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Genetic and pharmacological antagonism of NK₁ receptor prevents opiate abuse potential

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Abstract

Development of an efficacious, non-addicting analgesic has been challenging. Discovery of novel mechanisms underlying addiction may present a solution. Here, we target the neurokinin system, which is involved in both pain and addiction. Morphine exerts its rewarding actions, at least in part, by inhibiting GABAergic input onto substance P (SP) neurons in the ventral tegmental area (VTA), subsequently increasing SP release onto dopaminergic neurons. Genome editing of the neurokinin 1 receptor (NK₁R) in the VTA renders morphine non-rewarding. Complementing our genetic approach, we demonstrate utility of a bivalent pharmacophore with dual activity as a μ/δ opioid agonist and NK₁R antagonist in inhibiting nociception in an animal model of acute pain while lacking any positive reinforcement. These data indicate that dual targeting of the dopaminergic reward circuitry and pain pathways with a multifunctional opioid agonist-NK₁R antagonist may be an efficacious strategy in developing future analgesics that lack abuse potential.

Introduction

The U.S. faces two simultaneous and associated epidemics. First, the widespread prevalence of pain over the last 20 years has led to the very well-publicized opioid epidemic¹. Yet, 100 million Americans suffering from chronic pain still need efficacious analgesics, leaving the medical community in a conundrum. While guidelines for prescribing opioids have been put forth by the Centers for Disease Control and Prevention², it is likely that understanding how opioids drive reward pathways may lead to development of efficacious, non-addictive pain relievers.

Substance P (SP) has a storied role in propagating both the nociceptive and reward pathways in the peripheral and central nervous systems³. As an undecapeptide with principle

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biological activity at the neurokinin-1 receptor (NK₁R), SP induces pronociceptive flinching and scratching behaviors when administered intrathecally (i.th.)⁴ and elicits positive conditioned place preference (CPP) when administered into multiple CNS nuclei⁵. Therefore, the neurokinin system is a very attractive pharmacological target for both pain intervention and addiction. While morphine can inhibit presynaptic SP release from dorsal root ganglia neurons in the spinal cord⁶, it also indirectly increases dopaminergic cell firing in the ventral tegmental area (VTA) of the midbrain by inhibiting tonically active, inhibitory GABAergic neurons⁷. Although NK₁Rs are located in the VTA amongst other CNS regions⁸, and activation therein with SP elicits dopaminergic firing⁹, the role of SP in mediating the rewarding action of drugs of abuse is unclear.

Here, we show that genomic editing of the NK₁R, specifically in the VTA, of adult, male rats renders morphine non-rewarding. Additionally, we demonstrate that morphine exerts its rewarding actions, at least in part, by inhibiting GABAergic input onto SP neurons in the VTA, subsequently increasing SP release onto dopaminergic neurons. Finally, we demonstrate utility of a bivalent pharmacophore with dual activity as a μ/δ opioid agonist and NK₁R antagonist in inhibiting nociception in an animal model of acute pain while lacking any positive reinforcement.

Materials and Methods

Animals

Male Sprague Dawley rats (250–300g) and male ICR (20–25g) mice purchased from Harlan (Indianapolis, IN) were housed in a climate controlled room on a regular 12 hour light/dark cycle with lights on at 7:00 am and food and water *ad libitum*. Rats were housed 3 per cage and used for all microdialysis, CPP, and SNL studies. Mice were housed 5 per cage and used for acute tail flick in a hot water bath and flinching studies. All procedures were performed during the 12-hour light cycle and according to the policies and recommendations of the International Association for the Study of Pain, the NIH guidelines for laboratory animals, and were approved by the IACUC of the University of Arizona.

Microdialysis guide cannula implantation

Rats were anesthetized with 80% ketamine/20% xylazine and stereotaxically fixed (Stoelting, Wood Dale, IL). For ventral tegmental area (VTA) microdialysis experiments, a guide cannula (AG-10, EiCom, San Diego, CA) was lowered 7.5 mm through a burr hole drilled in the skull at P: 5.0 mm, L: 0.7 mm from the bregma. The guide cannula was fixed in place with dental cement and a dummy probe (AD-10, EiCom) was screwed in place to maintain guide cannula patency, and all animals were given prophylactic subcutaneous antibiotic (gentamycin, 1 mg/kg). Animals were allowed to recover for 3 days before microdialysis experiments.

For the NAc, a microdialysis guide cannula (AG-8, EiCom) was lowered 6.0 mm through a hole drilled in the skull at A: 1.7 mm, L: 1.0 mm from the bregma. A dummy probe (AD-8, EiCom) was screwed in place to maintain guide cannula patency. The VTA microinjector guide cannula (22GA, 10 mm from pedestal, PlasticsOne) was lowered 7.5 mm through a

hole drilled at P: 5.0 mm, L: 0.7 mm from the bregma. The NAc guide cannulas were fixed in place with dental cement, and all animals were given prophylactic subcutaneous antibiotic (gentamycin, 1 mg/kg). Rats were then allowed to recover for 3 days before undergoing microdialysis experiments.

Microdialysis for VTA and NAc sampling

Microdialysis experiments were performed 3 days after surgical implantation of the guide cannulas. For the VTA microdialysis experiments, the dummy probe was removed from the guide and a microdialysis probe (AZ-10-2, EiCom) was inserted and screwed into place. The probe was attached to a cannula swivel at the top of the cage to allow for real-time sampling in awake, freely moving rats. The microdialysis probe was perfused with artificial cerebral spinal fluid (aCSF, NaCl 147 mM, KCl 2.8 mM, CaCl₂ 1.2 mM, MgCl₂ 1.2 mM) at a rate of 0.5 µL/min. VTA dialysate was collected in microcentrifuge tubes containing 1 µL aprotinin (1 µM) placed in an automated, cooled, timed turnstile set to 1 hour bins. BL microdialysis was run for 2 hours before any pharmacological manipulations. Morphine (10 mg/kg, i.p.) or vehicle (saline, i.p.) were injected and 4 dialysate samples were collected over 4 hours. After each hour, the microcentrifuge tubes were snap frozen in liquid nitrogen and subsequently stored at -80 °C until the dialysates were analyzed for SP content using a commercial SP ELISA (R&D Systems, Minneapolis, MN).

For the NAc microdialysis experiments, the dummy probe was removed from the guide cannula and a microdialysis probe (AZ-8-2, EiCom) was inserted and screwed into place. Perfusing the probe at a rate of 2 µL/min, dialysate was collected in cooled microcentrifuge tubes in 30 min bins. BL microdialysis was run for 2 hours before any pharmacological manipulations in the VTA. Drugs (either morphine 5 µg, SP 1 µg, L-732,138 1 µg, TY032 2 µg, or vehicle) were administered in 0.5 µL volumes over 50 sec (0.01 µL/sec) directly into the VTA via the surgically installed VTA guide cannula. Briefly, the dummy probe was removed at the time of drug administration and a prefilled 2 µL Hamilton syringe was attached to the VTA guide cannula. Drugs were administered slowly to ensure minimal disruption of the VTA parenchyma. Dialysate was collected every 30 min for 3 hours; cocaine 20 mg/kg was then injected i.p. as a positive control for NAc extracellular DA content. Microcentrifuge tubes were stored in -80 °C until HPLC analysis for DA content. NAc dialysate was analyzed using HPLC (Agilent 1100, Coulochem III 5014B electrochemical detector).

