Differential behavioral tolerance to the delta-opioid agonist SNC80 in Sprague-Dawley rats

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Abbreviations: [(+)-4-[(αR)-α-[(2S,5R)-2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]-(3-methoxyphenyl)methyl]-N,N-diethylbenzamide (SNC80); [(+)-4-[(αR)-α-[(2S,5R)-2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]-(3-hydroxyphenyl)methyl]-N,N-diethylbenzamide ((+)BW373U86); frontal cortex (FrCtx); prelimbic cortex (PCtx), cingulate cortex (CCtx); nucleus accumbens (NAcc); caudate putamen (CP); hippocampus (HPC); amygdala (Amyg); piriform cortex (PirCtx); thalamus (Thal); hypothalamus (hypothal); substantia nigra (SN); periaqueductal gray (PAG); pontine nuclei (PN)

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Abstract

Nonpeptidic delta-opioid agonists produce a number of behaviors, such as antidepressant-like effects, locomotor stimulation, antinociception, and convulsions. To consider this class of compounds as potential therapeutics for humans, the effects of delta-opioid agonists following repeated administration must be evaluated. Therefore, the present study investigated the effects of repeated delta-opioid agonist (SNC80) administration on its antidepressant-like effects in the forced swim test, locomotor activity, and convulsions in male Sprague-Dawley rats. Tolerance developed rapidly to the convulsive and locomotor-stimulating effects of SNC80, but not to the antidepressant-like effects. In addition, tolerance was evaluated at the level of the receptor-G protein interaction by measuring $[^{35}S]GTP\gamma S$ binding in brains from rats that were pretreated with SNC80. With various exposure durations to SNC80, some brain regions demonstrated tolerance at different times, suggesting that adaptations in the delta-opioid system may occur during agonist exposure. Overall, the lack of observable tolerance to the antidepressant-like effects of SNC80 indicates that this class of compounds has potential as a novel antidepressant therapy.

Introduction

Nonpeptidic delta-opioid agonists have antidepressant-like effects in the forced swim test in rats (Broom et al., 2002a; Jutkiewicz et al., 2004). The selective delta-opioid antagonist, naltrindole, attenuates the antidepressant-like effects of nonpeptidic delta-opioid agonists, demonstrating that these effects are mediated through the delta-opioid receptor. As compared with typical antidepressants such as desipramine and fluoxetine, these compounds produce a greater magnitude of antidepressant-like activity in the forced swim test (Broom et al., 2002a). Moreover, the nonpeptidic delta-opioid agonists produce antidepressant-like effects following acute agonist administration, suggesting a faster onset of action than known antidepressants. In addition, delta-opioid receptor agonists produce a number of other behavioral effects, including convulsions in mice, rats, and monkeys (Comer et al., 1993; Hong et al., 1998; Broom et al., 2002a, Broom et al., 2002b; Dykstra et al., 1993; Pakarinen et al., 1995; Negus et al., 1994), antinociception in hyperalgesic or allodynia models (Hong et al., 1998; Broom et al., 2001; Butelman et al., 1995; Brandt et al., 2002), locomotor stimulation in rats (Spina et al., 1998; Fraser et al., 2000; Broom et al., 2002a; Jutkiewicz et al., 2004), and discriminative and other control of operant behavior in rats and monkeys (Stevenson et al., 2002; Negus et al., 1994; Dykstra et al., 1993; Pakarinen et al., 1995).

Following repeated administration of peptidic or nonpeptidic delta-opioid agonists, tolerance develops to convulsive effects in mice and rats (Comer et al., 1993; Broom et al., 2002c), antinociception in mice (Zhao and Bhargava, 1997; Tseng et al., 1997; Broom et al., 2002c), and rate decreasing effects on food-maintained responding in

monkeys (Brandt et al., 2001). *In vitro* and *ex vivo* studies demonstrate that prolonged delta-opioid agonist treatment rapidly desensitizes and downregulates delta-opioid receptors, providing possible mechanisms for decreased behavioral and cellular responses (Breivogel et al., 1997; Okura et al., 2000; Bot et al., 1997; Okura et al., 2003; Remmers et al., 1998; Law et al., 2000). For example, mice treated with intracerebroventricular [D-Pen^2,D-Pen^5]-enkephalin (DPDPE) or [D-Ala^2,Glu^4]-deltorphin II (deltorphin II) for 2 or 4 d displayed diminished antinociceptive responses and decreased receptor density in brain homogenates as measured by [^{3}H]DPDPE binding (Zhao and Bhargava, 1997). Likewise, repeated administration of the delta-opioid receptor-preferring ligand [D-Ala^2,D-Leu^5]-enkephalin decreased delta-opioid receptor density in several brain regions (Tao et al., 1993).

The effect of repeated administration of delta-opioid agonists could be critical in the development of an antidepressant therapy based on delta-opioid receptor activation. Depressed patients need to take antidepressants on a chronic basis; therefore, tolerance development would limit the therapeutic utility of delta-opioid agonists. As previously proposed (Jutkiewicz et al., 2004), the behavioral effects associated with delta-opioid receptor activation may have different efficacy requirements and, therefore, different receptor reserves (Broom et al., 2002c). Drawing from receptor theory, the present study tests the hypothesis that repeated administration of nonpeptidic delta-opioid agonists produces tolerance to high efficacy-requiring behaviors, such as convulsions, but not to the low efficacy-requiring behaviors, such as locomotor activity and antidepressant properties. For example, a previous study demonstrated that, following a single treatment

in rats, tolerance developed to convulsive effects of (+)BW373U86, but not to its antidepressant-like effects (Broom et al., 2002b).

The present study investigated the effects of repeated administration of the nonpeptidic delta-opioid agonist SNC80 on convulsive, locomotor-stimulating, and antidepressant-like effects in the forced swim test in rats. In addition, SNC80-induced, brain region-specific loss of receptor function was evaluated using *ex vivo* [^{35}S]GTPγS binding in brain slices from rats pretreated with SNC80. Overall, these data demonstrated that differential tolerance developed to the behavioral effects and that delta-opioid agonist exposure produced cellular changes across brain regions. However, following repeated SNC80, changes in G protein activation alone does not account for all the tolerance observed to the behavioral effects of SNC80. These findings may identify receptor populations in different brains regions that contribute to the delta-opioid agonist-induced behaviors as well as receptor populations that are differentially regulated.

Methods

Subjects. Male Sprague-Dawley rats (250-350g) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed in groups of three rats per cage. All animals were fed on a standard laboratory diet and maintained on a 12 h light/dark cycle with lights on at 6:30 AM at an average temperature of 21°C. Experiments were conducted during the light cycle between 9:00 AM and 5:00 PM. Studies were performed in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The

experimental protocols were approved by the University of Michigan University Committee on the Use and Care of Animals.

