

GABA_B RECEPTOR ACTIVATION IN THE VENTRAL TEGMENTAL AREA INHIBITS
THE ACQUISITION AND EXPRESSION OF OPIATE-INDUCED MOTOR SENSITIZATION

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RUNNING TITLE- GABA_B receptors in the VTA inhibit opiate sensitization

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Non-standard abbreviations: aCSF, artificial cerebral spinal fluid; NAc, nucleus accumbens;

NAcS, nucleus accumbens shell; NAcC, nucleus accumbens core; PFC, prefrontal cortex;

GABA, γ -aminobutyric acid; VTA, ventral tegmental area; SNK, Student-Newman-Keuls.

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Abstract

Opiate-induced motor sensitization refers to the progressive and enduring motor response that develops after intermittent drug administration, and results from neuroadaptive changes in ventral tegmental area (VTA) and nucleus accumbens neurons. Repeated activation of μ -opioid receptors localized on γ -aminobutyric acid (GABA) neurons in the VTA enhances dopaminergic cell activity and stimulates dopamine release in the nucleus accumbens. We hypothesize that GABA_B receptor agonist treatment in the VTA blocks morphine-induced motor stimulation, motor sensitization, and accumbal Fos immunoreactivity by inhibiting the activation of accumbal neurons. First, C57BL/6 mice were co-administered a single subcutaneous injection of morphine with intra-VTA baclofen, a GABA_B receptor agonist. Baclofen produced a dose-dependent inhibition of opiate-induced motor activation that was attenuated by 2-hydroxysaclofen, a GABA_B receptor antagonist. Next, morphine was administered on days 1, 3, 5 and 9 and demonstrated sensitization to its motor stimulant effects and concomitant induction of Fos immunoreactivity in the NAc shell (NAcS) but not NAc core. Intra-VTA baclofen administered during morphine pretreatment blocked the acquisition of morphine-induced motor sensitization and Fos activation in the NAcS. Intra-VTA baclofen administered only on day 9 blocked the expression of morphine-induced motor sensitization and Fos activation in the NAcS. A linear relationship was found between morphine-induced motor activity and accumbal Fos in single- and repeated-dose treatment groups. In conclusion, GABA_B receptor stimulation in the VTA blocked opiate-induced motor stimulation and motor sensitization by inhibiting the activation of NAcS neurons. GABA_B receptor agonists may be useful pharmacological treatments in altering the behavioral effects of opiates.

The ventral tegmental area (VTA) in the midbrain is composed of dopamine neurons and γ -aminobutyric acid (GABA) neurons that have a role in mediating opiate-induced motor stimulation and sensitization. The VTA dopaminergic neurons ascend to the nucleus accumbens (NAc) and prefrontal cortex (PFC), and VTA GABAergic neurons ascend to the NAc (Van Bockstaele and Pickel, 1995) and PFC (Carr and Sesack, 2000). In addition, the VTA receives GABAergic projections from the ventral pallidum and NAc (Kalivas et al. 1993). Mu-opioid receptors are localized on inhibitory GABAergic projection neurons and interneurons that synapse on dopamine neurons in the VTA. Activation of μ -opioid receptors decreases GABA release that consequently disinhibits dopamine neurons and increases extracellular dopamine release in the NAc and PFC (Di Chiara and Imperato, 1988). Morphine administered systemically or microiontophoretically into the VTA inhibits the firing of GABAergic interneurons and increases the firing of dopamine neurons (Gysling and Wang, 1983; Matthews and German, 1984; Johnson and North, 1992). Furthermore, Fos the protein product of the immediate early gene *c-fos* is a known marker of dopaminergic neuronal transmission. The expression of Fos was enhanced in the NAc following morphine (10 mg/kg s.c.) treatment (Leite-Morris et al., 2002) and is reflective of dopaminergic neuronal activation in the NAc (Barrot et al., 1999). Thus, morphine mediates its acute effects by activating μ -opioid receptors located on VTA GABAergic neurons that result in enhanced firing of dopaminergic neurons, increased postsynaptic dopamine release in the NAc, and resultant activation of accumbal cells.

Activation of GABA_B receptors localized on dopaminergic neurons (Wirtshafter and Sheppard, 2001) and glutamatergic neurons (Wu et al., 1999) in the VTA modulate the stimulant and motivational properties of drugs of abuse by regulating mesolimbic circuitry and its afferent inputs. Electrophysiologic studies show that GABA_B receptors in the VTA hyperpolarize dopamine neurons

(Lacey, 1993) and inhibit NMDA receptor-induced activation of these cells (Seutin et al., 1994; Wu et al., 1999). The net effect of VTA GABA_B receptor activation was reduction of both somatodendritic dopamine release in the VTA (Klitenick et al., 1992) and the synaptic dopamine release in the NAc (Westerink et al., 1996). Administration of the selective GABA_B receptor agonist baclofen into the VTA was reported to reduce extracellular dopamine levels in the NAc and block opiate's reinforcing effects (Xi and Stein, 1999, Tsuji et al, 1996). In another study, intra-VTA administration of baclofen attenuated opiate-induced motor activation and dopamine release in the NAc (Kalivas et al. 1990). VTA GABA_B receptors modulate the motor stimulant and rewarding effects of opiates (Kaplan et al., 2003) via their inhibition of mesolimbic dopaminergic systems.

In rodents, when drugs of abuse are administered repeatedly, a progressive enhancement of motor activity known as sensitization is produced. Opiate-induced motor sensitization appears to be mediated by neuroplastic changes in VTA GABAergic, dopaminergic, and glutamatergic transmission (reviewed by Vanderschuren and Kalivas, 2000). Because of the potential importance of drug sensitization during addiction, novel studies were performed and the role of VTA GABA_B receptors throughout the acquisition and expression of morphine-induced motor sensitization was investigated. This study also examined whether morphine-induced motor stimulation and sensitization was temporally coordinated with NAc cell activation as measured by Fos protein expression in NAc. In addition, the inhibition of intra-VTA GABA_B receptor activation upon these morphine-induced effects was also evaluated.