Microdialysis probe verification

Following microdialysis experiments, animals were sacrificed; brains were harvested and fixed in 4% paraformaldehyde. After cryoprotection in 30% sucrose, VTA/NAc blocks were sectioned in 40-µm increments on a cryostat (Microm HM 525) and mounted. Slides were viewed microscopically (Olympus) for visual identification of NAc and VTA cannula placements. Terminations of the guide cannulas were plotted on a rat brain atlas¹⁰ for verification.

gRNA design for *Tacr1* gene targeting

Our strategy was to target the tachykinin receptor 1 (*Tacr1*) gene using a gRNA designed to match the 5' end of the protein sequence that is most likely to result in a complete loss of the protein. We identified and extracted the sequence of the *Tacr1* gene from the genomic sequence of chromosome 4 from Sprague Dawley rats (AC_000072.1). To design the gRNA sequence, we screened for potential off-targets using the gRNA design tool (<http://crispr.mit.edu>)¹¹. No potential gRNA sequence met our criteria of selectivity in the first exon from the *Tacr1* gene; thus, the gRNA selected for knockout was located in exon 2 (on the reverse strand: GATGACCACTTTGGTAGCCG, score 89; Figure 3a). As a control, we chose a gRNA targeting the same gene but in a non-coding region located in intron 1 of the *Tacr1* gene (on the reverse strand: gTAAAATGGATATTTTCGGTGC, score 89, a g was added at the 5' end to ensure better expression downstream of the U6 promoter)¹².

Cloning of gRNAs

We used the pSpCas9(BB)-2A-GFP plasmid (PX458, Cat#48138, Addgene, Cambridge, MA)¹³ which allows for simultaneous expression of the Cas9 enzyme with the gRNA and green fluorescent protein (GFP) to control for transfection efficiency. The plasmid was cut using *BbsI* restriction enzyme according to the manufacturer's instructions (Cat#FD1014; Thermo-Fisher Scientific; Waltham, MA). The digested plasmid was extracted from a 1% agarose gel (Cat#K0691; Thermo-Fisher Scientific). Oligonucleotides were designed according to Ran et al¹³ for the cloning of either exon 2 targeting gRNA (*Tacr1* forward: 5' - CACCGATGACCACTTTGGTAGCCG-3'; *Tacr1* reverse: 5' - AAACCGGCTACCAAAGTGGTCATC-3') or intron 1 targeting gRNA (*Tacr1* control forward: 5' -CACCGTAAAATGGATATTTTCGGTGC-3'; *Tacr1* control reverse: AAACGCACCGAAATATCCATTTTAC) and were obtained from Eurofins. A 10 μ M solution of forward and reverse oligonucleotides was annealed in a thermocycler using the following protocol: 37 °C for 30 min, 95 °C for 5 min, with a cooling to 25 °C at a rate of 5 °C/min. Then, 100 ng of the digested pSpCas9(BB)-2A-GFP plasmid was set up for ligation with 50 nM of the annealed oligonucleotides at 22 °C for 5 min using Rapid DNA ligation Kit (Cat#K1422; Thermo-Fisher Scientific). The ligation products were transformed into *E. coli* DH5 α competent bacteria (Cat#C2987; New England Biolabs; Ipswich, MA) according to the manufacturer's instructions. The integrity of all plasmids was confirmed by Sanger sequencing (Eurofins, Louisville, KY) and 100% efficiency was observed for the insertion of gRNA sequences into the pSpCas9(BB)-2A-GFP plasmid. Plasmids were purified from DH5 α *E. coli* using the NucleoBond® Xtra Maxi kit (Cat# 740414, Macherey-Nagel, Germany).

In vivo transfection of CRISPR plasmids

For *in vivo* transfection, the plasmids pSpCas9(BB)-2A-GFP-Tacr1-gRNA or pSpCas9(BB)-2A-GFP-Tacr1-control-gRNA were diluted to 0.5 μ g/ μ l in a 5% sterile glucose solution. Then, Turbofect *in vivo* transfection reagent (Cat#R0541, Thermo-Fisher Scientific) was added following the manufacturer's instructions. Finally, 0.5 μ L of the plasmid complexes were injected bilaterally into the VTA of Sprague Dawley rats.

CRISPR-Cas9 to knockdown the NK₁ receptor in the VTA

Rats were anesthetized with 5% isoflurane in O₂ (2.5 L/min) and maintained at 2.5% isoflurane while fixed in a stereotaxic frame. Animals were microinjected with NK₁R gRNA/Cas9 or control directly into the VTA bilaterally, but on each side individually, for a total volume of 0.5 μL over 5 min per side. The cannula was slowly removed and the skull burr hole was filled with bone wax (Medline Industries, Mundelein, IL); animals were given prophylactic subcutaneous antibiotic (gentamycin, 1 mg/kg) and were used for microdialysis, CPP, or IHC studies 3 weeks later.

Conditioned place preference to morphine

Three weeks post-VTA Cas9 injections, animals' baselines (BLs) were obtained in a modified, three chambered, rat-conditioned place preference/aversion (CPP/CPA) apparatus (San Diego Instruments, San Diego, CA) as previously reported¹⁴: 1) an end chamber with rough floor and horizontal black/white striped walls; 2) an end chamber with smooth floor and solid black walls; and 3) a center chamber with solid grey walls and metal rod flooring. Rats were placed in the center chamber and a 15-min recording period indicated entrance into chambers and time spent in chambers. Any rat with >80% preference to any chamber during BL was excluded from the study. The rats were subdivided into morphine (10 mg/kg, i.p.) or saline (1 mL/kg, i.p.) groups and trained to associate one end chamber with morphine and the other with saline for 15 min, alternating days for a total of five drug exposures and five saline exposures. After ten days of training and 24 hrs after the last drug or saline administration, animals are placed in the center chamber. The number of entries and time spent in all three chambers are recorded over 15 min. CPP score is calculated as:

$$\text{CPP score} = \text{time spent in drug paired chamber}_{\text{exp}} - \text{time spent in drug paired chamber}_{\text{BL}}$$

IHC to verify NK₁ receptor knockdown

Following CPP experiments, rats were sacrificed via transcardial perfusion with 0.1 M PB and 4% paraformaldehyde for tissue fixing. Next, the brains were removed; the VTA was blocked and placed in 4% paraformaldehyde for 30 min followed by 30% sucrose in 0.1 M PB for 2 days or until the specimen sunk. The VTA was sliced in coronal, 40-μm thick sections and placed in cooled 0.1 M PB in a 12-well plate. The sections were placed in 1% sodium borohydride for 30 min followed by a copious rinse with 0.1 M PB 3 X 10 min. Sections were then rinsed in 0.1 M Tris saline 2 X 5 min followed by an incubation in 0.5% BSA in Tris saline for 30 min for blocking. Tissue specimens were transferred to scintillation vials with a fine brush and incubated in anti-NK₁ receptor primary antibody (1:5,000; RA25001, Neuromics, Edina, MN) in 0.1% BSA/0.25% Triton in Tris saline for 2 days at 4 °C. Following incubation, tissue was transferred to a 12-well plate with fine brush and rinsed with 0.1 M Tris saline 3 X 10 min. The slices were then incubated and light protected in anti-rabbit secondary antibody (Alexa-Flour goat anti-rabbit, 1:800, Thermo-Fischer Scientific) in 0.1% BSA/0.1 M Tris saline for 2 hours at room temperature. With continued protection from light, the tissue was rinsed in 0.1 M Tris saline 3 X 10 min and then 0.1 M PB 3 X 10 min. The tissue was mounted onto slides in 0.05M PB and cover-

slipped with Prolong Gold Antifade w/DAPI (Thermo-Fischer Scientific). Images were viewed on a Olympus microscope and merged.