Procedures

Locomotor activity measurements. To measure changes in locomotor activity, rats were surgically implanted with transmitters (model ER-4000 E-Mitter, Mini Mitter Co. Inc., Bend, OR) under ketamine (100 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.) anesthesia. The transmitter was implanted inside the peritoneal cavity of a rat at least 6 days before conducting an experiment and, following the surgical procedure, rats were singly-housed. The transmitter broadcast changes in location that were sent to a receiver (model ER-4000 Receiver, Mini Mitter Co., Inc.) placed under the home cage of each rat. Data were collected and processed simultaneously by the Vital View data acquisition system (Mini Mitter Co., Inc.). Counts of locomotor activity were summed every five minutes for each rat and recorded by Vital View software (Mini Mitter Co., Inc., Bend, OR). Baseline locomotor activity counts were collected for at least 40 minutes prior to handling or injection. Following an injection of vehicle or the delta-opioid agonist SNC80 (n=6 per treatment), locomotor activity measurements were collected for each rat for approximately 5 hr, but graphical representations of the data include post-injection locomotor counts until activity levels returned to baseline. For tolerance studies, baseline and post-injection locomotor activity counts were recorded at the same time daily for 7 days.

Forced Swim Test. To measure antidepressant-like activity, 6-8 rats per condition (n=6-8/condition) were subjected to a modified forced swim test as previously described (Broom et al., 2002a). Briefly, rats were placed gently in a clear cylindrical acrylic

container (46 cm tall X 20 cm in diameter) filled with 30 cm of 25°C ($\pm$ 1°C) water for a single, 15-min swim session. SNC80 (3.2, 10, or 32 mg/kg) was administered as a single subcutaneous (s.c.) injection 60 min prior to the forced swim test. For tolerance studies, rats were injected once daily with the same dose (either 0.32, 1.0, 3.2, 10, or 32 mg/kg SNC80) for 1-7 days, and then were evaluated in the forced swim test 60 min after the last injection. In other studies, rats were administered once daily injections of increasing doses of SNC80 over three consecutive days (3.2, then 10, then 32 mg/kg SNC80), and then were evaluated in the forced swim test 60 min after the last injection. Cylinder water was changed after every rat. Following the swim period, rats were removed from the water, towel-dried, and placed in a heated cage for 15 min.

Videotaped 15-min test swims were scored for immobility, swimming, and climbing behaviors (Detke et al., 1995). The individuals scoring the videotapes were blind to the drug treatments received by each rat. Every 5 sec, the observer scored the rat's behavior as one of the three categories: immobility, swimming, or climbing. The total counts of each behavior during the 15-min swim were averaged within treatment groups. These behaviors were defined as: *immobility*- floating in the water without struggling and using only small movements to keep the head above water; *swimming*- moving limbs in an active manner (more than required to keep head above water) causing movement between quadrants of the cylinder; *climbing*- making active movements with the forepaws in and out of the water, often directed at the wall of the swim tank.

 Convulsion observations. Immediately after s.c. injection, rats were placed in an observation chamber for 20 min to observe for convulsions and cataleptic behaviors. Time to onset of convulsion, duration of convulsion, and duration of catalepsy were

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recorded. Catalepsy duration was defined as the time to remove forelimbs from a rod

elevated approximately 2 inches off the ground. Following the 20-min observation

period, rats were returned to the home cage. For tolerance studies, rats were injected

daily as described above.

Ex vivo agonist-stimulated [^{35}S]GTPγS autoradiography. Agonist-stimulated

[^{35}S]GTPγS autoradiography was performed as previously described (Sim et al., 1995).

Rats (N= 6-8/condition) were decapitated 24 h after a single injection of vehicle or 32

mg/kg SNC80 or 24 h after the last of 6 daily injections of vehicle or 32 mg/kg SNC80.

Brains were removed and fresh frozen in 2-methylbutane (Sigma, St Louis, MO) on dry

ice. Coronal sections (20 μm) were cut throughout the brain on a cryostat maintained at –

18ºC, mounted on gelatin-subbed slides, and stored at -80ºC for less than four weeks until

use. Coronal sections were rinsed in TME buffer (50mM Tris-HCl, 3mM $MgCl_2$, 0.2

mM EGTA, 100mM NaCl, 0.1% BSA, pH 7.4) at room temperature for 10 min.

Following this first incubation, slices were incubated in TME buffer containing 2mM

GDP and 9.5 mU/ml adenosine deaminase for 15 min at room temperature. Then, slices

were incubated in TME buffer with GDP, adenosine deaminase, 0.04 nM [^{35}S]GTPγS,

and 1nM (+)BW373U86 or 10μM cold GTPγS (to determine non-specific binding) for 2

h at room temperature. As determined from previous studies (Jutkiewicz et al., 2005),

1nM (+)BW373U86 was determined to be the EC_{50}. For some experiments, frontal

cortex slices only were incubated with 0.32 nM (+)BW373U86, and some hippocampal

slices were incubated with 10 μM (+)BW373U86. After the 2 h incubation, slices were

rinsed twice for 2 min in cold 50 mM Tris buffer (pH 7.4) and once in distilled water for

1 min. Slides were air-dried for a few hours and exposed to film for 48 h in film cassettes

together with ^{14}C standards (Amersham Biosciences, NJ, USA). Images were digitized

and densitometric analysis was conducted using NIH Image J software. Abbreviations

are as follows: frontal cortex (FrCtx), prelimbic cortex (PCtx), cingulate cortex (CCtx),

nucleus accumbens (NAcc), caudate putamen (CP), hippocampus (HPC), amygdala

(Amyg), piriform cortex (PirCtx), thalamus (Thal), hypothalamus (hypothal), substantia

nigra (SN), periaqueductal gray (PAG), pontine nuclei (PN).

Data Handling and Statistical Analysis. Data from the forced swim test were averaged

within each treatment. Totals for each behavioral measure (immobility, swimming, or

climbing) were analyzed by one-way ANOVA, and Dunnett's post hoc tests were used to

compare drug-treated groups to vehicle-treated rats (Graphpad Prism Software). For

studies involving increasing doses of SNC80, data were analyzed by one-way ANOVA

with a significance level of $p<0.05$ and Tukey's post hoc test.

Locomotor activity data were summed over 5 min intervals beginning at least 40

min prior to injection with drug and continuing for at least 5 h after an injection of a test

compound. Locomotor activity counts were averaged across rats in each treatment group

and were calculated as time after injection $\pm$ 2 min. Timecourse data for each rat was

also converted to a positive area under curve value relative to the average vehicle data for

the corresponding test day and, subsequently, these values were averaged for each dose

(or vehicle treatment) (Graphpad Prism software). Positive area under curve values were

compared for each dose across days of injections or for each test day across drug

treatments by one-way ANOVA with Tukey's post hoc test.