Materials and Methods

Animals and housing conditions. Male C57BL/6 mice weighing 24-26 g were obtained from Charles River Laboratories (Raleigh, NC). C57BL/6 mice were chosen because of their demonstrated sensitivity to the motor stimulatory effects of morphine (Weisberg and Kaplan, 1999). The animals were housed in a facility approved by the Association for the Assessment and Accreditation of Laboratory Animal Care. Animals were kept under a 12-hour light/dark cycle (light on 0600 h to 1800 h) in a temperature- and humidity-controlled environment where they were allowed food and water *ad libitum*. All mice acclimated to the environment for 5 days prior to treatment. Drug treatment and behavioral monitoring began every morning after 0700 h and ended before 1700 h in order to remain within the bounds of the light period and to avoid the transition hour of the light/dark cycle. All animal use procedures were approved by the Veterans Affairs Medical Center Animal Care and Use Committee and were conducted in accordance with the NIH 'Guide for the Care and Use of Laboratory Animals'.

Stereotaxic surgery. Mice were anesthetized with 70 mg/kg (i.p.) of pentobarbital sodium (Wyeth-Ayerst; Philadelphia, PA) before undergoing stereotaxic surgery and the inhalant methoxyflurane (Pitman Moore; Mundelein, IL) was supplemented as needed during surgery. During and after surgery the mice were maintained on a heated (40°C) pad to prevent hypothermia. Animals were placed in a Kopf Model 900 stereotaxic instrument (David Kopf Instruments, Tujunga, CA) and were held in position with ear bar adapters that eliminated damage to the eardrum. Guide cannulae (13.4 mm) were constructed from 26 gauge stainless steel tubing (Small Parts Inc., Miami Lakes, FL) and implanted bilaterally into the VTA using the following stereotaxic coordinates (Franklin and Paxinos, 1997) relative to Bregma: AP = -3.0 mm, DV = -3.3 mm, ML = ± 0.5 mm. The guide cannulae were implanted 1.5 mm above the VTA and fixed to the skull with

dental acrylic. Vetbond (3M Company, St. Paul, MN) acrylic was used to close the wound. Stylets constructed from 33-gauge stainless steel tubing (Small Parts Inc., Miami Lakes, FL) were inserted into the guide cannulae to maintain patency. Stainless steel injection cannulae (33 gauge) were designed for placement 0.5 mm above the VTA to allow drug diffusion into the VTA. Mice were allowed to recover from surgery for at least 4 days before drug delivery and behavioral monitoring. The survival rate for surgery was 100%.

Drug administration & behavioral monitoring procedure. Mice were acclimated to the activity monitor for 30 minutes before drug injections. Locomotor activity over a 180-minute period was quantified using sixteen-beam Digiscan 'Micro' animal activity monitors (Omnitech Electronics, Columbus, OH). The GABA_B receptor agonist baclofen or antagonist 2-hydroxysaclofen (Sigma Chemical Co., St. Louis, MO) was dissolved in 80% artificial cerebral spinal fluid/20% Alkamuls (aCSF).

The first set of experiments examined the dose-dependent effects of intra-VTA GABA_B receptor agonist baclofen on morphine-induced motor stimulation, and reversal of the agonist effects by a GABA_B receptor antagonist 2-hydroxysaclofen. Mice received two treatments during these studies: 1) a bilateral intra-VTA injection of baclofen, or baclofen plus 2-hydroxysaclofen, or aCSF, immediately followed by 2) a subcutaneous injection of either morphine (10 mg/kg) or sterile saline. All intra-VTA infusions were injected bilaterally via injection cannulae in a volume of 0.5 µl per hemisphere over 15 seconds using a computerized microinfusion pump (CMA, Solona, Sweden). The injection cannulae were kept in place for 45 seconds after infusion to avoid any backflow.

In the second set of experiments, we examined the effects of intra-VTA administration of baclofen on the acquisition and expression of morphine-induced motor sensitization. In these studies, two injections were co-administered on days 1, 3, 5, and 9: 1) a bilateral intra-VTA

injection of baclofen, or aCSF followed immediately by 2) a subcutaneous dose of morphine (10 mg/kg) or saline. The timing and dosage required for morphine-induced sensitization was based on previous reports (Weisberg and Kaplan, 1999). In all experiments, following drug treatment, mice were placed in acrylic monitoring cages (44 x 22.5 x 15.5 cm) and motor activity was assessed according to the number of successive infrared beam crossings in the horizontal plane within 15-minute intervals over 180 minutes. The monitors were located in a room with the same lighting and atmosphere conditions as the animals' living environment. A white noise producer in the center of the monitoring room attenuated any novel auditory stimuli.

Verification of cannulae placement. Mice underwent intracardiac perfusion (see below) immediately following activity monitoring. Brains were removed from the skull, rinsed in ice cold phosphate buffered saline (PBS), and blocked on ice. The midbrain block was frozen separately and mounted in O.C.T. compound (Sakura Finetek; Torrance, CA). Coronal sections through the VTA (42 μ m) were obtained and Nissl stained to verify correct placement of injection cannula tips. Behavioral and neurochemical data from mice with correctly positioned VTA cannulae were incorporated into the analysis.