Acute thermal nociception in mice

Warm water bath (52 °C) was used as a nociceptive stimulus to assay acute thermal nociception in mice. The thermal tail withdrawal latency (TWL) was defined as the time (sec) from immersion of 2/3rds distal portion of the tail in water to its withdrawal. BL values for all animals were obtained prior to drug administration; animals were excluded if their TWL was greater than 7 sec at BL. Administration of compounds in mice were performed via intrathecal (i.t.) injection using a slightly modified Hylden and Wilcox technique^{4,15}. A 10 µL Hamilton injector fitted with a 30-gauge needle was used to administer 5 µL. Tail-flick times were then measured every 20 min for 2 hours. A cut-off latency of 10.0 sec was implemented to prevent tissue damage to the distal third of the tail.

Maximal percent efficacy was calculated and expressed as:

$$\% \text{ Antinociception} = 100 * (\text{test latency after drug treatment} - \text{BL latency}) / (\text{cut-off} - \text{BL latency})$$

Acute thermal nociception in rats

Rats were assessed for thermal nociception using a modified method developed by Hargreaves and colleagues.¹⁶ Animals were allowed to acclimate for 30 min in a Plexiglas enclosure fixed on a clear glass plate. A mobile radiant infrared (IR) heat source was focused onto the left hindpaw from underneath the glass plate. A standard IR intensity was used for all animals and an automatic cut-off point of 32.4 sec was set to prevent tissue damage. The heat source and timer were activated simultaneously by the experimenter and cut-off by a motion detector on the apparatus focused directly on the plantar surface of the left hindpaw. BL was assessed prior to i.t. compound administration. PWL values were collected every 15 min for 2.5 hours after i.t. compound administration. PWL values were converted to percent-antinociception.

Maximal percent efficacy was calculated and expressed as:

$$\% \text{ Antinociception} = 100 * (\text{test latency after drug treatment} - \text{BL latency}) / (\text{cut-off} - \text{BL latency})$$

SP-induced nocifensive behaviors in mice

SP i.t. elicits nocifensive behaviors such as flinching as previously described¹⁷. We injected mice i.t. with TY032 (3 µg/5 µL) and SP (0.3 µg/5 µL) and observed flinching behavior for 5 min to determine if the NK₁ antagonist blocked these elicited behaviors. Naloxone (1 mg/kg, i.p.) or vehicle was injected 15 min prior to TY032 or vehicle to remove opioid activity¹⁸, which was subsequently injected 5 min prior to SP administration.

Lumbar i.t. catheter implant surgery for spinal drug delivery in rats

Male Sprague-Dawley rats were prepared with i.t. catheters as described by Yaksh and Rudy for direct central drug administration¹⁹. Rats were anesthetized with 80% ketamine/20%

xylozine (1 mg/kg, i.p.) and placed in a stereotaxic frame (Stoelting). The cisterna magna was opened and a 7.5 cm catheter (PE10) was implanted in the spinal column, terminating in the lumbar region. The catheter was sewn into the cervical muscle to maintain its position and animals were given prophylactic subcutaneous antibiotic (gentamycin, 1 mg/kg). Control animals received sham surgeries. The rats were allowed to recover for 7 days before SNL surgeries. Animals exhibiting paralysis were excluded from further surgeries or testing.

Spinal nerve ligation for induction of neuropathic pain in rats

Spinal nerve ligation (SNL) was utilized to induce neuropathic pain as described by Chung's group²⁰. Animals were anesthetized with 5% isoflurane in O₂ (2.5 L/min) and subsequently maintained with 2.5% isoflurane. The left lumbar/sacral region of the dorsal vertebral column was exposed and the left L₆ vertebra was removed. Both L₅ and L₆ nerves were individually ligated with 4-0 suture silk. The incision was closed and the animals were allowed to recover for 7 days. Animals exhibiting motor deficiencies or a lack of tactile allodynia were excluded from further testing. Sham control animals underwent the same procedures excluding the ligation of the L₅/L₆ nerves.

Thermal hypersensitivity

Rats were assessed for thermal hypersensitivity using a modified method developed by Hargreaves and colleagues¹⁶. Animals were allowed to acclimate for 30 min in a Plexiglas enclosure fixed on a clear glass plate. A mobile radiant infrared (IR) heat source was focused onto the left hindpaw from underneath the glass plate. A standard IR intensity was used for all animals and an automatic cut-off point of 32.4 sec was set to prevent tissue damage. The heat source and timer were activated simultaneously by the experimenter and cut-off by a motion detector on the apparatus focused directly on the plantar surface of the left hindpaw. Pre-injury was assessed prior to SNL; post-injury BL was assessed prior to i.t. drug administration. PWL values were collected every 20 min for 3 hours after i.t. drug administration.

Mechanical allodynia

Rats were assessed for mechanical allodynia one week after SNL-induced neuropathic pain using calibrated von Frey filaments (0.4–15.0 g). First, rats were allowed to acclimate in suspended wire-mesh cages for 30 min. Then, von Frey filaments were used to probe perpendicularly the plantar surface of the left hindpaw. A response was elicited when the animal retracted the hindpaw from the wire-mesh floor. Pre-injury values were assessed prior to SNL; post-injury BL was assessed prior to i.t. drug administration via the surgically installed i.t. catheter. von Frey filament probing was repeated every 20 min for 3 hours after i.t. administration.

Rotarod motor impairment

Male Sprague-Dawley rats were trained to walk on a spinning rotarod device (Columbus Instruments International, Columbus, OH) as described by Vanderah's group²¹ until all animals were able to remain on the rotating platform for 180 sec at a speed of 10 revolutions per min. Following i.t. administration of TY032 or vehicle via the surgically installed i.t.

catheter, the length of time the rats were able to remain on the device without falling was recorded at 20-min intervals for 120 min.

Statistical analyses

Microdialysis time courses were analyzed by nonparametric two-way analysis of variance (ANOVA; with multiple comparisons, post-hoc Student-Newman-Keuls) with the AUC analyzed with one-way ANOVA, and the Student's t-test when appropriate. CPP and flinching studies were analyzed by one-way ANOVA. Nociception time courses were analyzed by two-way ANOVA. Data were plotted in GraphPad Prism 7 (GraphPad Software, La Jolla, CA) and represent the mean \pm SEM with statistical significance represented by $p < 0.05$. Blinding was used for receptor density and nociception assays. No randomization was used. Predetermine sample size was based on our previous publications and literature review^{22,23}. The variance was similar in the groups being compared.

