, 43], UFP-512 [44], and AZD2327 [45] decreased signs of anxiety measured in behavioral tests. Anxiolytic effects were observed following intracerebroventricular injection of UFP-512 [44] as well as direct administration of an enkephalin derivative directly into the CA1 region of the dorsal hippocampus [46] or the central nucleus of the amygdala [47].

The anxiolytic effects of chronic SNC80 treatment measured in the open field test in the present study were confounded by increased exploratory activity. Only a few previous studies evaluating the action of a single administration of SNC80 put attention to total locomotor activity. A single injection of SNC80 led to no change [5, 6] or hyperactivity [48, 49]. Based on the results of the present study, the stimulatory effect of SNC80 on locomotor activity may be explained, at least in part, by the stimulatory effect of this ligand on the excitability of hippocampal glutamate and VTA dopamine neurons. Though the increased movements induced by a single treatment of DOR agonists are not generally confronted with their anxiolytic effect, Perrine and colleagues [43] provided results supporting the statement that the effects of SNC80 on reducing anxiety are independent of its effects on locomotion. As no further supporting data are available, the anxiolytic effects of single and repeated treatment with DOR agonists should be interpreted with caution.

We found that chronic (14 days) SNC80 did not alter the characteristics of the anxiety behavior in the EPM test, such as s number of entries and time spent in the open arms. This is consistent with the results of a previous study, reporting a lack of effect of repeated (5 days) SNC80 treatment on these behavioral characteristics in intact rats [9]. We did, however, find a slight anxiolytic effect of the chronic SNC80 treatment as shown by changes in the ethological parameters, such as protected head dipping. The weak anxiolytic action of repeated SNC80 treatment observed in the present study may be the result of the development of tolerance described for analgesic, locomotor, and anxiolytic effects of DOR agonists [50–52]. Since hippocampal glutamate [53] and mesolimbic dopamine [54] systems have an impact on anxiety, the residual anxiolytic effect, observed after chronic SNC80, may be based on glutamatergic and/or dopaminergic mechanisms.

The habituation process in a new environment, which represents a cognitive process of learning not to respond to redundant non-significant stimuli [35], has not been investigated in relation to treatment with DOR agonists so far. In the present study, in spite of a significant increase in exploratory activity induced by chronic treatment with SNC80, the habituation process failed to be modified. Apparently, chronic SNC80 treatment in the dose used in the present experiments had no influence on learning and coping with the stress of a new environment.

A limitation of the present study is the possible development of tolerance to repeated injections of SNC80. There are several studies in the literature showing tolerance to behavioral and analgetic effects of repeatedly administered DOR agonists [50–52]. The present results provide supporting evidence on the possible development of tolerance to some but certainly not all effects of repeated treatment with SNC80. Unlike the results of a previous study using the administration of 3.2–32 mg/kg of SCN80 for 7 days [50], we observed

hyperlocomotion after 14-day treatment with 3 mg/kg of SNC80. Similarly to the results of the present study, Pradhan and colleagues [51] observed hyperlocomotion following 5 days of mice treatment with 5 mg/kg of SNC80. Moreover, the present results clearly show the stimulatory effect of repeated SNC80 on the excitability of hippocampal glutamate and VTA dopamine neurons. On the other hand, the finding of no changes in 5-HT neuronal excitability can be related to the concomitant weak anxiolytic effect. Both mentioned observations could be the result of the development of tolerance. However, reports on sub-chronic and chronic treatment with DOR agonists are still scarce and further studies are needed to understand the relationship between DOR stimulation and tolerance development.

In conclusion, the results of the present study suggest that chronic treatment with SNC80 enhances hippocampal glutamate and mesolimbic dopamine transmission, via a mechanism involving increased excitability and burst firing of CA1/3 glutamate and VTA dopamine neurons. The obtained results are likely to be stimulating especially for scientists studying opioid substances and the neurobiology of opioid receptors. This increase in hippocampal glutamate and mesolimbic dopamine putatively underlines the rise in locomotion and exploratory activity, observed after chronic SNC80 treatment. The anxiolytic effects induced by SNC80 have to be interpreted with caution with respect to the effect of this drug on locomotion and the possible development of drug tolerance.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical standards *Experiments with Animals:* All experimental procedures were approved by the Animal Health and Animal Welfare Division of the State Veterinary and Food Administration of the Slovak Republic (Permit number Ro 3592/15-221) and confirmed to the Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes.

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