Immunohistochemistry. Multiple studies have utilized c-Fos immediate-early gene expression as a measure of NAc cell activation after single-dose (Liu et al., 1994; Bontempi and Sharp, 1997; Barrot et al., 1999) and repeated-dose morphine treatments (Curran et al., 1996, Pontieri et al., 1997). Morphine-induced increases in extracellular dopamine levels in the NAc are associated with increases in Fos immunoreactivity (Barrot et al., 1999). We utilized this approach to measure postsynaptic dopaminergic activation of accumbal neurons. Mice were deeply anaesthetized with 70 mg/kg (i.p.) of sodium pentobarbital for transcardial perfusion. Heparin (1000 units/ml) in cold 0.05 M PBS (pH 7.4) was infused for two minutes followed by 20 ml of 4% paraformaldehyde in

PBS. The brains were quickly removed and post-fixed in a series 4% paraformaldehyde, 12.5% sucrose PBS, and 25% sucrose PBS (24 hours each). The brains were quickly frozen in isopentane and mounted in O.C.T. on dry ice and stored at -70°C . Coronal sections ($42\ \mu\text{m}$; from bregma 1.42 mm to 0.86 mm) of the NAc were cut at -15°C using a cryostat. The sections immediately anterior and posterior to the bregma coordinates were stained with a Nissl stain to verify the anatomical localization of the NAc according to the atlas of Franklin and Paxinos (1997).

Immunohistochemistry was performed as described by Hathaway and coworkers (1995). Fresh coronal sections were floated and shaken gently in all solutions. Sections were first incubated in PBS for several hours at 4°C and then floated in 1.5 % normal goat serum diluted in PBS for 30 minutes at room temperature. Subsequently, tissues were incubated in a rabbit affinity-purified polyclonal antibody raised against a peptide mapped at the amino terminus of c-Fos (Santa Cruz Biotechnology, Inc. Santa Cruz, CA). The antibody was diluted 1:10,000, with 1% blocking serum in PBS at 4°C . Sections were washed 3 x 5 minutes in PBS, incubated for 1 hour in biotinylated goat anti-rabbit secondary antibody diluted 1:2000 in PBS and washed again. All corresponding sections from a given study were processed in parallel and immunoreactivity was visualized by the biotin-streptavidin technique (ABC Staining System, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) using Immunopure Metal Enhanced DAB (3,3'-diaminobenzidine) as the chromagen (Pierce, Rockford, IL). Brain sections were washed in PBS and allowed to dry completely before mounting the cover slips with Permout (Sigma Chemical Co., St. Louis, MO). In control sections, c-Fos antibody binding to antigen was inhibited by pre-absorption of this antibody with a blocking peptide. The time chosen (180 min) for perfusion coincides with the peak levels of morphine-induced Fos-positive nuclei immunoreactivity as determined by pilot studies.

Quantification of Fos-positive nuclei. Fos immunoreactivity was quantified using a computer-assisted image analysis system. The system consists of a Nikon Eclipse E400 bright field microscope (Nikon Instruments Inc., Melville, NY) equipped with a digital color CCD camera interfaced to a dedicated PC. The image was captured, and analyzed using Optimas version 6.5 (Imaging Processing Solutions, Woburn, MA) to count Fos-positive nuclei. Four sections from the nucleus accumbens shell (NAcS) and core (NAcC) were taken from each animal and the Fos-positive nuclei were counted bilaterally, based on a randomization procedure. Fos-positive nuclei were considered to be signals when showing gray levels ranging between 140 and 160 (total range was from 0-255) in order to eliminate very lightly stained nuclei constitutively present in the brain. A computer-generated rectangle (200 x 500 μm) was placed in a fixed area of the NAcS and NAcC of each section, and the analysis software counted stained nuclei over the gray-scale threshold within the area (Fig. 5D). In the NAcS, the right vertical length of the rectangle was placed lateral to the major islands of Calleja and the top horizontal width was placed with the dorsal edge at equal height with the anterior commissure. In the NAcC, the left vertical length of the rectangle was placed medial to the anterior commissure and the top horizontal width was placed above the height of the anterior commissure.

Statistical evaluations. During immunohistochemical procedures, the Fos-positive nuclei counts of four sections from each animal were expressed as the mean \pm SEM. Results from multiple subjects within the different treatment groups were compared using one-way analysis of variance procedures (ANOVA). Similar statistics were performed on the mean number of beam crossings. When significance was found using ANOVA procedures, Student-Newman-Keuls (SNK) post-hoc testing was used to compare individual treatment groups. Linear regression analysis was used to evaluate any relationship between beam crossings and Fos immunoreactivity in NAc in both the

single-dose and repeated-dose morphine studies. Significance for all analyses was defined at the $p < 0.05$ level unless otherwise specified.

Results

Verification of cannula placement. Coronal sections (42 μm) of the VTA were Nissl stained to determine anatomical localization (Fig. 1A) and mapping (Fig. 1B) of the bilateral injection cannula tips. Following behavioral monitoring, each brain was perfused and frozen for sectioning, as described in the methods. In each section, the most ventral point of the cannula track showing a decrease in staining intensity was defined as the injection site. The injection cannula tips were plotted on a stereotaxic map of the VTA (Fig. 1B) -2.92, -3.08, -3.16, and -3.28 mm posterior to Bregma, according to the atlas of Franklin and Paxinos (1997). Injection cannula tips were correctly placed at the VTA in 75% of the subjects. The tissue surrounding the guide and injection cannulae did not appear to have any excessive structural damage or necrosis.

Drug diffusion into the VTA. In order to examine the extent of drug diffusion and to verify that the drug was confined to the VTA, toluidine blue solution was microinfused bilaterally into the VTA according to injection protocol as described in the methods. Toluidine blue dye was chosen because it has a molecular weight in the same range as baclofen and 2-hydroxysaclofen. Coronal sections (42 μm) through the VTA were visualized under light microscopy (figure not shown). The dye indicated that the extent of diffusion was confined to the VTA over the 180-minute study period, suggesting that the drug would remain within the VTA. Ventral tegmental anatomy was verified using the atlas of Franklin and Paxinos (1997).