Results

Systemic morphine acutely increases intra-VTA SP release via disinhibition of GABAergic neurons

To examine the role SP plays in opiate activation of the reward circuit, we employed microdialysis in the VTA of male Sprague Dawley rats (Fig. 1a). Intraperitoneal (i.p.) administration of morphine sulfate (10 mg/kg) significantly increased VTA SP release in the first hour compared to vehicle ($+76.1 \pm 42.9\%$ of baseline (BL), $n=7$, versus $+5.3 \pm 12.4\%$ of BL, $n=10$, respectively; $p<0.005$; Fig. 1b). SP release returned to BL following the first hour of increased release in the morphine-treated animals. To assess morphine's direct effect on SP release in the VTA, we administered morphine (35 mM) into the VTA via reverse microdialysis and analyzed the dialysate for SP release. Intra-VTA administration of morphine significantly increased VTA SP release in the first hour compared to vehicle ($+36.8 \pm 8.4\%$ of BL, $n=16$, versus $-6.2 \pm 9.1\%$ of BL, $n=11$, respectively; $p<0.05$; Fig. 1d). Morphine administration into the VTA via reverse microdialysis continued to significantly increase SP release in the VTA compared to vehicle in the 2nd hour ($+72.0 \pm 14.2\%$ versus $+8.7 \pm 13.1\%$, respectively; $p<0.005$). After the morphine induced increase at 1 hour of SP release, a second set of animals received the GABA-A agonist muscimol (10 μ M)²⁴ via the VTA microdialysis probe, in these animals there was a significant attenuation of morphine-induced SP release by 2 h as compared to morphine only treated animals ($16.1 \pm 19.4\%$ versus $72.0 \pm 14.2\%$, respectively; $p<0.01$) (Fig. 1d).

It is established that morphine inhibits the firing of GABAergic neurons in the VTA, preventing the release of the inhibitory neurotransmitter GABA onto dopaminergic neurons⁷. As such, opioid-mediated disinhibition has been recognized as the primary mechanism of DA firing in the VTA. To test the hypothesis that GABAergic tone additionally inhibits SP firing and subsequent release, we administered the GABA-A receptor antagonist bicuculline (10 μ M) directly into the VTA via reverse microdialysis and sampled the dialysate for SP release. During the first hour of VTA administration of bicuculline ($n=9$), SP release was significantly increased to $+99.3 \pm 23.3\%$ of BL compared to vehicle-treated animals ($-19.9 \pm 3.2\%$ of BL, $n=8$; $p<0.0001$; Figure 1f). Throughout this

manuscript, only animals verified to have the guide cannula correctly positioned in the VTA were used in the analyses (Fig. 1c, e, g).

Intra-VTA administration of NK₁ antagonist blocks morphine-induced DA release in the NAc

Following the studies investigating how morphine impacts SP release in the VTA, we sought to determine how SP in the VTA affects NAc DA release *in vivo* using microdialysis (Figure 2a). Intra-VTA administration of SP (1 µg/0.5 µL (n=9)) significantly increased NAc DA release, peaking at 90 min post-injection (+103.6 ± 33.1%), compared to vehicle-treated animals (n=7, p<0.05; Fig. 2b), and this was blocked by co-administration of the NK₁R antagonist L-732,138 (1 µg/0.5 µL). Intra-VTA administration of morphine (5 µg/0.5 µL, n=9) significantly increased NAc DA release, peaking at 30 min post-injection (+81.5 ± 26.8%), compared to vehicle-treated animals (n=7, p<0.05; Fig. 2c). To determine if the NK₁R antagonist could attenuate intra-VTA morphine-induced DA release, we administered the NK₁R antagonist L-732,138 (1 µg/0.5 µL) in conjunction with morphine (5 µg/0.5 µL, n=8) directly into the VTA and measured DA release in the NAc. The combination did not significantly increase DA release compared to vehicle-treated animals (n=7; Fig. 2c). For comparison amongst groups, the area under the curve (AUC) for percent-change in DA release was calculated (Fig. 2d). SP and morphine were each significantly greater than their respective vehicle controls (402.60 ± 124.20 vs -49.9 ± 37.6, and 326.1 ± 111.1 vs -72.6 ± 32.3, respectively). Both SP- and morphine-induced DA release was blocked by co-administration with L-732,138. Only animals with proper VTA microinjections and NAc microdialysis guide cannula placements were included in data analyses (Supplemental Fig. 1a, b).

CRISPR-Cas9 knockout of NK₁R in the VTA prevents morphine CPP

We next investigated whether selectively knocking out *Tacr1*, the gene encoding NK₁R, specifically in the VTA of male Sprague-Dawley rats would block conditioned place preference (CPP) to morphine. We injected clustered regularly interspaced short palindromic repeats (CRISPR) associated protein-9 nuclease (Cas9) plasmids bilaterally into the VTA with the gRNA targeting either exon 2 (i.e., NK₁R KO) or intron 1 (i.e., control CRISPR) of the *Tacr1* gene (Fig. 3a). After three weeks, NK₁R densities in the VTA were compared between KO and control animals using immunohistochemistry (IHC; Fig. 3b). Control animals displayed a NK₁R density of 2.7 ± 0.7 while KO animals had significantly decreased receptor densities (0.8 ± 0.2, p<0.05; Fig. 3c). Three weeks after CRISPR-Cas9 plasmid injections, we began a training paradigm for morphine (10 mg/kg) and assessed the animals' CPP after 10 days. CRISPR-Cas9 control animals trained with morphine (n=18) had significantly elevated CPP scores compared to vehicle-treated animals (144.9 ± 50.2 and -13.5 ± 46.6, n=11, respectively, p<0.05; Fig. 3d). VTA-NK₁R KO animals (n=16) exhibited no difference in CPP score to morphine compared to vehicle-treated animals (n=11, -25.6 ± 34.0 and -30.5 ± 46.6 respectively), while those trained with morphine were significantly different between CRISPR KO and CRISPR control groups (p<0.01, Fig. 3d). To examine the chemical correlate of this reward signature of NK₁R KO animals, microdialysis was performed in the NAc after systemic administration of morphine or vehicle in NK₁R CRISPR KO and control animals. The time course of DA release was evaluated and

converted to AUC values. In CRISPR-Cas9 control animals, morphine (10 mg/kg i.p.) significantly increased DA release in the NAc during the three-hour time course (n=9, 380.4 ± 149.5) compared to vehicle (n=8, 40.6 ± 51.3 ; $p < 0.05$; Fig. 3e). Amongst the CRISPR KO groups, morphine treatment did not significantly increase DA release in the NAc compared to vehicle treatment (98.0 ± 95.2 , n=7 and -89.8 ± 48.4 , n=8 respectively). For 55 days post-CRISPR-Cas9 injection, both NK₁R KO and control animals gained body weight at the same rate (Fig. 3f), indicating the reward processing for food intake/appetite was still intact while the reward processing of opioids was rendered nonfunctional.

TY032 results in antinociception in naïve animals and in a model neuropathic pain

TY032, a μ/δ -opioid agonist and NK₁R antagonist was designed to inhibit neuropathic pain while reducing both opioid dose escalation and unwanted side effects²⁵. To determine the functional pharmacological properties of TY032, we investigated the compound's ability to inhibit SP-induced flinching in mice. SP (0.3 $\mu\text{g}/5 \mu\text{L}$; n=7) significantly increased flinching and scratching to 19.4 ± 3.5 in 5 min compared to vehicle (n=7, 1.1 ± 0.5 in 5 min, $p < 0.005$; Fig. 4a). Intrathecal TY032 3 $\mu\text{g}/5 \mu\text{L}$ (n=6) significantly reduced SP-induced flinching to 2.8 ± 1.3 in 5 min ($p < 0.05$). Additionally, with pretreatment of naloxone 1 mg/kg, TY032 (n=7) significantly reduced SP-induced flinching and scratching to 4.9 ± 1.8 in 5 min ($p < 0.05$). With naloxone already on board, this suggests TY032 is inhibiting SP-induced flinching primarily through its actions at the NK₁R and not by way of its opioidergic activity.

