

**INHIBITION OF OPIOID INDUCED REWARD VIA
GENETIC AND PHARMACOLOGICAL MANIPULATIONS**

By

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Abstract

Chronic pain affects approximately 100 million Americans for whom opioids are a popular therapy. While physicians and patients alike are apprehensive about using opioids due to their side effects including respiratory depression and addiction, 259 million opioid prescriptions were written in 2012. Although opioids are the best available analgesics, they increase both positive and negative reinforcement, ultimately leading to addiction. The pro-nociceptive or pain promoting, neurotransmitter Substance P (SP) and its corresponding receptor (NK₁R) are found associated with dopamine neurons in the ventral tegmental area (VTA). Evidence exists suggesting that SP potentiates positive reinforcement of opiates leading to reward. Using *in vivo* microdialysis, we show that systemic morphine significantly increases SP release in the VTA, an effect mediated by inhibition of VTA GABAergic neurons. SP administered in the VTA significantly increases levels of dopamine in the nucleus accumbens (NAc), a reward processing center in the ventral striatum. Using CRISPR-Cas9 knockdown of NK₁R in the VTA, we show this prevents the activation of opiate reward, corroborating the results from a conditioned place preference paradigm (CPP). Finally, we developed a novel opioid agonist/NK₁R antagonist, TY032, which was shown to inhibit acute and chronic pain in male rats. Importantly, our study found TY032 microinjection into the VTA did not result in an increase in extracellular dopamine release in the NAc. These data support the idea that targeting the dopamine reward circuitry and pain pathways with multifunctional opioid-NK₁R

compounds could be a path to developing analgesics that lack the potential for abuse.

Introduction

According to the U.S. Department of Health and Human Services, drug overdose is the leading cause of injury death in the U.S., with opioids accounting for 6 out of 10 of these deaths, or 30,000 per year. Unfortunately, despite this growing epidemic, nearly 11% of Americans suffering from chronic pain are still in need of adequate relief. The United States faces an ever-increasing need to address chronic pain as well as the opioid epidemic that has followed its suit. According to the NIH, over 115 Americans die each day due to opioid overdose¹. This national crisis has followed an increase in prescribed opioids during the last two decades due to a misunderstanding about their truly addictive nature². This epidemic encompasses prescription pain relief, heroin and synthetic opioids such as fentanyl. This difficult situation has led to the search for an effective, non-addictive pain reliever and a need to better understand components of the reward pathway.

Our lab has identified Substance P (SP) as a possible target to explore due to its known role in both nociception and the reward pathway of the brain. SP activates the neurokinin-1 receptor (NK1) and has been demonstrated to induce pronociceptive flinching³ and scratching, as well as eliciting a positive conditioned place preference⁴ (CPP) response. It is known that morphine inhibits presynaptic SP release in the dorsal root ganglia neurons of the spinal cord,⁵ it also indirectly leads to an increase in dopamine in the nucleus accumbens by inhibiting the inhibitory GABAergic neurons of the ventral tegmental area (VTA). We wished to

explore the role of SP in the VTA due to the combined presence of NK1 receptors and mu opioid receptors. A more developed understanding of components of the reward pathway will be key to identifying an effective and non-addictive compound.

Through the use of microdialysis and CPP we were able to demonstrate that editing of the NK1R of the VTA using CRISPR-Cas9 of adult, male rats reduced dopamine release and reward seeking behavior elicited by morphine administration. We also demonstrate the morphine acts to inhibit GABAergic neurons, which in turn allows SP to act as a driver of dopamine release.⁵

Background

In order to come up with a solution to this public health crisis, it has been critical to understand the mechanism by which these drugs operate. Opioids act in both the central (CNS) and peripheral (PNS) nervous systems. “In the peripheral

nervous system, actions of opioids in both the myenteric plexus and submucous plexus in the wall of the gut are responsible for the powerful constipating effect of opioids. In peripheral tissues such as joints, opioids act to reduce inflammation.^{2”} As shown in Diagram 1⁸, within the CNS, opioids act the level of the spinal

cord. Most commonly, the opioids prescribed as pain management act at the mu opioid receptor⁸.