For the [^{35}S]GTPγS autoradiography experiments, nonspecific binding (NSB)

[^{35}S]GTPγS binding was subtracted from basal- and drug-stimulated levels, and drug-

stimulated [^{35}S]GTPγS binding was averaged across rats for each brain region in each treatment group and calculated as percent stimulation over basal. Stimulation levels between vehicle- and SNC80-treated rats were compared by Student's t-test for each brain region evaluated.

Drugs. All test compounds were injected s.c. in a volume of 1 ml/kg. SNC80 was dissolved in an 8% 1M HCl solution. (+)BW373U86 was dissolved in sterile water. Ketamine hydrochloride (Vedco Inc., St. Joseph, MO) and xylazine hydrochloride (Fermenta Animal Health Co., Kansas City, MO) were all dissolved in sterile water.

Results

Acute (s.c.) injection of 32 mg/kg SNC80 produced convulsions in 25 to 33% of rats (i.e., 2 rats of 6, or 2 rats of 8 in two separate determinations). The SNC80-induced convulsions were brief, occurring 6-14 min after injection, and lasted 10-30 s. The convulsions were manifested as clonic contractions involving the head, forelimbs, and neck. After a convulsion, rats were cataleptic for approximately 2 min, during which the rats also failed to demonstrate a righting reflex. These data are consistent with previous studies measuring the convulsive effects of SNC80 (Jutkiewicz et al., 2004). With subsequent administrations, SNC80 did not produce convulsions in any rats at any dose tested (data not shown), such that rats either convulsed following the first administration or they did not convulse at all.

In rats implanted with locomotor activity probes, baseline levels of activity before time 0 were low (Fig. 1a). Rats receiving vehicle injections demonstrated increased locomotor activity immediately after the injection, but activity levels returned to baseline

within approximately 40 min. With an acute dose of 3.2 mg/kg SNC80, locomotor

activity was stimulated for 100-120 min. Single doses of 10 and 32 mg/kg SNC80

increased initial activity counts above vehicle levels immediately after the injection, and

locomotor activity remained elevated for approximately 160-180 min. In Figure 1b,

locomotor stimulation is graphed as positive area under curve relative to locomotor

activity levels induced by vehicle injection for each day tested. A dose of 10 mg/kg

SNC80 produced a robust increase in locomotor activity, more than doubling the level of

locomotor activity produced by 3.2 mg/kg; however, 32 mg/kg SNC80-induced

locomotor activity was slightly lower than that produced by 10 mg/kg. With 32 mg/kg

SNC80, all rats became unstable and demonstrated uncoordinated movements, and some

rats convulsed and experienced catalepsy, potentially contributing to the slightly

decreased, more variable activity observed.

　　　　With successive vehicle injections, injection-induced activity decreased,

demonstrating adaptation or behavioral tolerance to the injection effect over time (data

not shown). Locomotor activity produced by subsequent daily injections of the same

dose of SNC80 was represented as positive area under curve relative to averaged vehicle

levels for each day (Fig. 1b). All tested doses of SNC80 produced less locomotor activity

following repeated injections as compared with the first injection (for 3.2 mg/kg:

[$F_{(6,35)}=5.3$, $p<0.001$]; for 10 mg/kg: [$F_{(6,35)}=35.42$, $p<0.001$]; for 32 mg/kg:

[$F_{(6,35)}=12.59$, $p<0.001$]); however, 3.2 and 10 mg/kg SNC80 produced small, but

significant, increases above vehicle levels even after 7 d of administration ($p<0.05$,

$p<0.01$, respectively). For example, with a second injection of 10 mg/kg SNC80, the

level of activity was decreased more than 300% from the initial injection. Day 4 was the only day where there were no statistical differences between the treatment conditions.

Antidepressant-like effects of SNC80 were evaluated in the forced swim test following an acute injection or following 2, 3, or 7 d of injections. An acute injection of SNC80 significantly decreased immobility [$F_{(4,29)}=9.54$, $p<0.001$], increased swimming [$F_{(4,29)}=3.33$, $p<0.05$], and increased climbing [$F_{(4,29)}=4.08$, $p<0.01$] (Fig. 2a). At 3.2 mg/kg SNC80, immobility was significantly decreased as compared with vehicle ($p<0.01$). A single injection of 10 mg/kg SNC80 decreased immobility ($p<0.01$) and increased climbing ($p<0.05$). With 32 mg/kg SNC80, immobility was significantly decreased ($P<0.01$), and swimming ($p<0.05$) and climbing ($p<0.01$) were increased as compared with vehicle treatment. Following two days of injections, SNC80 significantly decreased immobility [$F_{(4,29)}=4.39$, $p<0.01$], produced a trend to increase swimming [$F_{(4,29)}=2.35$, $p=0.08$], but did not alter climbing (Fig. 2b). At doses of 10 and 32 mg/kg, SNC80 significantly decreased immobility as compared with vehicle ($p<0.05$ for both). In addition, SNC80 dose-dependently increased swimming; however, this effect was not statistically reliable. Following 3 d of injections, SNC80 decreased immobility [$F_{(4,28)}=10.72$, $p<0.001$] and increased swimming [$F_{(4,28)}=12.25$, $p<0.001$], but did not alter climbing behaviors (Fig. 2c). Doses of 10 and 32 mg/kg SNC80 significantly decreased immobility ($p<0.01$) and increased swimming ($p<0.01$). Following 7 d of agonist administration (Fig. 2d), SNC80 decreased immobility [$F_{(4,28)}=6.47$, $p<0.001$], increased swimming [$F_{(4,28)}=6.54$, $p<0.001$], and increased climbing [$F_{(4,28)}=3.04$, $p<0.05$]. Doses of 10 and 32 mg/kg SNC80 decreased immobility ($p<0.01$) and increased

swimming (p<0.05). In contrast to other drug administration regimens, 1.0 mg/kg

SNC80 decreased immobility (p<0.05) and increased climbing (p<0.05).

Antidepressant-like effects of SNC80 were also evaluated in the forced swim test

following administration of increasing doses of SNC80 over 3 d. Rats received either 3 d

of vehicle injections, or 2 d of vehicle injections and 32 mg/kg SNC80 on Day 3, or an

injection of 3.2 mg/kg SNC80 on Day 1, 10 mg/kg SNC80 on Day 2, and 32 mg/kg on

Day 3 (Fig. 3). Following 2 d of vehicle injections and 32 mg/kg SNC80 on Day 3, 5 out

of 6 rats demonstrated convulsions; however, rats that received increasing doses of

SNC80 over 3 days did not convulse. SNC80 administered either as a single injection of

32 mg/kg SNC80 or increasing doses over 3 d significantly decreased immobility

(F[2,17]=47.8, p<0.001), increased swimming (F[2,17]=11.22, p<0.001), and increased

climbing (F[2,17]=6.36, p<0.01) as compared with rats that received only vehicle

injections. There were no statistical differences between rats receiving a single dose or 3

increasing doses of SNC80.