To assess the opioidergic activity of TY032 *in vivo*, we performed a tail flick assay in a hot water bath (52°C) with mice and evaluated the degree to which naloxone inhibits the precipitated antinociception of TY032. TY032 (3 $\mu\text{g}/5 \mu\text{L}$, i.th., n=5) significantly increased tail withdrawal latency (TWL) to 7.72 ± 1.33 sec 40 min post-injection, compared to vehicle-treated animals (n=7, 2.70 ± 0.40 sec, $p < 0.0005$; Fig. 4b). Pretreatment with naloxone (1 mg/kg, i.p., n=7) reduced TY032 antinociception by about 50% but was not significantly different from vehicle-treated animals. TY032 (10 mg/kg, i.p., n=6) significantly increased hindpaw withdrawal latencies 15 min post-injection to $+55.0 \pm 13.0\%$ of BL compared to vehicle-treated animals (n=6, $+16.9 \pm 10.5\%$ of BL, $p < 0.005$; Fig. 4c). Percent-antinociception remained significantly elevated compared to vehicle 2 hours post-injection ($p < 0.05$) and returned to BL by 2.5 hours.

We next assessed if TY032 was efficacious in reversing neuropathic pain in rats with spinal nerve ligation (SNL) – a surgical model of neuropathic pain. We evaluated pre-injury BL to innocuous mechanical stimuli with von Frey filaments and to thermal nociception with the IR radiant heat source as described above, followed by SNL and a re-evaluation of mechanical and thermal thresholds one week later. To mechanical stimuli, animals were reliably neuropathic with a post-injury BL paw withdrawal threshold (PWT) of 2.1 ± 0.6 g. TY032 (0.3 $\mu\text{g}/5 \mu\text{L}$, i.th., n=11) produced a significant anti-allodynia compared to vehicle, peaking at 80 min post-administration (9.7 ± 1.8 g) and returning to BL after 180 min (Fig. 4d). We administered two additional doses of TY032 (0.1 and 0.2 $\mu\text{g}/5 \mu\text{L}$) and constructed a dose-response curve for its effect on reversing mechanical allodynia. The A₅₀ was calculated to be 0.17 $\mu\text{g}/5 \mu\text{L}$. Exposure to a thermal stimulus produced a post-injury BL paw

withdrawal latency (PWL) of 8.6 ± 0.8 s. TY032 ($1.0 \mu\text{g}/5 \mu\text{L}$, i.th., $n=7$) produced a significant anti-hyperalgesia compared to vehicle peaking 20 min post-administration (22.4 ± 3.2 s) and lasting 80 min (Fig. 4e). Two additional doses of TY032 were administered (0.1 and $0.3 \mu\text{g}/5 \mu\text{L}$) and a dose-response curve was constructed for its anti-hyperalgesic effect to thermal stimuli; the A_{50} was calculated to be $0.12 \mu\text{g}/5 \mu\text{L}$.

To assess if TY032 induces motor impairment, we used the rotarod test to evaluate the latency with which rats will maintain their walk on a rotating cylinder. TY032 ($1 \mu\text{g}/5 \mu\text{L}$ i.t., $n=6$) did not significantly alter the latency to fall of the rod compared to vehicle-treated animals ($n=8$, Supplementary Fig. 2).

TY032 neither results in elevated levels of NAc DA release nor in CPP

With TY032 demonstrating antinociceptive efficacy in mice and rats, we next investigated whether it has the potential for abuse. We first assessed the DAergic response to intra-VTA TY032 via microdialysis in the NAc and converted to AUC. TY032 ($2.0 \mu\text{g}/0.5 \mu\text{L}$, $n=6$) did not significantly change DA release compared to vehicle ($n=7$) treated animals (Fig. 4f). We next used the CPP paradigm described above wherein we trained male Sprague-Dawley rats with TY032 $10 \text{ mg}/\text{kg}$, i.p./once per day. After ten days, we assessed their preference for morphine and TY032. The vehicle for morphine was saline while the vehicle used for TY032 was 10% DMSO, 10% Tween, 80% saline. Saline training produced a CPP score of 4.1 ± 40.6 ($n=15$) while the positive control, morphine ($10 \text{ mg}/\text{kg}$, i.p., $n=13$) produced a significantly increased CPP score of 128.0 ± 41.8 ($p<0.05$; Fig. 4g). The vehicle for TY032 produced a CPP score of -13.9 ± 38.4 ($n=13$) while TY032 $10 \text{ mg}/\text{kg}$ ($n=14$) did not produce a statistically significant difference in CPP score (1.1 ± 49.1) compared to vehicle. These data indicate that while TY032 does produce antinociception when administered both centrally and systemically, it does not produce characteristics of reward.

Discussion

Chronic pain affects 100 million Americans or 30% of the US population²⁶. About 2.5 million Americans suffer from opioid abuse²⁷ from prescription narcotics or heroin, placing researchers at an interesting crossroads of the two epidemics²⁸. It is the view of many that the health profession is responsible for the opioid epidemic we face, and as such, it is the responsibility of the health profession to confront it. Deciphering possible dual acting mechanisms of inhibiting narcotic reward activation while enhancing pain relief may lead to the development of novel, non-addictive pain therapies.

Here, we show the role substance P (SP) plays in mediating opioid activation of the reward pathway. Mu opioid agonists inhibit GABAergic input directly onto DA neurons^{7,29} (the canonical model); yet we demonstrate that they also relieve tonic inhibition of SP containing neurons that promote dopamine (DA) firing, as evidenced by the bicuculline administration induced SP release in the VTA and the SP-induced increase in dopamine release in the nucleus accumbens. Studies have shown that the VTA is a highly heterogeneous brain region, containing dopamine (DA), GABA, and glutamate neurons^{30,31,32}. GABAergic inputs from other areas of the CNS make connections onto non-dopaminergic neurons of the VTA and are sensitive to mu opioids³³. Interestingly, a few reports suggest that mu opioid

agonist DAMGO may directly stimulate some DA neurons of the VTA³⁴. The activity of the DA cells in the VTA is also driven by glutamate³⁵. Substance P is often contained in glutamatergic neurons and adds to the excitability of the postsynaptic neuron via neurokinin receptors (Gα_q-protein coupled receptors)³⁶. This glutamate-SP is found in primary nociceptive fibers to drive the spinal pain signal³⁷, as well as in the rostral ventromedial medulla where activation of NK₁ receptors enhances responses of neurons evoked by NMDA suggesting an ensuing sensitization³⁸. There is strong evidence that a similar system may drive activation of dopamine neurons in the VTA^{39,40}. We provide compelling evidence that SP is, at the very least, a co-driver of DA neurons. Yet, the inability to block glutamate receptors to inhibit chronic pain or opioid-induced rewarding activity is encumbered with severe side effects of glutamate inhibition. A study by Ren and colleagues demonstrated that substance P has the ability to evoke a “slow” excitation in the dopamine cells of the VTA that can be blocked by an NK₁ receptor antagonist⁴¹. Studies using KO mice identified the NALCN (neuronal cation channel carrying a small background leak Na⁺ current) as the cation channel activated by SP in the VTA. Most importantly, studies demonstrated that SP could drive action potentials of the dopamine cells of the VTA in neurons dissociated from wildtype mice but not in *Nalcn*^{-/-} mice⁴¹. Using VTA-microdialysis, we now show that morphine-induced release of SP can be significantly attenuated by adding a GABA-A agonist, muscimol, demonstrating the disinhibition of SP release by morphine.