GABA_B receptor agonist dose-dependently inhibits morphine-induced motor stimulation. Baclofen, a selective GABA_B receptor agonist, was used to determine if midbrain GABA_B receptors have a role in modulating morphine-induced motor activity. In these single dose treatment studies, mice were given bilateral intra-VTA baclofen (0, 0.01, 0.05, 0.1, and 1.0

nmol/hemisphere) or aCSF (vehicle) and subcutaneous morphine (10 mg/kg) or saline (vehicle). The data represent the mean number of beam crossings (\pm SEM) over 180 minutes (Fig. 2). ANOVA demonstrated significant differences in the mean number of beam crossings between the different treatment groups ($F_{5, 36} = 14.81$; $p < 0.0001$). Systemic morphine co-injected with intra-VTA aCSF produced an increase in beam crossings compared to control mice (subcutaneous saline plus intra-VTA aCSF, Fig. 2), as previously reported (Leite-Morris et al., 2002). Baclofen administration into the VTA (0.05, 0.1, and 1.0 nmol/hemisphere) produced a significant dose-dependent inhibition of morphine-induced locomotor stimulation ($p < 0.001$). Based on this dose-response curve, baclofen at 0.1 nmol per hemisphere was used in all subsequent experiments. Locomotor activity in the control groups receiving intra-VTA infusion of baclofen (0.1 nmol/hemisphere) plus subcutaneous saline (4654 beam crossings, $n = 4$) were almost at baseline compared to the aCSF plus saline control group (2878 beam crossings, $n = 6$).

GABA_B receptor antagonist reverses the inhibitory effects of baclofen upon morphine-induced motor stimulation. The GABA_B receptor antagonist 2-hydroxysaclofen was used to assess the pharmacological specificity of baclofen's effect (Fig. 3A). In this group of mice, bilateral intra-VTA injections of baclofen (0.1 nmol/hemisphere), baclofen plus 2-hydroxysaclofen (0.1 nmol/hemisphere each) or aCSF (vehicle) followed by subcutaneous morphine (10 mg/kg) or saline (vehicle) were administered. We chose the 2-hydroxysaclofen dose based on effectiveness during pilot studies (data not shown). The locomotor stimulant effect of morphine (10 mg/kg, s.c.) peaked at 90 minutes and baclofen inhibited this effect (Fig. 3A). ANOVA demonstrated significant differences in the mean number of beam crossings between the different treatment groups ($F_{4, 45} = 25.38$; $p < 0.0001$, Fig. 3B). SNK post-hoc analysis demonstrated that intra-VTA 2-hydroxysaclofen and baclofen plus morphine

significantly reversed baclofen's inhibition of morphine-induced motor stimulation ($p < 0.05$). Stereotypy counts, as measured by the repetitive breaking of the same beam, were minimal in these subjects (data not shown).

Baclofen blocks morphine-induced Fos immunoreactivity in the NAc shell. Coronal sections of the brain were obtained and Fos-positive nuclei were imaged of the NAcS (shell) and NAcC (core) of drug-treated mice (Fig. 4). Fos-positive nuclei were quantified inside a rectangle (200 μm x 500 μm Fig. 5D) placed over each region. Intra-VTA aCSF plus morphine (10 mg/kg s.c.) increased Fos immunoreactivity compared to control mice in the NAc, and differential effects were found in the NAcS and NAcC (Fig. 4A vs. Fig. 4C). The Fos-positive nuclei counts in the NAcS were higher than in the NAcC after morphine administration (Figs. 5A and 5B). ANOVA demonstrated significant differences between treatment groups in the number of Fos-positive nuclei in the NAcS ($F_{2,9} = 14.4$; $p < 0.005$). The number of Fos-positive nuclei in the NAcS was higher in morphine (vehicle/morphine) versus control (vehicle/vehicle) treated subjects, and SNK post hoc analysis revealed that this difference was significant ($p < 0.01$, Fig. 5A). No significant differences in NAcC Fos-positive nuclei counts between the different treatment groups were found.

Mice receiving bilateral intra-VTA baclofen (0.1 nmol/hemisphere) plus morphine (10 mg/kg s.c.) showed a significant reduction of Fos-positive nuclei in the NAcS (Figs. 4B and 5A) compared to morphine treatment (plus intra-VTA aCSF). Immunoreactivity appeared specific to the c-Fos protein product because neutralization of the c-Fos antibody with a blocking peptide (Fig. 4D) produced a complete blockade of Fos protein expression in the NAc. The specificity of the Fos immunolabeling was also confirmed by the finding that no labeling was observed in the control brain sections incubated in secondary antibody alone (data not shown). In addition,

no differences in the number of Fos-positive nuclei were found when comparing the control group receiving intra-VTA infusion of baclofen plus subcutaneous saline (79, $n = 3$) with the group receiving aCSF plus saline (77, $n = 5$).

Regression analysis was used to determine the relationship between number of beam crossings and number of Fos-positive nuclei in the NAcS (Fig. 5C). There was a significant positive relationship between Fos-positive nuclei in NAcS and the number of beam crossings over 180 minutes ($r = +0.74$; $p = 0.0063$; $df = 11$). No such linear relationship was found in the NAcC (data not shown).