To measure G protein activation in different brain regions, brain slices from rats

treated with vehicle or 32 mg/kg SNC80 24 h prior to sacrifice were compared in *ex vivo*

[^{35}S]GTPγS binding experiments. In brain slices from vehicle-pretreated rats, the

approximate EC_{50} of (+)BW373U86 (1 nM; Jutkiewicz et al., 2005) produced high levels

of [^{35}S]GTPγS binding in forebrain regions, including the frontal cortex, nucleus

accumbens, and caudate putamen, and in a one hindbrain region, the pontine nuclei (Fig.

4a). Intermediate to low levels of [^{35}S]GTPγS binding were measured in the prelimbic

cortex, cingulate cortex, amygdala, piriform cortex, and thalamus in vehicle-pretreated

rats. Some brain regions had minimal, if any, agonist-induced [^{35}S]GTPγS binding at 1

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nM (+)BW373U86, such as the hippocampus, hypothalamus, substantia nigra, and periaqueductal gray, in vehicle-pretreated rats. In brain slices from 32 mg/kg SNC80-pretreated rats, 1 nM (+)BW373U86 produced similar levels of [^{35}S]GTPγS binding in all brains regions as compared with that observed in slices from vehicle-pretreated rats, except in the pontine nuclei. In the pontine nuclei, [^{35}S]GTPγS binding was absent and, therefore, significantly lower in slices from SNC80-pretreated rats as compared with vehicle-treat rats ($p < 0.05$) (Fig. 4a and 4e). In SNC80-pretreated rats, there was also a trend for less [^{35}S]GTPγS binding in the nucleus accumbens as a whole as compared with slices from vehicle-treated rats ($p = 0.07$). More detailed analysis demonstrated that 1 nM (+)BW373U86 produced significantly less [^{35}S]GTPγS binding in the nucleus accumbens core ($p = 0.023$) and a trend to less [^{35}S]GTPγS binding in the nucleus accumbens shell ($p = 0.096$) from SNC80-treated rats as compared with vehicle-treated rats (Fig. 4b). Conversely, there was a trend of increased [^{35}S]GTPγS binding in the hippocampus from SNC80-treated rats as compared with vehicle-treated rats ($P = 0.09$).

[^{35}S]GTPγS binding was evaluated in the frontal cortex at the EC$_{50}$ for (+)BW373U86 in this region (0.32 nM; Jutkiewicz et al., 2005), and no differences were observed in 0.32 nM (+)BW373U86-stimulated [^{35}S]GTPγS binding between slices from vehicle- and SNC80-treated rats (Fig. 4d). A maximal concentration of (+)BW373U86 (10 μM) was used to evaluate [^{35}S]GTPγS binding in brain regions with low stimulation at 1 nM (+)BW373U86 (hippocampus, amygdala, piriform cortex, thalamus, and hypothalamus) from vehicle- and SNC80-treated rats (Fig. 4c); however, [^{35}S]GTPγS binding did not differ between these two pretreatment conditions in any brain region tested.

Following 6 d of vehicle or 32 mg/kg SNC80 injections, lower levels of

$[^{35}S]$GTPγS binding were found only in the caudate putamen of SNC80-treated rats as

compared with vehicle-treated rats (p<0.05) (Fig. 5a and 5c). Although all sub-regions of

the caudate putamen demonstrated less $[^{35}S]$GTPγS binding in slices from SNC80-treated

rats as compared with vehicle-treated rats (Fig. 5b), a significant decreases in $[^{35}S]$GTPγS

binding was observed in the dorsolateral caudate putamen (p<0.05), and a nearly-

significant decrease was observed in the dorsomedial caudate putamen (p=0.059). There

was no statistical difference between the rostral and caudal measurements of $[^{35}S]$GTPγS

binding in the caudate putamen (data not shown). The loss of $[^{35}S]$GTPγS stimulation in

the pontine nuclei observed 24 h following an acute injection was no longer observed

after 6 d of SNC80 administration.

Discussion

The present study investigated the effects of repeated administration of the nonpeptidic

delta-opioid agonist SNC80 on convulsive, locomotor-stimulating, and antidepressant-

like effects in Sprague-Dawley rats. With repeated SNC80 treatment, tolerance

developed to the convulsive and locomotor stimulating effects of SNC80, but not to the

antidepressant-like effects.

In the forced swim test, acute administration of SNC80 dose-dependently

decreased immobility, indicating an antidepressant-like effect. These antidepressant-like

effects did not dissipate after 2, 3, or 7 d of once daily injections. In contrast, tolerance

developed to the convulsive effects of SNC80, such that 20 to 33% of rats convulsed after

the first injection, but no rats convulsed with any subsequent injection. In the locomotor

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activity experiments, acute SNC80 dose-dependently increased locomotor activity in the home cage. With repeated SNC80 administration, SNC80-induced locomotor activity was decreased as compared with acute activity levels at all doses tested. Finally, attentuated [^{35}S]GTPγS binding suggests possible mechanisms for the observed tolerance to the behavioral effects of the delta-opioid agonist SNC80.

The results of the present study support previous findings demonstrating that tolerance to the convulsive effects of delta-opioid agonists developed rapidly (Comer et al., 1993; Broom et al., 2002b; Broom et al., 2002c). Based on pharmacological principles, tolerance should develop to the behaviors requiring high drug efficacy, such as convulsions and antinociception (Broom et al., 2002c), before lower efficacy-related behaviors. Therefore, it might be expected that tolerance development to the locomotor-stimulating and antidepressant properties would require longer treatments or more agonist exposure, thereby causing increased receptor desensitization and/or downregulation.