However, despite being effective in blocking pain, mu opioid agonists, such as morphine and heroin, are carried in the blood to the brain, attaching to specific opioid receptors located on neurons in the dopamine producing region of the brain, the ventral tegmental area (VTA). The activation of these receptors results in the release of dopamine, leading to euphoria. After multiple exposures and bouts of

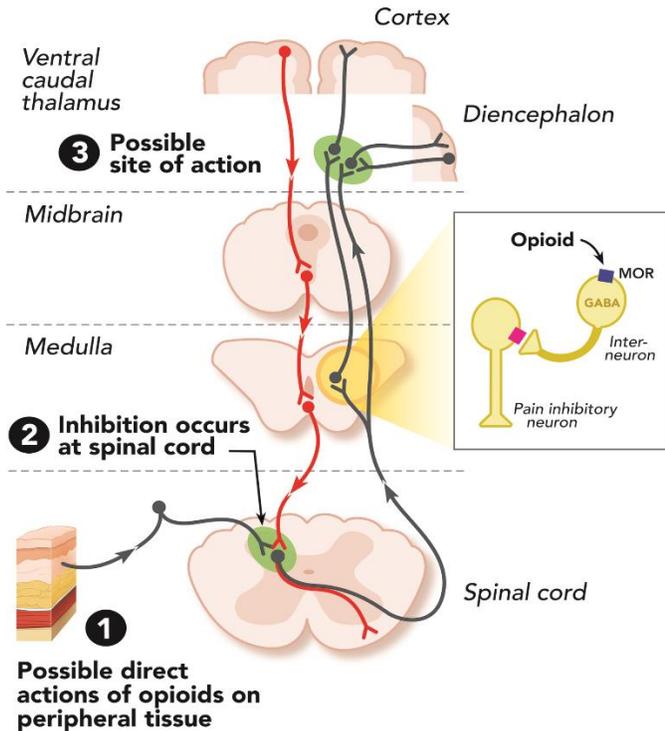


Diagram 1 from Al-Hasani and Bruchas, (2011) shows possible sites of action of opioids.

euphoria, a phenomenon called positive reinforcement drives the user to seek and take the opioid again.

Results

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

In order to better understand the role of SP in the reward pathway, we used microdialysis (see methods) in the VTA to measure SP levels in the cerebral-spinal fluid (Fig 1a). Intraperitoneal (i.p) injection of morphine sulfate (10mg/kg) significantly increased SP release within the VTA within the first hour compared to animals administered vehicle (Fig 1b). SP levels returned to baseline after one hour of morphine administration.