Intermittent repeated morphine administration produces motor sensitization. To study morphine-induced motor sensitization, mice received two injections on treatment days 1, 3, 5, and on challenge day 9; the first injection was intra-VTA aCSF followed immediately by subcutaneous morphine. Morphine's motor stimulant effects were maximal between 60 and 90 minutes on each day of treatment (Fig. 6A). Behavior consisted of constant perimeter running in the open field activity monitor and low counts of stereotyped movement, which are characteristic of motor sensitization. The cumulative number of beam crossings over 180 minutes (+ SEM) is shown for each treatment day (1, 3, 5, and 9) and a progressive enhancement of motor activity was found (Fig. 6B and 6C). One-way ANOVA was performed to evaluate the effects of repeated days of morphine treatment on motor activity in this paradigm and demonstrated a significant effect ($F_{3, 24} = 9.69$, $p < 0.0005$). Post-hoc testing of individual comparisons using the SNK analysis showed a significant increase in the number of beam crossings compared to mice given a single morphine dose (day 1 vs. day 9 $p < 0.001$). A significant increase in motor activity was also found on day 5 vs. day 1 ($p < 0.05$). Since the

morphine dose (10 mg/kg) was held constant, the enhanced motor stimulant effect on day 9 suggests that this treatment approach produced motor sensitization.

Baclofen co-administered with morphine blocks the acquisition and expression of morphine-induced motor sensitization. Mice received intra-VTA aCSF (vehicle) and morphine (10 mg/kg i.p.) on days 1, 3, 5, and 9, and were placed in activity monitors for 180 minutes (Fig. 6A). ANOVA testing revealed a significant difference in motor activity on the different days of treatment ($F_{3,24} = 9.69$; $p < 0.0005$) and locomotor activity was significantly higher on day 9 compared to day 1 (Fig. 6B). These results confirmed that motor sensitization developed in our behavioral model.

The effects GABA_B receptor stimulation on the acquisition (B/M V/M) and expression (V/M B/M) of morphine-induced motor sensitization (Fig. 6C) was evaluated by comparing these treatment groups to sensitized mice (V/M V/M). On days 1, 3, 5, and 9 mice were treated either with intra-VTA aCSF (vehicle) or baclofen (0.1 nmol/hemisphere, “B”) plus subcutaneous morphine (10 mg/kg, “M”) or saline (vehicle, “V”). One-way ANOVA revealed significant differences in motor activity between all drug treatment groups on day 9 ($F_{5,36} = 39.84$, $p < 0.0001$). SNK post-hoc testing was performed for comparison of individual treatment groups. On day 9, morphine treatment resulted in a significant enhancement in locomotor activity (V/V V/V vs. V/M V/M, $p < 0.001$, compared to mice that only received vehicle (Fig. 6C). Similarly, morphine treatment on days 1, 3, and 5 significantly augmented morphine-induced locomotor activity on day 9 compared to mice treated with vehicle on days 1, 3 and 5, and morphine only on day 9, confirming locomotor sensitization to morphine (V/M V/M vs. V/V V/M, $p < 0.01$, Fig. 6C). In the BM/VM group, baclofen treatment reduced morphine-induced motor activity on day 9 compared to the V/M V/M group ($p < 0.001$, Fig. 6C). This suggests that intra-VTA

GABA_B receptor activation blocks the acquisition of morphine-induced motor sensitization. In the VM/BM group, baclofen treatment reduced morphine-induced motor activity on day 9 compared to the V/M V/M group ($p < 0.001$, Fig. 6C). This suggests that intra-VTA GABA_B receptor activation blocks the expression of morphine-induced motor sensitization.

Baclofen blocks Fos immunoreactivity in the NAc shell during acquisition and expression of morphine sensitization. The effects of repeated morphine treatment and bilateral intra-VTA injection of baclofen on accumbal Fos immunoreactivity was examined in C57BL/6 mice on day 9 of treatment. Figure 5D is a stereotaxic illustration of a coronal section through the NAc. The placement of a rectangle in the NAcS and NAcS depicts the area where Fos-positive nuclei were counted using computer assistance. Figure 7 illustrates the distribution pattern of Fos-positive nuclei in the NAcS and NAcC 180 minutes after injection. Cumulative Fos immunoreactivity from multiple subjects for each treatment group is shown in Figures 8A and B. ANOVA demonstrated significant differences in the number of Fos-positive nuclei between the drug treatment groups in the NAcS on day 9 of treatment ($F_{5, 16} = 4.81$, $p < 0.01$). SNK post-hoc testing of individual treatment groups in the NAcS showed significant differences between treatment groups. Morphine treatment on days 1, 3, 5 and 9 (V/M V/M) produced a significant increase in the number of Fos-positive nuclei compared to mice receiving only vehicle treatment ($p < 0.01$, Fig. 8A and Fig. 7B vs. 7E). Morphine treatment on days 1, 3, 5, and 9 also produced a significant increase in Fos immunoreactivity on day 9 compared to mice receiving only a single dose of morphine (V/M V/M vs. V/M D1, $p < 0.05$, Fig. 8A, Fig. 7B vs. 7A).

Baclofen pretreatment significantly reduced morphine-induced Fos immunoreactivity in the NAcS (B/M V/M vs. V/M V/M, $p < 0.05$; Fig. 8A and Fig. 7D vs. 7B). Baclofen treatment on

challenge day significantly attenuated morphine-induced Fos immunoreactivity in the NAcS (V/M B/M vs. V/M V/M, $p < 0.01$, Fig. 8A and Fig. 7C vs. 7B). The number of Fos stained neurons in the control groups for baclofen (B/V V/V) were at baseline levels (data not shown). ANOVA showed no significant differences between the different treatment groups in Fos immunoreactivity the NAcC (Fig. 8B).

In the repeated-dose morphine studies, the mean number of beam crossings and the number of Fos-positive nuclei on day 9 were analyzed using a linear regression analysis (Fig. 8C). There is a significant and positive linear relationship between motor activity (the number of beam crossings over 180 min) and the number of Fos-positive nuclei in the NAcS ($r = + 0.76$; $p = 0.0001$; $df = 19$). No such linear relationship between the number of beam crossings and the number of Fos-positive neurons were found using Fos data from the NAcC.