A single administration of SNC80 drastically decreased the amount of locomotor activity measured with subsequent SNC80 injections, suggesting rapid tolerance development to the locomotor-stimulating properties of SNC80. Each dose of SNC80 produced statistically similar levels of locomotor activity between Day 2 to 7; however, SNC80-induced locomotor activity levels on Days 2 to 7 were frequently, but not always, greater than that observed with vehicle-treated rats. For example, activity induced by 32 mg/kg SNC80 was statistically greater than vehicle levels on Days 1 and 5 only, whereas activity induced by 3.2 mg/kg SNC80 was statistically greater than vehicle levels on Days 1, 2, 5, 6, and 7. These findings are consistent with the idea that more tolerance occurs with administration of larger doses of SNC80. Locomotor activity following

vehicle injection also decreased with subsequent injections, suggesting behavioral

tolerance occurred with repeated handling and injection experiences. These data might

suggest that the initial administration of SNC80 may have slowed or prevented the

progression of behavioral tolerance with successive injections, such that SNC80-induced

activity may not return to vehicle levels as vehicle-induced activity is continually

changing. Alternatively, complete tolerance to SNC80-induced locomotor increases may

never develop, potentially signifying that some portion of delta-opioid receptors related

to the locomotor-stimulating properties fail to desensitize or downregulate. For example,

following a single SNC80 administration, (+)BW373U86-induced [^{35}S]GTPγS binding

did not change in the caudate putamen, a brain region generally associated with

locomotor stimulation. However, following 6 d of SNC80 treatment, (+)BW373U86-

induced [^{35}S]GTPγS binding decreased by approximately 50% in the caudate putamen as

compared with vehicle-treated rats. Overall, these data demonstrated that tolerance

developed rapidly to the robust locomotor-stimulating properties of an acute injection of

SNC80.

Although tolerance developed to the convulsive and locomotor-stimulating effects

of the delta-opioid agonist SNC80, rapid tolerance did not develop to the antidepressant-

like effects of SNC80. These data support previous findings that tolerance failed to

develop to the antidepressant-like effects of the delta-opioid agonist (+)BW373U86

following 2 d of injections (Broom et al., 2002b; Torregrossa et al., 2004). In the present

studies, the immobility level produced by 32 mg/kg SNC80 was similar across different

treatment regimens, such that SNC80 engendered similar immobility levels between 113-

118 counts following acute, 2, 3, or 7 daily injections. Immobility levels differed slightly

between the different vehicle-treated groups. Previously, various stressors have been

shown to alter immobility levels in the forced swim test, such as restraint stress (Platt and

Stone, 1982), and repeated injections might have had a similar effect in the present study.

Despite these small differences, high doses of SNC80 still produced statistically

significant and consistent decreases in immobility, indicating sustained antidepressant-

like effects.

It might be suggested that the portion of locomotor stimulation remaining

following repeated SNC80 contributed to the increased activity in the forced swim test,

thus falsely identifying persistent antidepressant-like properties. However, previous

studies demonstrated that delta-opioid agonist-induced antidepressant effects are

separable from locomotor-stimulating effects of SNC80 (Broom et al., 2002a). In

addition, the amount of locomotor stimulation was dramatically different between the

first and subsequent injections; however, the highest dose of SNC80 produced similar

levels of immobility in the forced swim test between acute, 2, and 3 d treatments, as

mentioned earlier. These data indicate that the locomotor stimulation was not related to

increased activity in the forced swim test.

Ex vivo [^{35}S]GTPγS binding experiments were used to evaluate tolerance

development at the level of delta-opioid receptors in various brain regions (Fig. 4), such

that tolerance would be manifested as reduced [^{35}S]GTPγS binding as a result of

desensitized receptors (i.e., decreased functional coupling of receptors and G proteins) or

of a downregulated receptor population. In these experiments, agonist-induced G protein

activation was completely eliminated in the region of the pontine nuclei in rats that

received 32 mg/kg SNC80 24 h earlier as compared with vehicle-treated rats. The

pontine nuclei were demonstrated to have a high delta-opioid receptor density (Mansour et al., 1995) and are involved relaying information between the cortex and the cerebellum. Additionally, this brain region was demonstrated to be involved in simple motor tasks, motor conditioning, and motor and spatial learning, as demonstrated by lesions in the ponto-cerebellar pathway that impaired motor coordination and equilibrium (Gasbarri et al., 2003). In relation to the present studies, acute administration of high SNC80 doses produced unstable and uncoordinated activity even in rats that did not convulse; however, this effect was not observed following subsequent drug administrations. Therefore, delta-opioid receptor activation in the pontine nuclei might contribute to the SNC80-induced impaired motor function, but with repeated agonist exposure, delta-opioid receptors in the pontine nuclei became uncoupled from the G protein, resulting in less [^{35}S]GTPγS binding in brain slices and potentially more coordinated motor behavior in rats. However, following 6 d of SNC80 treatment, [^{35}S]GTPγS binding in the pontine nuclei recovered, suggesting an adaptation of the system to repeated SNC80 exposure.

In addition to the pontine nuclei, a 24 h pretreatment with SNC80 decreased G protein activation in the nucleus accumbens. The nucleus accumbens is generally associated with the reinforcing effects of drugs. Under some conditions, rats intracranially self-administered the peptidic delta-opioid agonist [D-Pen2,D-Pen5]-enkephalin (Devine and Wise, 1994), but neither monkeys nor rats (unpublished observations) self-administered the nonpeptidic delta-opioid agonist SNC80 (Negus et al., 1998) or BW373U86 (Negus et al., 1994). Rapid tolerance development in the nucleus accumbens may contribute to the lack of self-administration of delta-opioid

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agonists. In addition to the reinforcing effects of drugs, striatal brain regions are generally associated with locomotor stimulation. The initial decrease in [^{35}S]GTPγS binding in the nucleus accumbens followed by a subsequent decrease in the caudate putamen might contribute to the loss of SNC80-induced locomotor activity following repeated SNC80 administration.

Finally, in rats pretreated with SNC80, there was either no change or a trend to increase [^{35}S]GTPγS binding in the hippocampus. The hippocampus is generally thought to be involved in delta-opioid agonist-induced convulsive activity (DeSarro et al., 1992; Haffmans and Dzoljic, 1983). Based on these findings, it was predicted that [^{35}S]GTPγS binding would be decreased in rats that received SNC80 24 h earlier. However, at the maximal concentration tested (10 μM), no difference was observed between vehicle- and SNC80-treated rats. More recently, the piriform cortex was proposed as a brain region related to delta-opioid agonist-induced convulsion (Torregrossa et al., 2004); however, the present study did not observe differences in [^{35}S]GTPγS binding between vehicle- and SNC80-treated rats. The present data suggest that another brain region, cellular mechanism, or downstream effector must contribute to observed tolerance to the convulsive effects of SNC80.

In conclusion, the present study investigated the effects of repeated administration of the delta-opioid agonist SNC80 on antidepressant-like effects, convulsions, and locomotor activity. Rapid tolerance developed to the convulsive and locomotor-stimulating effects of SNC80, but not to the antidepressant-like effects. In addition, uncoupling of the receptor from the G protein might contribute to some of the tolerance observed, but clearly, tolerance to the convulsive and locomotor effects of SNC80

observed in this study cannot be explained solely by G protein-receptor uncoupling.

Finally, these results suggest that the antidepressant-like effects of the delta-opioid

agonist SNC80 are separable from other SNC80-induced behavioral effects as measured

by differential tolerance development. Moreover, a lack of observable tolerance to the

antidepressant activity of SNC80 indicate that delta-opioid agonists could be effective on

a long-term basis for antidepressant therapy.