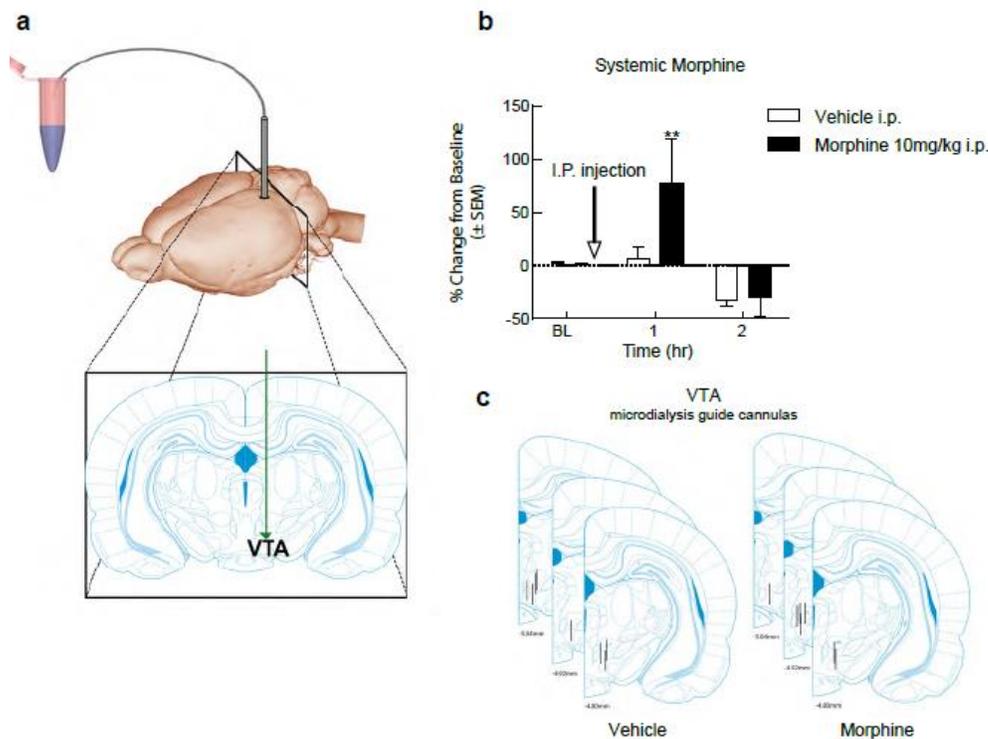


Figure 1a and 1b: Morphine acutely increases intra-VTA Substance P via GABAergic disinhibition. (a) Illustration of microdialysis studies, location is VTA. **(b)** Administration of morphine 10 mg/kg via intraperitoneal (I.P.) injection (n=7), significantly increases SP release ($+76.1 \pm 42.9\%$) in the VTA after 1 hour compared to vehicle-treated animals (n=8, $+5.3 \pm 12.4\%$). **(c)** Microdialysis guide cannula placement verification from studies done in (b).

In order to determine the effect of morphine on SP release, morphine was administered via reverse microdialysis in the VTA, cerebral-spinal fluid (CSF) collected, and analyzed for SP. Administration of morphine directly to the VTA resulted in a significant increase in SP release compared to the vehicle (Fig 1d).

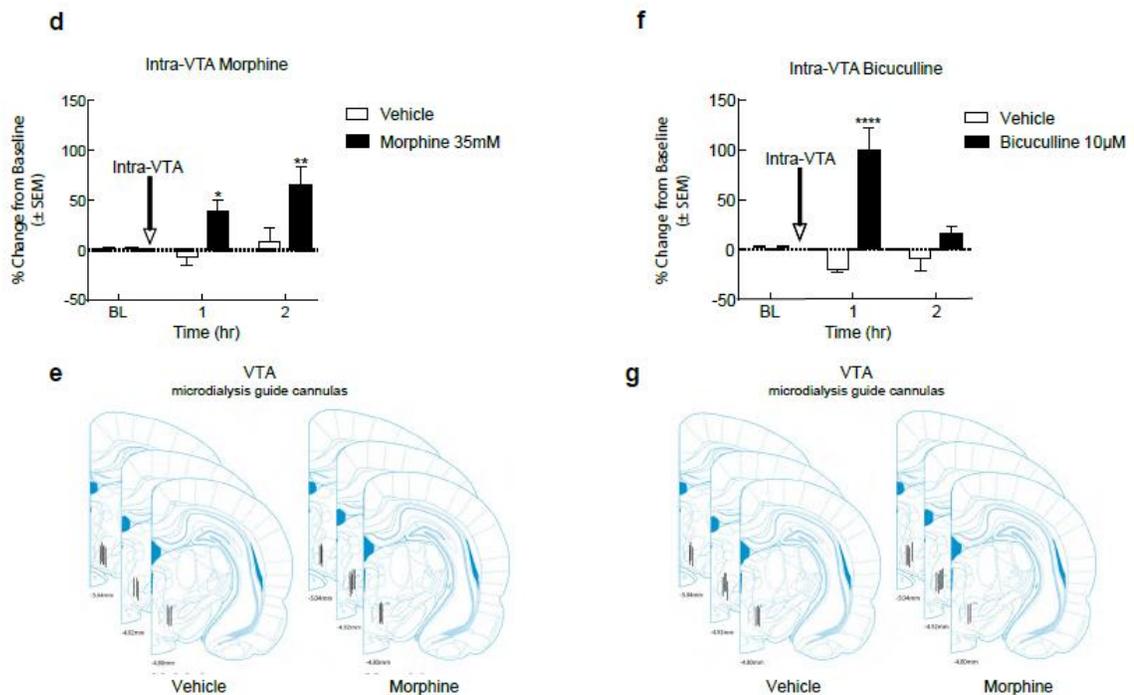


Figure 1d, 1e, 1f: Morphine acutely increases intra-VTA Substance P via GABAergic disinhibition. (d) Reverse microdialysis intra-VTA administration of morphine 10 mg/mL (n=10) significantly increases SP release ($+37.8 \pm 12.5\%$) in

the VTA compared to vehicle-treated animals ($n=11$, $-6.2 \pm 9.1\%$) at 1 and 2 hours post-administration. **(e)** Microdialysis guide canula placement verification from studies done in (d). **(f)** Reverse microdialysis intra-VTA administration of bicuculline $10 \mu\text{M}$ ($n=9$) significantly increases SP release ($+99.3 \pm 23.3\%$) in the VTA 1 hour post-administration when 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