Discussion

These studies demonstrate that GABA_B receptors in the ventral tegmental area have a role in modulating the motor stimulant and motor sensitizing effects of morphine and associated activation of accumbal shell neurons. Mice receiving a single morphine treatment demonstrated motor stimulation and induction of Fos in the NAcS but not NAcC. Intra-VTA injection of the GABA_B receptor agonist baclofen produced a significant dose-dependent inhibition of this stimulant effect and blockade of Fos stimulation. Baclofen's effects were reversed by treatment with a GABA_B receptor antagonist. Mice receiving repeated morphine injections were sensitized to the motor stimulant effects of morphine, as exhibited by a two-fold increase in beam crossings on day 9 versus day 1. Moreover, in opiate-sensitized subjects, there was a dramatic increase of Fos immunoreactivity in the NAcS but not NAcC. Intra-VTA baclofen administration blocked both the development and expression of morphine-induced sensitization as well as morphine's associated activation of NAcS neurons. However, the effects of baclofen administration on day 9 of opiate administration may reflect its blockade of acute morphine effects on Fos immunoreactivity in the NAcS in addition to effects on sensitization responses. Finally, there was a significant positive linear relationship between Fos immunoreactivity in the NAcS and motor activity levels in both single and repeated morphine dose treatment groups. The acute and repeated dose effects of morphine on motor stimulation appear to relate to the activation of NAcS neurons presumably through mesolimbic dopaminergic systems and VTA GABA_B receptor activation blocks morphine's actions.

VTA neurotransmitter systems in opiate sensitization. Dopaminergic transmission plays a role in the expression of opiate sensitization (Vanderschuren and Kalivas, 2000). In rodents, repeated and intermittent morphine treatment produces an enhancement of motor activity

associated with increases in mesolimbic dopaminergic cell firing (Kalivas, 1985; Spanagel et al., 1993). Repeated opiate pretreatment produces a long-lasting enhancement of motor responses to dopaminergic agents such as amphetamine, cocaine, and dopamine uptake blockers (Cunningham et al., 1997; Kalivas, 1985). VTA GABA_B receptor activation inhibits the firing of mesocorticolimbic dopaminergic neurons (Lacey, 1993), via increases in potassium conductance in these neurons, and reduces somatodendritic and synaptic extracellular dopamine release (Klitenick et al., 1992; Westerink et al., 1996). In this study, intra-VTA baclofen treatment may inhibit morphine-induced motor sensitization and activation of NAcS neurons by blocking the development and expression of neuroadaptive changes in dopaminergic transmission in the VTA.

Prior research has shown opiate sensitization to involve multiple neurotransmitter systems including changes in glutamatergic neurotransmission in the midbrain (Vanderschuren and Kalivas, 2000). VTA dopamine neurons may be regulated via a combined mechanism, a direct excitatory (glutamate) corticofugal input and an indirect inhibitory (GABA) input (Wang and French, 1995). GABA_B receptors are located presynaptically on these glutamatergic afferents and synapse onto VTA dopamine neurons. The activation of presynaptic and postsynaptic GABA_B receptors in the VTA reduces NMDA postsynaptic potentials on dopaminergic cells (Seutin et al., 1994; Wu et al., 1999). Enhanced VTA GABA_B receptor inhibition of glutamate levels in amphetamine-sensitized rats versus control subjects supports this role of VTA GABA_B receptors in glutamatergic transmission in drug-sensitized subjects (Giorgetti et al., 2002). In order to determine whether VTA GABA_B receptor activation is altering postsynaptic glutamate or dopamine transmission in opiate sensitized mice requires further investigation.

Role of the NAc shell in opiate sensitization. Single- and multiple-dose morphine treatment regimens produced dramatic increases of Fos immunoreactivity in the NAcS but not NAcC regions. These results are consistent with other reports that morphine treatment produced an increase in accumbal Fos-like immunoreactivity that corresponded with extracellular dopamine levels in the NAcS but not NAcC regions (Barrot et al., 1999, Pontieri et al., 1995). Accumbal Fos immunoreactivity may result from activated dopaminergic projections to this region. The findings of acute and repeated morphine on postsynaptic NAc activation likely relate to presynaptic activation of dopaminergic cells and are confirmed by others (Curran et al., 1996; Pontieri et al., 1997).

These findings are consistent with other reports that preferential activation of NAcS neurons plays a critical role in the motor sensitizing and reinforcing effects of drugs of abuse. The NAc is a heterogeneous structure and has been subdivided into a medioventral shell region and a dorsolateral core region based upon anatomy, neurochemistry and functionality (Heimer et al., 1991). The core is considered a part of the striatal complex while the shell has more limbic connectivity and has been described as part of the extended amygdala. NAc cells receive dopaminergic and GABAergic innervations but also receive differing glutamatergic efferent projections from prefrontal cortex and other regions (Pinto and Sesack, 2000). Drugs of abuse preferentially stimulate dopaminergic transmission in the NAcS and this effect does not habituate with repeated treatment (Di Chiara, 2002). Rodents self-administer drugs preferentially to the NAcS but not NAcC (Carlezon and Wise, 1996). Repeated activation of dopaminergic and glutamatergic transmission by opiates resulting in postsynaptic activation of NAcS neurons may enhance associations between drug and associated conditioned environmental stimuli resulting in conditioned motor sensitizing effects.