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Footnotes Section

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Legends for Figures

Figure 1. (a) Time course of effects of SNC80 on locomotor activity in Sprague-Dawley rats (*n*=6 per dose) following an acute injection of vehicle, 3.2, 10, or 32 mg/kg. Locomotor activity counts before time 0 were baseline levels of activity before the injection (s.c.) that occurred at time 0. (b) The effects of repeated administration of SNC80 on locomotor activity in Sprague-Dawley rats. Data are represented as positive area under curve relative to averaged vehicle activity counts for each day and statistical analysis is compared across subsequent days of injections for a single dose of SNC80 by one-way ANOVA with Tukey's post hoc test. *** $p<0.001$ as compared with all subsequent days of injections with either 10 or 32 mg/kg SNC80 by post-hoc comparison. * $p<0.05$ as compared with days 2, 3, 5, and 6 of 3.2 mg/kg SNC80 by post-hoc comparison, # $p<0.001$ as compared with day 4 with 3.2 mg/kg SNC80 by post-hoc comparison, and & $p<0.01$ as compared with day 7 with 3.2 mg/kg SNC80 by post-hoc comparison.

Figure 2. The effects of SNC80 in the forced swim test following acute (a), 2 days (b), 3 days (c), or 7 days (d) of drug administration in Sprague-Dawley rats (*n*=6 per dose). Drug was injected 60 min prior to the forced swim test. The bars and vertical lines above each bar represent the mean and standard error of the mean (sem) for immobility (open bars), swimming counts (single-hatched bars), and climbing counts (double-hatched bars). * $p<0.05$ and ** $p<0.01$ as compared with vehicle only as determined by Dunnett's post-hoc test.

Figure 3. The effects of increasing doses of SNC80 on convulsions (a) and antidepressant-like effects (b) in Sprague-Dawley rats (*n*=6 per dose). Either vehicle or one dose of SNC80 was injected once daily and, 60 min after the final injection, rats were evaluated in the forced swim test. The bars and vertical lines above each bar represent the mean and standard error of the mean (sem) for immobility (open bars), swimming counts (single-hatched bars), and climbing counts (double-hatched bars). * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ as compared with vehicle only as determined by Dunnett's post-hoc test.

Figure 4. (a) Percent [^{35}S]GTPγS stimulation by 1 nM (+)BW373U86 as compared with basal [^{35}S]GTPγS stimulation in various brain regions from rats that received vehicle (open bars) or 32 mg/kg SNC80 (hatched bars) 24 h prior to sacrifice. Abbreviations are as follows: frontal cortex (FrCtx), prelimbic cortex (PCtx), cingulate cortex (CCTX), nucleus accumbens (NAcc), caudate putamen (CP), hippocampus (HPC), amygdala (Amyg), piriform cortex (PirCtx), thalamus (Thal), hypothalamus (hypothal), substantia nigra (SN), periaqueductal gray (PAG), pontine nuclei (PN). * $p<0.05$ as compared with vehicle as determined by Student's t-test for each brain region. (b) Percent [^{35}S]GTPγS stimulation by 1 nM (+)BW373U86 as compared with basal [^{35}S]GTPγS stimulation in selected sub-regions from rats that received vehicle (open bars) or 32 mg/kg SNC80 (hatched bars) 24 h prior to sacrifice. Abbreviations are as follows: nucleus accumbens core (core), nucleus accumbens shell (shell), dorsolateral caudate putamen (DL), dorsomedial caudate putamen (DM), ventrolateral caudate putamen (VL), ventromedial

caudate putamen (VM). (c) Percent [^{35}S]GTPγS stimulation by 10 μM (+)BW373U86 as compared with basal [^{35}S]GTPγS stimulation in various brain regions from rats that received vehicle (open bars) or 32 mg/kg SNC80 (hatched bars) 24 h prior to sacrifice. Abbreviations are same as listed above. (d) Percent [^{35}S]GTPγS stimulation by 0.32 nM (+)BW373U86 as compared with basal [^{35}S]GTPγS stimulation in one brain region from rats that received vehicle (open bars) or 32 mg/kg SNC80 (hatched bars) 24 h prior to sacrifice. Abbreviations are same as listed above. (e) Autoradiograms of rat brain slices containing the pontine nuclei following stimulation by 1) basal-, 2) nonspecific binding (NSB)-, 3) 1 nM (+)BW (in vehicle-treated rat brain slice) and 4) 1 nM (+)BW373U86 (in 32 mg/kg SNC80-treated rat brain slice) treatments.

Figure 5. (a) Percent [^{35}S]GTPγS binding by 1 nM (+)BW373U86 as compared with basal [^{35}S]GTPγS binding in various brain regions from rats that received vehicle (open bars) or 32 mg/kg SNC80 (hatched bars) every 24 h for six days and were sacrificed 24 h after the sixth injection. Abbreviations are as follows: frontal cortex (FrCtx), prelimbic cortex (PCtx), cingulated cortex (CCTX), nucleus accumbens (NAcc), caudate putamen (CP), hippocampus (HPC), amygdala (Amyg), piriform cortex (PirCtx), thalamus (Thal), hypothalamus (hypothal), substantia nigra (SN), periaqueductal gray (PAG), pontine nuclei (PN). * $p<0.05$ as compared with vehicle as determined by Student's t-test for each brain region. (b) Percent [^{35}S]GTPγS binding by 1 nM (+)BW373U86 as compared with basal [^{35}S]GTPγS binding in selected sub-regions from rats that received vehicle (open bars) or 32 mg/kg SNC80 (hatched bars) every 24 h for six days and were sacrificed 24 h after the sixth injection. Abbreviations are as follows: nucleus accumbens core (core), nucleus accumbens shell (shell), dorsolateral caudate putamen (DL),

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dorsomedial caudate putamen (DM), ventrolateral caudate putamen (VL), ventromedial

caudate putamen (VM). (c) Autoradiograms of striatal rat brain slices following

stimulation by 1) basal-, 2) NSB-, 3) 1 nM (+)BW (in vehicle-treated rat brain slice) and