The link between SP neurons and their cognate receptor in the reward pathway has been established^{38,39}. Global NK₁R^{-/-} mice exhibited a lack of CPP⁴² and self-administration⁴³ to morphine but not cocaine, indicating a crucial role for the neurokinin system in mediating the reward to opiates and their mechanism of action in the VTA. Yet, a global deletion of the NK₁ receptor might suggest compensatory loss of an indirect mechanism that may result in the loss of the opioid rewarding behavior. Studies using a saporin toxin conjugated to SP administered into the amygdala of adult mice resulted in a similar reduction in opioid CPP behavior⁴⁴. Hence our studies help identify substance P and NK₁ receptors directly in the VTA are responsible for opioid-mediated increase in dopamine release into the nucleus accumbens.

Our study is the first to use a clustered regularly interspaced short palindromic repeats (CRISPR) associated protein-9 nuclease (Cas9) approach to edit a gene in the adult rat brain to block a part of the reward pathway. Here, we edited the NK₁R in the VTA; these receptors localize to DA cells⁴⁵. Removing the NK₁R by CRISPR-Cas9 editing was sufficient to prevent DA release in the NAc in response to morphine. In addition, the CRISPR-Cas9 NK₁R editing in rats resulted in the lack of a positive morphine-induced CPP. Yet, the selective NK₁R CRISPR-Cas9 edited animals continued to gain weight normally over 2 months, much like naïve or control animals, suggesting that their appetite drive was intact. These animals had no unusual behaviors and demonstrated no motor impairment when placed on a slow rotating rod. These studies strongly support the notion that NK₁ receptors in the VTA play a role in rewarding behavior and the idea that opioids can result in an increase in SP by inhibiting the release of GABA onto the SP containing neurons.

These findings along with our previous work in neuropathic pain^{4,14} supported the development of a novel multifunctional opioid that would act as an agonist at mu opioid

receptors while inhibiting NK₁ receptors. TY032, a mu and delta opioid agonist/NK₁ antagonist, significantly attenuates behavioral signs of chronic neuropathic pain while not demonstrating rewarding behavior in a model of CPP. TY032 therefore represents the future of analgesics: multi-functional compounds designed to act at more than one molecular target to produce pain relief as well as endowed with properties to inhibit unwanted effects that so often limit dosing.

An unexpected finding from our microdialysis studies was the demonstration of a lack of a dopamine response to i.p. cocaine in CRISPR/Cas9 NK₁R KO mice or by NK₁R antagonism in the VTA. Histological verification of surgical placements was evident, making the lack of DA in response to cocaine intriguing. This outcome contrasts with numerous studies, which suggest that only morphine, not cocaine, is rendered non-rewarding in whole-animal NK₁R^{-/-} and amygdaloid SP⁺ neuron ablation^{42,43,44}. Our finding might suggest that a lack of initial dopaminergic neuronal excitation is due to low NK₁R availability, and may result in alterations in the neuronal cation channel carrying capacity as a background Na⁺ leak current. Hence, cocaine has less dopamine for which to block reuptake in the NAc and therefore result in less dopamine that can be measured at the end of our microdialysis experiments. There is a precedent for other drugs of abuse, namely alcohol, potentially being affected by NK₁R antagonism⁴⁶, making our unexpected finding all the more noteworthy and novel.

Our studies provide new insight into how opiates cause increases in dopamine in NAc and may heighten propensities towards addiction. Identifying SP as a common signaling factor in both pain and reward, we put forth the idea of rationally designing bifunctional drugs that target both the cellular pathology in chronic pain (i.e., upregulated SP in nociceptors) and the cellular pathology of reward (i.e., opioids in the VTA lead to the release of SP causing an increase in dopamine release in the nucleus accumbens) for better analgesic efficacy while purposefully reducing unwanted side effects such as addiction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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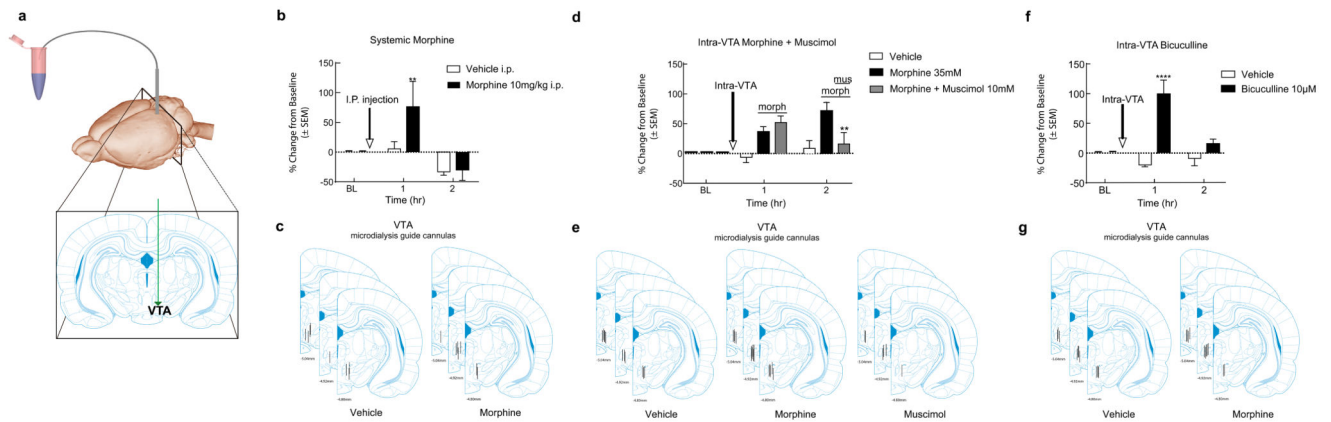


Figure 1. Morphine acutely increases intra-VTA Substance P via GABAergic disinhibition
(a) Schematic of microdialysis studies in the VTA. **(b)** Intraperitoneal administration of morphine 10 mg/kg (n=7) significantly increases SP release ($+76.1 \pm 42.9\%$) in the VTA after 1 hour compared to vehicle-treated animals (n=8, $+5.3 \pm 12.4\%$). **(c)** Anatomical verification of microdialysis guide cannula placements from studies done in (b). **(d)** Intra-VTA administration of morphine 10 mg/mL (n=16) significantly increases SP release ($+36.8 \pm 8.4\%$) in the VTA compared to vehicle-treated animals (n=11, $-6.2 \pm 9.1\%$) 1 hour post-administration and continues to significantly increase SP release in the VTA after 2 hours of administration. Morphine in the presence of Muscimol (10 μ M intra-VTA given after the 1st hour of dialysate collection, n=6) had significantly reduced SP release ($16.1 \pm 19.4\%$) compared to morphine only treated animals ($72.0 \pm 14.2\%$) at the 2hr time point. **(e)** Anatomical verification of microdialysis guide cannula placements from studies done in **d**. **(f)** Intra-VTA administration of bicuculline 10 μ M (n=9) significantly increases SP release ($+99.3 \pm 23.3\%$) in the VTA during the 1st hour post-administration compared to vehicle-treated animals (n=8, $-19.9 \pm 3.2\%$). **(g)** Anatomical verification of microdialysis guide cannula placements from studies done in (F). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$; analyses based on experimental vs vehicle groups, Two-way ANOVA.