Role of GABA_B receptor agonist in drug addiction. Robinson and Berridge (1993)

described the role of drug sensitization, or an increased responsiveness to the stimulant and motivational properties of drugs and drug-associated stimuli, in the development of addiction. The sensitization of dopaminergic systems may produce incentive motivational values attributed to the act of drug taking. Drug-induced sensitization increases the motivational value of drug-associated cues and contexts and may contribute to the development of compulsive drug taking, drug craving, and relapse. These sensitization phenomena are hypothesized to contribute to the development of addiction even with the loss of drug pleasure and with the aversive properties of drug abstinence (withdrawal). These findings and those of others suggest that GABA_B receptor agonist treatment inhibits the development and expression of sensitization phenomena to the stimulant and motivational effects of opiates and may be useful in the treatment of opiate addiction (Kaplan et al., 2003).

Some exciting preliminary work in humans demonstrates the efficacy of baclofen in the treatment of addictive disorders. In one such study (Addolorato et al., 2002), baclofen-treated alcoholics showed a significant improvement in abstinence, decreased obsessive and compulsive symptoms of craving and reduced drug intake compared to a placebo-treated group. In another trial in cocaine abusers (Brebner et al., 2002), baclofen treatment was given prior to drug cue presentation with imaging within a positron emission tomographic device. Patients who received baclofen prior to presentation of a cocaine drug video session showed blunting of cue-induced drug craving and a blockade of limbic activation. In contrast, cocaine-abusing subjects who did not receive medication experienced drug craving and limbic system activation.

In conclusion, single and repeated administration of opiates produced activation of NAcS, but not NAcC postsynaptic neurons. GABA_B receptor activation in the VTA blocked the acute

stimulant effects of opiates and inhibited activation of NAcS neurons. The acquisition and expression of opiate sensitization was also blocked by GABA_B receptor activation in the VTA. GABA_B receptor agonist treatment may represent a useful treatment approach for blocking the behavioral effects of opiates.

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Footnotes

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Fig. 1. Confirmation of cannula placement at the VTA. Coronal sections (42 μm) through the VTA were obtained from all mice. (A) A representative section stained with cresyl violet acetate is shown. Bilateral guide cannula tracks are visible on the dorsal surface of the brain and project to the most ventral point where the injection cannulae tips (arrowheads) represent the drug injection sites at the VTA. (B) Schematic illustration of injection cannula tips (black dots) within the VTA, representing all experiments ($n = 99$). The depicted serial coronal sections are adapted from the atlas by Franklin and Paxinos (1997).

Fig. 2. Effect of intra-VTA baclofen dose on morphine-induced motor stimulation. C57BL/6 mice ($n = 5-8$ per group) received bilateral intra-VTA injections of baclofen or aCSF immediately followed by subcutaneous morphine (10 mg/kg) or saline. Activity monitoring was conducted for 180 minutes following drug treatment and is depicted as the mean number of beam crossings (+ SEM). The dose of intra-VTA baclofen (nmol/side) and subcutaneous morphine is shown on the x-axis where “0” indicates that mice received aCSF or saline. SNK post hoc testing revealed significant differences as follows: *** $p < 0.001$ vs. aCSF (intra-VTA) plus saline (s.c.); ### $p < 0.001$ vs. aCSF (intra-VTA) plus morphine (10 mg/kg s.c.).

Fig. 3. Effect of intra-VTA 2-hydroxysaclofen treatment on baclofen’s inhibition of morphine-induced motor stimulation. C57BL/6 mice ($n = 5-8$ per group) received bilateral intra-VTA injections of baclofen (0.1 nmol/side, “B”), baclofen plus 2-hydroxysaclofen (0.1 nmol/side each, “B+S”), or aCSF (vehicle, “V”), immediately followed by morphine (10 mg/kg, s.c. “M”)

or saline (vehicle). Activity monitoring was conducted for 180 minutes following drug treatment. Locomotor activity is shown as (A) the mean number of beam crossings per 15 minutes (+SEM) and (B) the cumulative number of beam-crossings over 180 minutes. ▲ V/M, ▼ B+S/M, ◆ B/M, ■ V/V. *** $p < 0.001$ vs. V/V; +++ $p < 0.001$ vs. V/M; # $p < 0.05$ vs. B/M.

Fig. 4. Representative coronal sections showing Fos immunoreactivity in the NAc. C57BL/6 mice received (A) aCSF (intra-VTA) plus morphine (10 mg/kg s.c.), (B) baclofen (0.1 nmol/side intra-VTA) plus morphine (10 mg/kg s.c.), or (C) aCSF (intra-VTA) plus saline (s.c.), and were sacrificed 180 minutes after drug treatment. Note the differences in Fos immunoreactivity in the NAcS (see fig. 5 for quantitative analysis). (D) As a control, a blocking peptide was used to neutralize the primary antibody, and Fos immunoreactivity was not observed. LV: lateral ventricle; aca: anterior commissure; NAcS: nucleus accumbens shell; NAcC: nucleus accumbens core. Scale bar = 200 μ m.

Fig. 5. Quantitative analysis of Fos-positive nuclei in the (A) NAcS and (B) NAcC. C57BL/6 mice received intra-VTA injections of either baclofen (BAC; 0.1 nmol/side) or aCSF (VEH) immediately followed by either morphine (MOR; 10 mg/kg s.c.) or vehicle (VEH). Fos immunoreactivity on coronal sections through the NAc (see fig. 4) was quantified using computer-assisted methods. (A) In the NAcS, ** $p < 0.01$ vs. VEH/VEH; ## $p < 0.01$ vs. VEH/MOR. (B) In the NAcC no significant differences were found (C) Regression analysis using mice from all treatment groups demonstrated a significant linear relationship between number of beam crossings and number of Fos-positive nuclei in the NAcS ($r = +0.74$; $p = 0.0063$; $df = 11$). (D) A schematic coronal section through the NAc illustrating placement of the

200 x 500 μm rectangle in the NAcC and NAcS where Fos-positive nuclei were counted. ICjM: major islands of Calleja; aca: anterior commissure; NAcS: nucleus accumbens shell; NAcC: nucleus accumbens core. The depicted coronal section was adapted from the atlas by Franklin and Paxinos (1997).