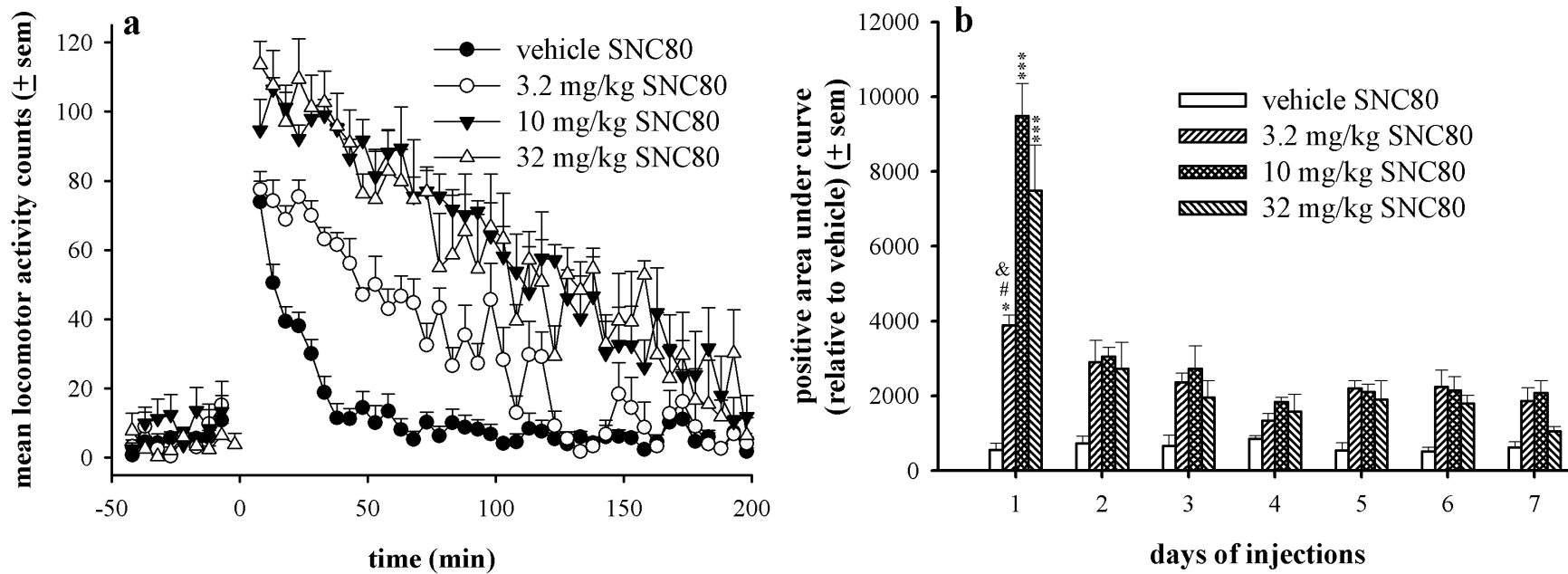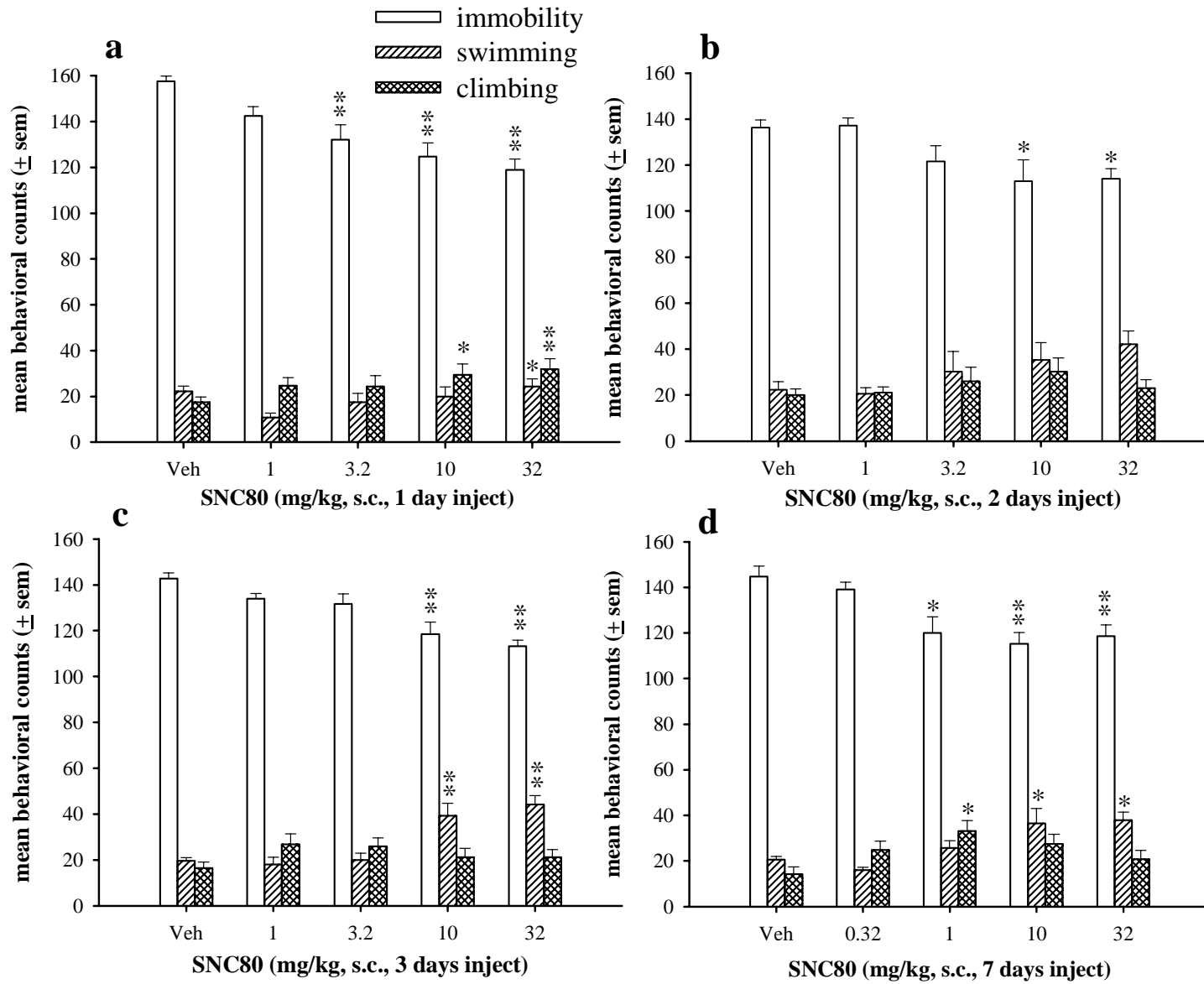4) 1 nM (+)BW373U86 (in 32 mg/kg SNC80-treated rat brain slice) treatments.