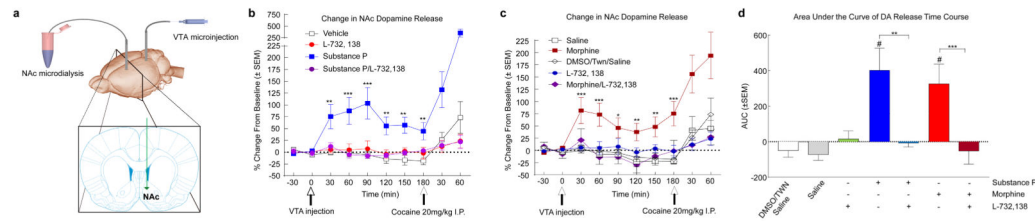


Figure 2. Intra-VTA NK₁ antagonist blocks morphine-induced DA release in the NAc
(a) Schematic of studies; compounds are microinjected into the VTA while microdialysis is performed in the NAc. **(b)** Intra-VTA L-732,138 1 μg/0.5 μL (n=9) did not produce a significant change in DA release compared to vehicle (n=7) during the 3-hour time course. Intra-VTA SP (n=9) produced a significant increase in DA release, peaking at 90 min post-administration (+103.6 ± 33.1%) compared to vehicle. The combination of L-732,138 and SP (n=7) produced no significant change compared to vehicle-treated animals. **(c)** Intra-VTA L-732,138 1 μg/0.5 μL (n=9) did not produce a significant change in DA release compared to vehicle (n=7) during the time course. Morphine-induced a significant increase in DA release peaking at the 30-min time point (+81.48 ± 26.76%, n=9) compared to saline (n=7). The combination of morphine and L-732,138 (n=8) did not significantly alter DA release in the NAc compared to vehicle. **(d)** Area under the curve was assessed for percent-change from BL from time 0–180 min. SP administration significantly increased AUC compared to vehicle (402.6 ± 124.2 vs -49.9 ± 37.6, respectively), which was reversed by co-administration of L-732,138. Intra-VTA morphine administration significantly increased AUC compared to vehicle (326.1 ± 111.1 vs -72.7 ± 32.3, respectively), which was reversed by co-administration of L-732,138. *p<0.05, **p<0.01, ***p<0.005, #p<0.05. Two-way ANOVA employed to compare experimental vs vehicle in time courses, One-way ANOVA for AUC statistical analyses.

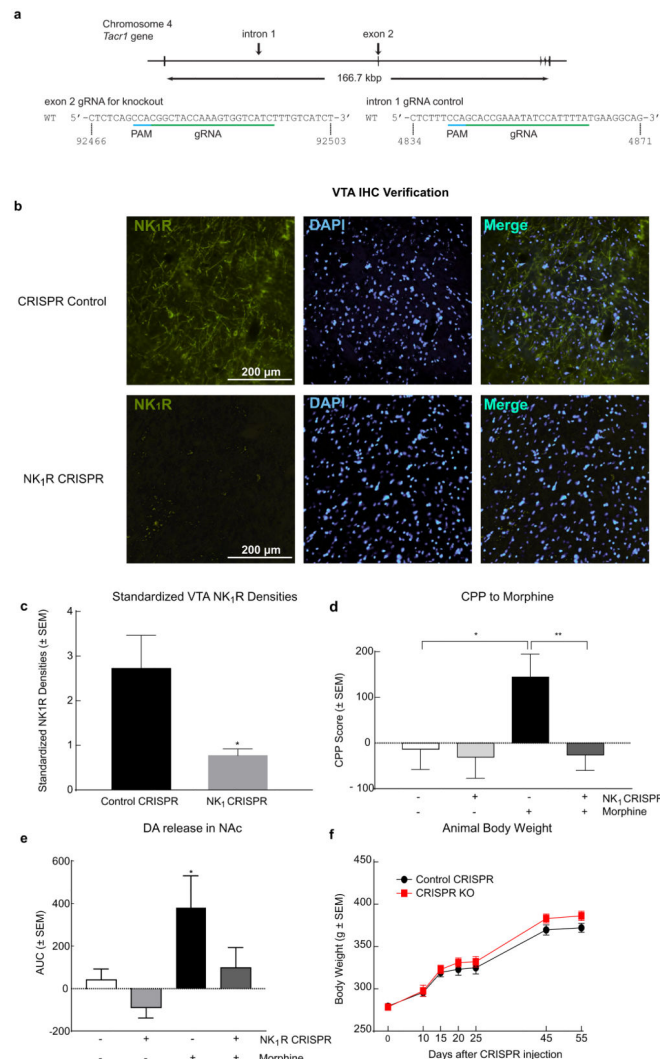


Figure 3. CRISPR-Cas9 knockdown of NK₁R in the VTA prevents rewarding activities to morphine

(a) Schematic showing exon/intron organization of the *Tacr1* gene in rat. To knockout NK₁R, we designed a gRNA targeting exon 2. We used a gRNA targeting a non-coding region in intron 1 of the *Tacr1* gene as a control. Both gRNAs are based on the reverse strand sequence and are indicated on the figure by the green line oriented from 5' to 3'. The protospacer adjacent motif (PAM sequence) is indicated by a blue line. The gRNA pairs with its DNA target followed by a 5' NGG sequence (PAM sequence). Cas9 catalyzes a double-stranded cleavage on the genomic DNA 3 bp before the PAM sequence. Nucleotide positions are indicated based on the DNA sequence on the *Tacr1* gene. (b) Representative immunohistochemistry images of the VTA in NK₁R KO and control CRISPR animals. (c) Standardized VTA NK₁R density was calculated from 24 CRISPR KO and 24 control CRISPR animals. (d) Conditioned place preference (CPP) to morphine is prevented by CRISPR mediated KO of NK₁R. Control CRISPR animals trained with morphine exhibited significantly increased preference compared to vehicle-treated animals (n=18, +144.9 ± 50.2 and n=11, -13.5 ± 46.6, respectively). VTA-NK₁R KO animals trained with morphine

(n=16, -25.6 ± 34.0) exhibited no significant difference from vehicle-treated animals (n=11, -30.5 ± 46.6). Within the groups trained with morphine, the CRISPR KO produced significantly reduced preference than the control CRISPR group. **(e)** Control CRISPR animals treated with morphine (10 mg/kg i.p.) had significantly increased DA release in the NAc (n=9, AUC: 380.4 ± 149.5) compared to vehicle (n=8, 40.61 ± 51.27). Within CRISPR KO groups, morphine treatment did not significantly increase DA release in the NAc compared to vehicle treatment (98.0 ± 98.0 , n=7 and -89.8 ± 48.4 , n=8 respectively). **(f)** Animals gained weight at the same rate over the course of 55 days following VTA CRISPR administration. *p<0.05, **p<0.01; student's t-test used for NK₁R density, One-way ANOVA employed for CPP, AUC of DA release, and body mass comparisons.