As demonstrated by Johnson and North (1992), it is known that morphine exerts its effects through the inhibition of GABAergic neurons of the VTA, which prevents the release of the inhibitory neurotransmitter GABA onto dopaminergic neurons, resulting in the release of dopamine in the NAc. To determine if GABA is additionally responsible for the inhibition of SP release, we administered bicuculline ($10\mu\text{M}$), a GABA-A receptor antagonist, to the VTA through direct reverse microdialysis. A significant increase in SP release was observed $+99.3 \pm 23.3\%$ of BL compared to vehicle-treated animals (Fig 1f). Only animals determined to have correct guide cannula placement were used in analyses (Fig 1c, 1e, 1g).

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

In order to determine effects that elevated SP levels in the VTA could have on levels of dopamine in the NAc, in-vivo microdialysis was performed in the NAc (Fig 2a). Collection of CSF from the NAc following intra-VTA administration of SP

(1ug/0.5 uL) resulted in significantly increased levels of dopamine release within the NAc (Fig 2b). This result was blocked through the co-administration of an NK1R antagonist, L-732,138 (1ug/0.5uL).

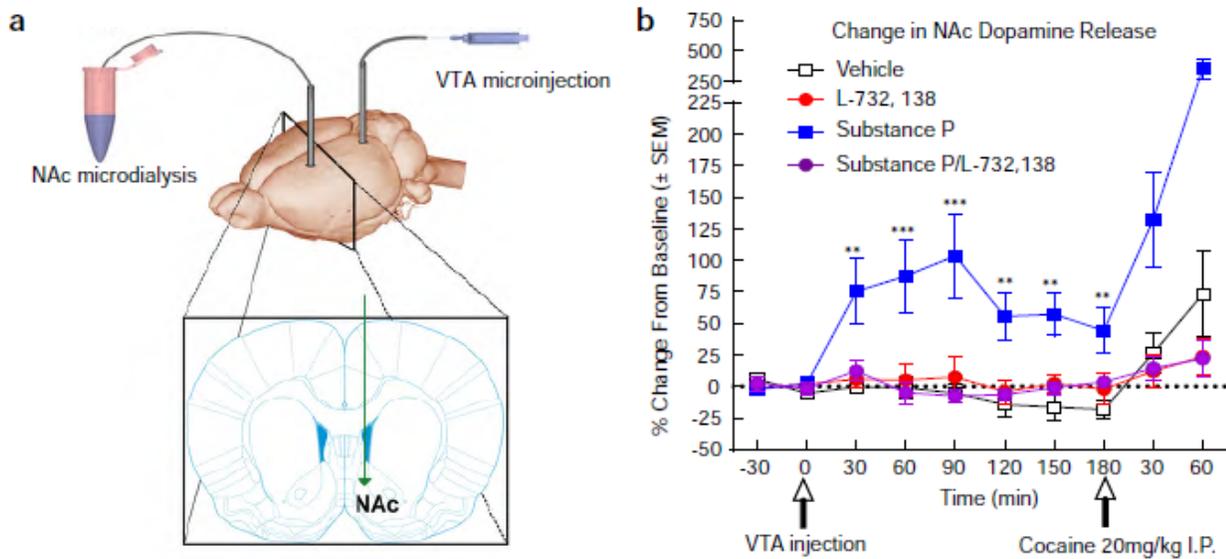


Figure 2: Intra-VTA NK₁ antagonist blocks morphine-induced DA release in the NAc. (a) Illustration of microdialysis studies; compounds are microinjected into the VTA while microdialysis is performed and collected from the NAc. (b) Intra-VTA L-732,138 (an NK1 receptor antagonist) 1 µg/0.5 µL (n=9) did 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 co-administration of L-732,138 and SP (n=7) produced no significant change in DA levels compared to control animals.