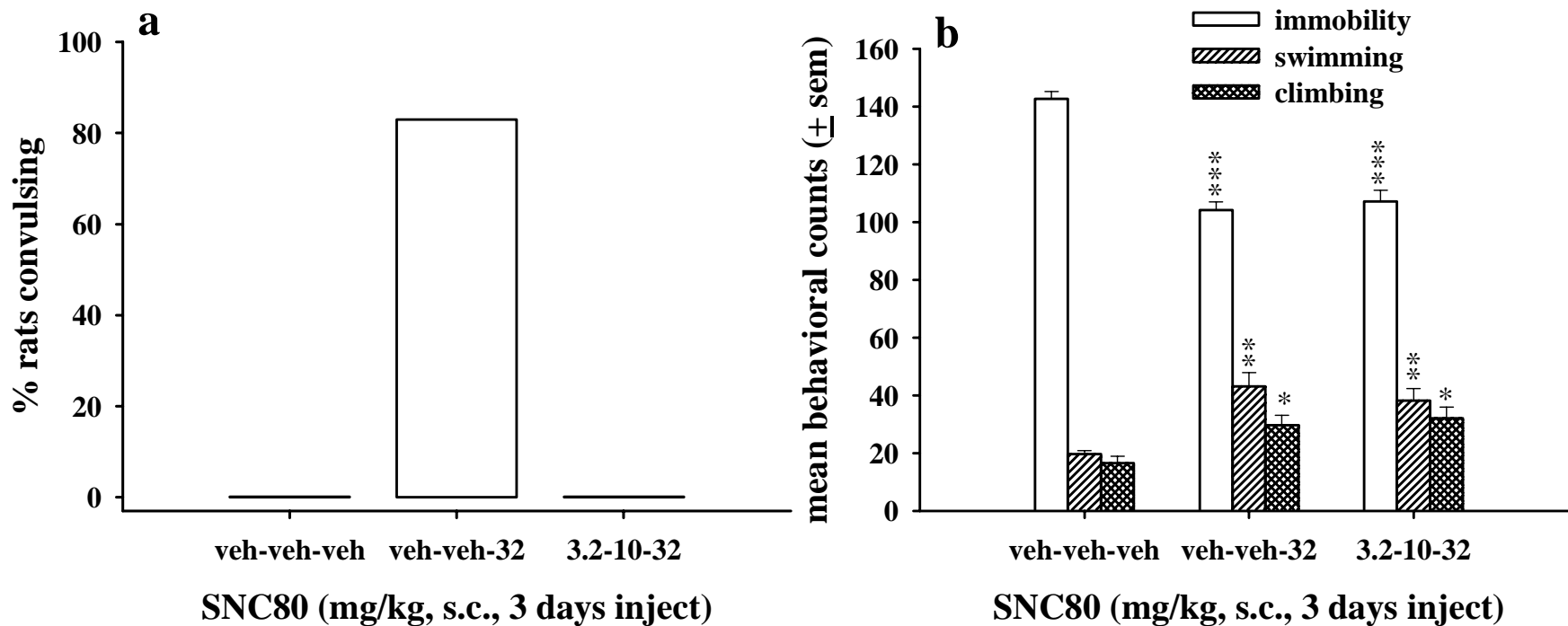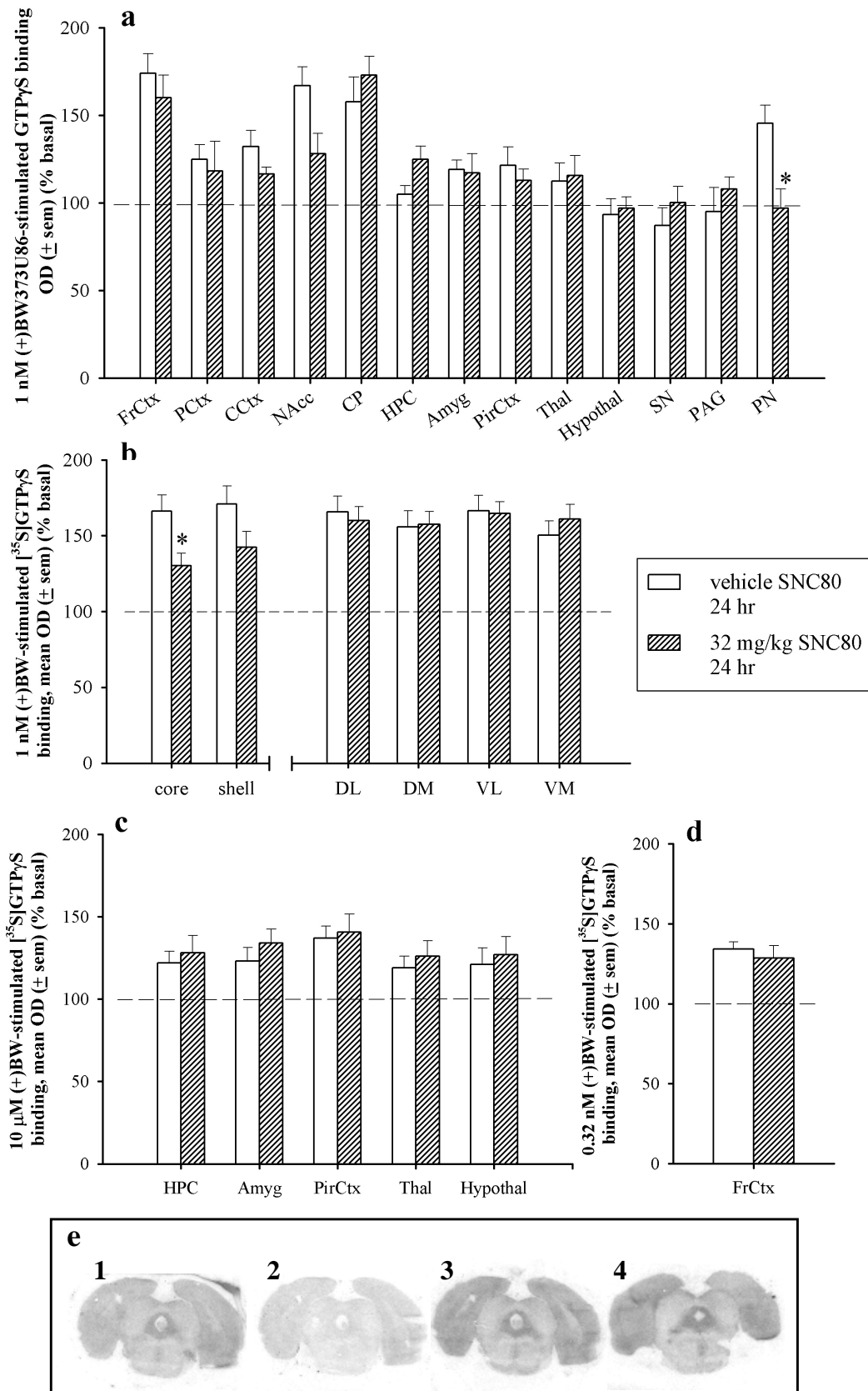
Figure 1.

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