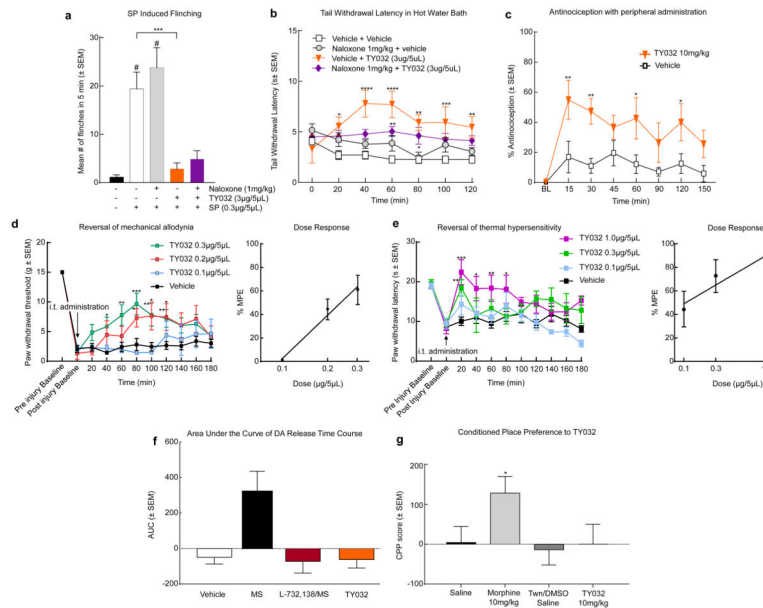


Figure 4. An opioid agonist/NK₁R antagonist inhibits nociception and lacks the potential for abuse in rodents

(a) TY032, an opioid agonist/NK₁R antagonist, inhibits intrathecal SP-induced flinching. SP significantly increased flinching and scratching to 19.4 ± 3.5 per 5 min ($n=7$), compared to vehicle-treated mice ($n=7$). TY032 by itself significantly reduced SP-induced flinching to $(2.8 \pm 1.3, n=6)$. TY032 + naloxone also reverses the significant increase in SP-induced flinching and scratching (4.9 ± 1.8 in 5 min, $n=7$), while naloxone alone does not significantly change SP-induced flinching ($23.8 \pm 4.5, n=6$). (b) In a model of acute thermal nociception in mice, intrathecal TY032 $3 \mu\text{g}/5 \mu\text{L}$ ($n=5$) significantly increased the tail withdrawal latency at every time point with a peak of 7.7 ± 1.3 sec at 40 min post-injection, compared to vehicle-treated animals ($n=7, 2.7 \pm 0.4$ sec). Pretreatment with naloxone $1 \text{ mg}/\text{kg}$ ($n=7$) rendered TY032 significant from vehicle at fewer time points (60 and 80 min post-administration); naloxone alone produced no significant change in PWL compared to vehicle at any time point. (c) In a model of acute thermal nociception in rats, intraperitoneal administration of TY032 $10 \text{ mg}/\text{kg}$ significantly increased percent-antinociception to $+55.0 \pm 13.0\%$ ($n=6$) compared to vehicle-treated animals ($n=6$). (d) TY032 dose-dependently reverses SNL-induced mechanical allodynia with peak effect 80 min post-i.t. administration ($9.7 \pm 1.8, n=11$). A dose-response curve was constructed with $0.1, 0.2,$ and $0.3 \mu\text{g}/5 \mu\text{L}$ doses. (e) TY032 dose-dependently reverses SNL-induced thermal hyperalgesia with the $1.0 \mu\text{g}/5 \mu\text{L}$ dose producing peak effect 20 min post-i.t. administration. 0.1 and $0.3 \mu\text{g}/5 \mu\text{L}$ doses were also administered and a dose-response curve was constructed. (f) When administered in the VTA, TY032 $2 \mu\text{g}/0.5 \mu\text{L}$ ($n=6$) did not significantly change NAc DA release for 180 min post-administration compared to vehicle-treated animals ($n=7$). (g) Systemic TY032 was assessed for preference after 10 days of the conditioned place preference paradigm. Morphine ($10 \text{ mg}/\text{kg}$, i.p.; $n=13$) produced a significantly positive CPP score of 128.0 ± 41.8 compared to vehicle-treated animals ($n=15$). TY032 $10 \text{ mg}/\text{kg}$ i.p. ($n=14$) did not significantly increase CPP score compared to vehicle (1.1 ± 49.1 and -13.9 ± 38.4 respectively). * $p<0.05$, ** $p<0.01$, *** $p<0.005$, **** $p<0.001$; One-way ANOVA used

for SP induced flinching, AUC of DA release and CPP studies. Two-way ANOVA employed for antinociception time courses with more than 2 groups.

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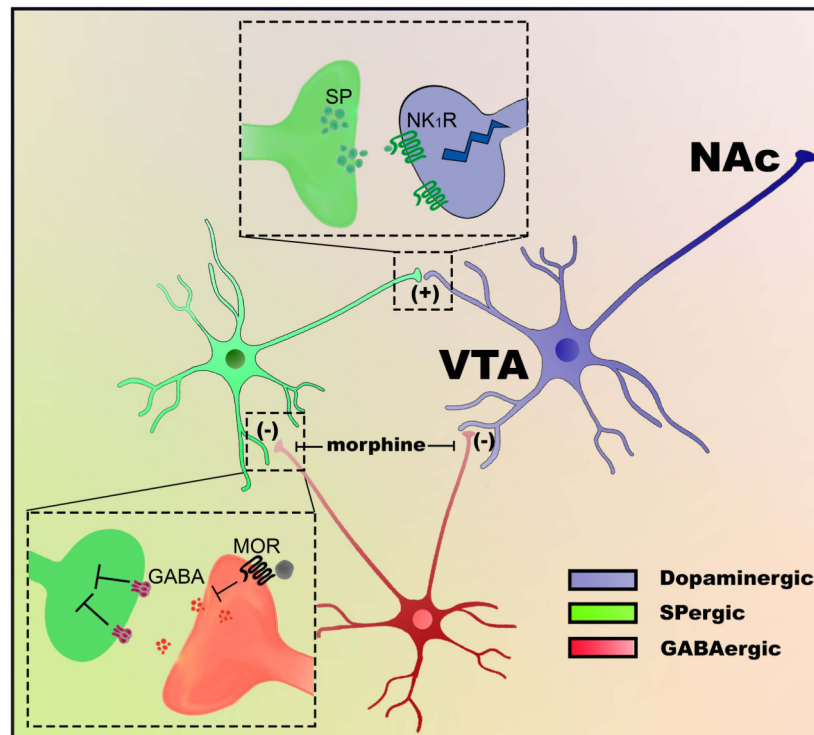


Figure 5. A model of VTA neuronal signaling in the reward pathway

Inhibitory GABAergic neurons (*red*) maintain tonic control over DA cells (*blue*) in the VTA of the midbrain, preventing their release of DA in the NAc of the ventral striatum. Opiates such as morphine inhibit GABAergic neurons, thus preventing the release of GABA onto DA neurons. This disinhibition allows DA cells to fire and release their contents into the NAc, which relays positive reinforcement. We hypothesize that GABAergic neurons also maintain inhibitory control over SP cells (*green*). When morphine inhibits GABAergic neurons, SPergic neurons fire and release their contents onto DA cells, acting as a “driver” of DA function.