Administration of morphine (5ug/0.5 uL) directly into the VTA significantly increased NAc dopamine levels when compared to vehicle treated animals. Importantly, this effect was not observed with co-administration of morphine and

the NK1R antagonist, L-732,138 (Fig 2c). For complete standardization and 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

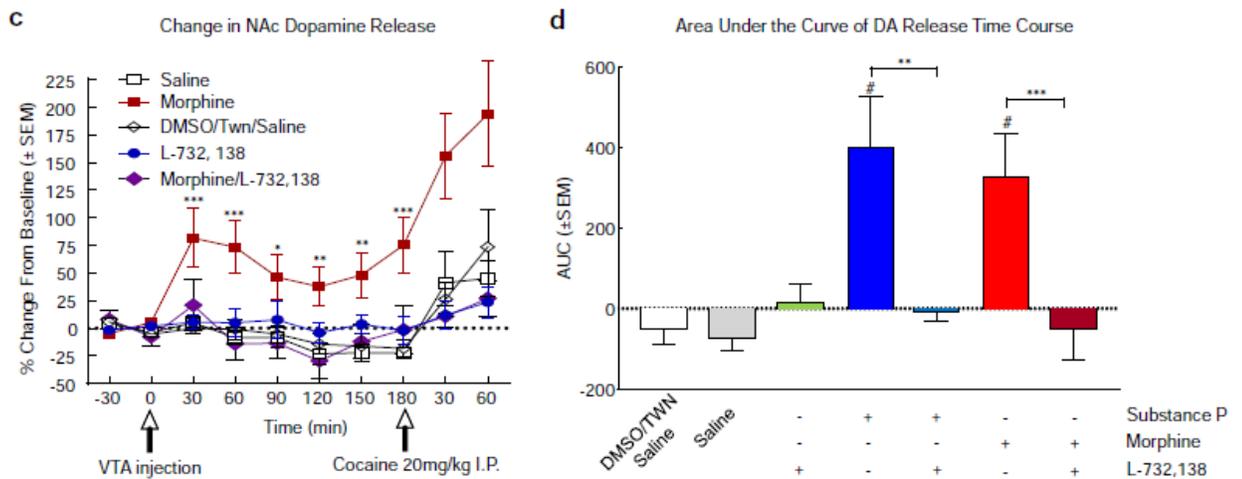


Figure 2c and 2d: Intra-VTA NK₁ antagonist blocks morphine-induced DA release in the NAc.

(c) Intra-VTA L-732,138 administration, 1 µg/0.5 µL (n=9), did not result in a significant change in DA release compared to vehicle (n=7). Morphine injection directly into the VTA resulted in 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 shows 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), this 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.

CRISPR-Cas9 knockout of NK1R in the VTA prevents morphine induced increase of dopamine release in the NAc.

Microdialysis was performed in the NAc after systemic injection of morphine or vehicle in NK1R CRISPR KO and control animals. In control animals, morphine (10mg/kg i.p.) significantly increased dopamine release in the NAc compared to vehicle. Importantly, morphine did not significantly increase dopamine release in CRISPR KO animals compared to those animals administered vehicle (Fig 3e). For 55 days post-CRISPR-Cas 9 injection, both NK1R KO and control animals gained body weight at the same rate (Fig 3f), demonstrating that the reward processing for food was still present while reward correlates for opioids were no longer observed.