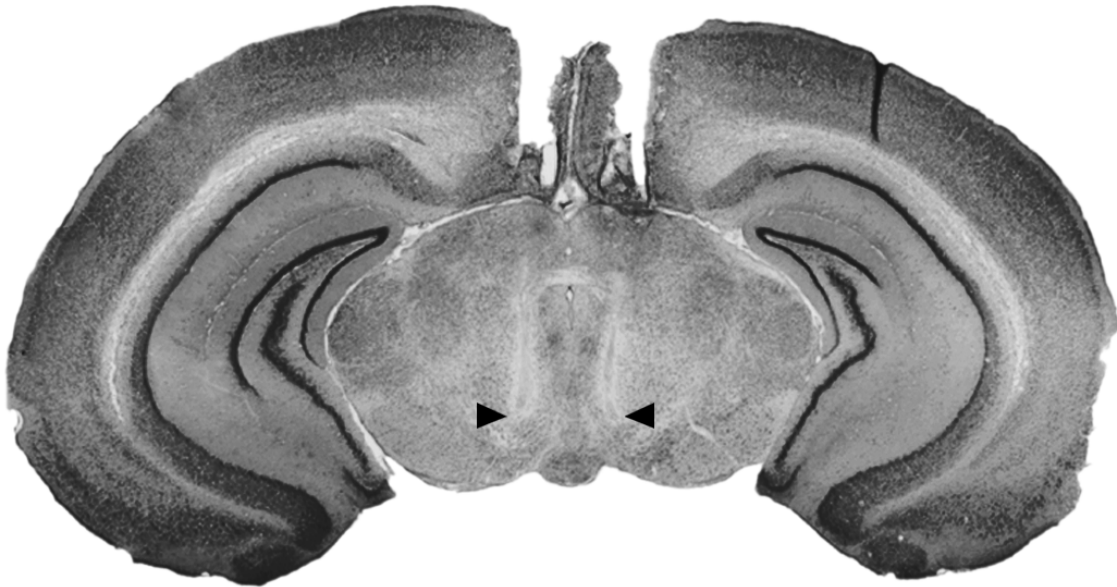
Fig. 6. Effects of the GABA_B receptor agonist, baclofen on the development and expression of morphine-induced motor sensitization. C57BL/6 mice received intra-VTA aCSF (vehicle) and morphine (10 mg/kg s.c.) on days 1, 3, 5, and 9, followed by 180 minutes of behavioral monitoring. Locomotor activity is illustrated as (A) mean number of beam crossings per 15 minutes (+ SEM) and (B) mean number of beam crossings cumulated over 180 minutes (+ SEM). * $p < 0.05$, *** $p < 0.001$ vs. day 1. (C) Mice ($n = 4-7$) received drug treatments on days 1, 3, and 5, and were challenged on day 9. The cumulative number of beam crossings per 180 minutes on day 9 is shown in the figure. V: vehicle; M: morphine; B: baclofen. The first letter represents intra-VTA drug treatment, while the second letter indicates subcutaneous administration. The first pair of letters represents the injections given on days 1, 3, and 5, and the second pair represents injections given on day 9 (challenge day). ### $p < 0.001$ vs. V/V V/V, ++ $p < 0.01$, +++ $p < 0.001$ vs. V/M V/M.

Fig. 7. Fos immunoreactivity on representative coronal sections through the NAc on challenge day (day 9). C57BL/6 mice received intra-VTA injections of either the GABA_B receptor agonist baclofen (0.1nmol/side, “B”) or aCSF (vehicle, “V”), followed by subcutaneous morphine (10 mg/kg, “M”) or saline (vehicle, “V”) on days 1, 3, 5, and 9. In the nomenclature the first pair of letters represent the injections given on days 1, 3, and 5, and the second pair represents

injections given on day 9 (challenge day). (A) V/M D1 (mice received a single dose of vehicle plus morphine on day 1 only) (B) V/M V/M (C) V/M B/M (D) B/M V/M (E) V/V V/V (F). Blocking peptide was used as a control in the V/M V/M group to neutralize the primary antibody, and no Fos immunoreactivity was observed. Note the differences in Fos immunoreactivity in the NAcS (see fig. 8 for quantitative analysis). LV: lateral ventricle; aca: anterior commissure; NAcS: nucleus accumbens shell; NAcC: nucleus accumbens core. Scale bar = 200 μ m.

Fig. 8. Quantitative analysis of Fos-positive nuclei in the (A) NAcS and (B) NAcC on challenge day (day 9). C57BL/6 mice received intra-VTA injections of either the GABA_B receptor agonist baclofen (0.1nmol/side, “B”) or aCSF (vehicle, “V”), followed by subcutaneous morphine (10 mg/kg, “M”) or saline (vehicle, “V”) on days 1, 3, 5, and 9. Fos immunoreactivity in a 200 x 500 μ m rectangle (see fig. 5D) placed over the NAc on coronal sections (see fig. 7), was quantified using a computer-assisted methods. In the nomenclature the first pair of letters represent the injections given on days 1, 3, and 5, and the second pair represents injections given on day 9 (challenge day). V/M* indicates that mice received only a single dose of intra-VTA vehicle plus subcutaneous morphine on day 1. ** $p < 0.01$ vs. V/V V/V; # $p < 0.05$, ## $p < 0.01$ vs. V/M V/M. (C) Regression analysis demonstrated a significant linear relationship between the number of beam crossings and the number of Fos positive nuclei in the NAcS during a 180 min period ($r = + 0.76$); $p = 0.0001$; $df = 19$).

A



B