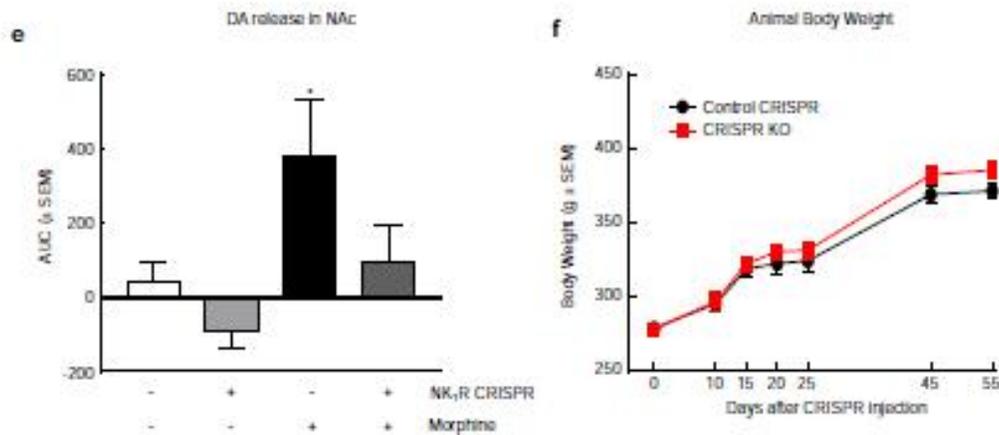


Figure 3: CRISPR-Cas9 knockdown of NK₁R in the VTA prevents DA release in NAc associated with reward seeking behaviors. (e) When administered morphine (10 mg/kg i.p.), control CRISPR animals had significantly increased DA release in the NAc (n=9, AUC: 380.4 \pm 149.5) compared to vehicle treated animals

(n=8, 40.61 ± 51.27). For CRISPR KO groups, morphine treatment did not result in significant increases in DA release in the NAc compared to vehicle treatment (98.0 ± 98.0 , n=7 and -89.8 ± 48.4 , n=8 respectively). **(f)** Weight gain over 55 days was the same rate for both groups of animals. *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.

TY032 does not result in increased levels of NAc dopamine release in rats

Developed by the Vanderah Laboratory with help from collaborators, TY032 is a compound which is a μ/δ -opioid agonist and NK₁R antagonist that has the intention of blocking pain at the level of the spinal cord while avoiding unwanted side effects. To determine its potential for abuse, intra-VTA administration of TY032 was followed by microdialysis in the NAc. TY032 (2.0ug/0.5 uL) did not significantly increase dopamine levels compared to vehicle (Fig 4f).

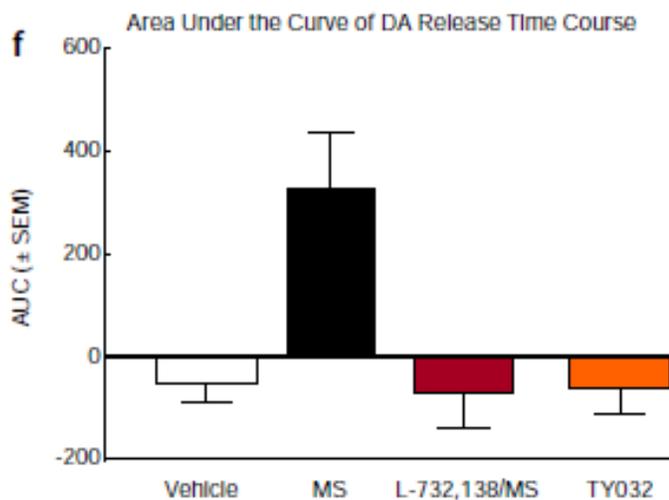


Figure 4: An opioid agonist/NK₁R antagonist lacks the potential for abuse in rodents. (f) When administered in the VTA via reverse microdialysis, TY032 2 μ g/0.5 μ L (n=6) did not significantly alter NAc DA

release for 180 min post-administration compared to vehicle-treated animals (n=7).

Discussion

According to the NIH, in 2012, around 25.3 million U.S adults (11.2 percent) experienced pain every day for at least three months.⁹ Unfortunately this growing awareness of our pain has also led to an increase in opioid-related addiction and overdose deaths, with drug overdose becoming the leading cause of accidental death in the US.⁷ Of the 52,404 drug overdose deaths in 2015, 20,101 were related to prescription opioids and 12,990 linked to heroin.⁷ In addition to the moral obligation to solve this crisis, there are objective economic incentives to address this issue as well. "Pain costs society at least \$560-635 billion annually (in 2010 dollars) in the United States, which combines the medical costs of pain care and economic costs related to disability days and lost wages and productivity."¹⁰ This value has likely raised and does not account for the money spent on emergency medical services directly dealing with the opioid crisis.

Our lab has proposed a unique explanation for the mechanism of action of opioids in the VTA. As shown in Figure 5 (Sandweiss et al), we hypothesize that inhibitory GABA neurons exert a tonic inhibition over not only dopaminergic neurons, but also SP-producing neurons. This would suggest that morphine's inhibition of the inhibitor GABA allows SP release which then acts as a driver to further increase dopamine release in the NAc.

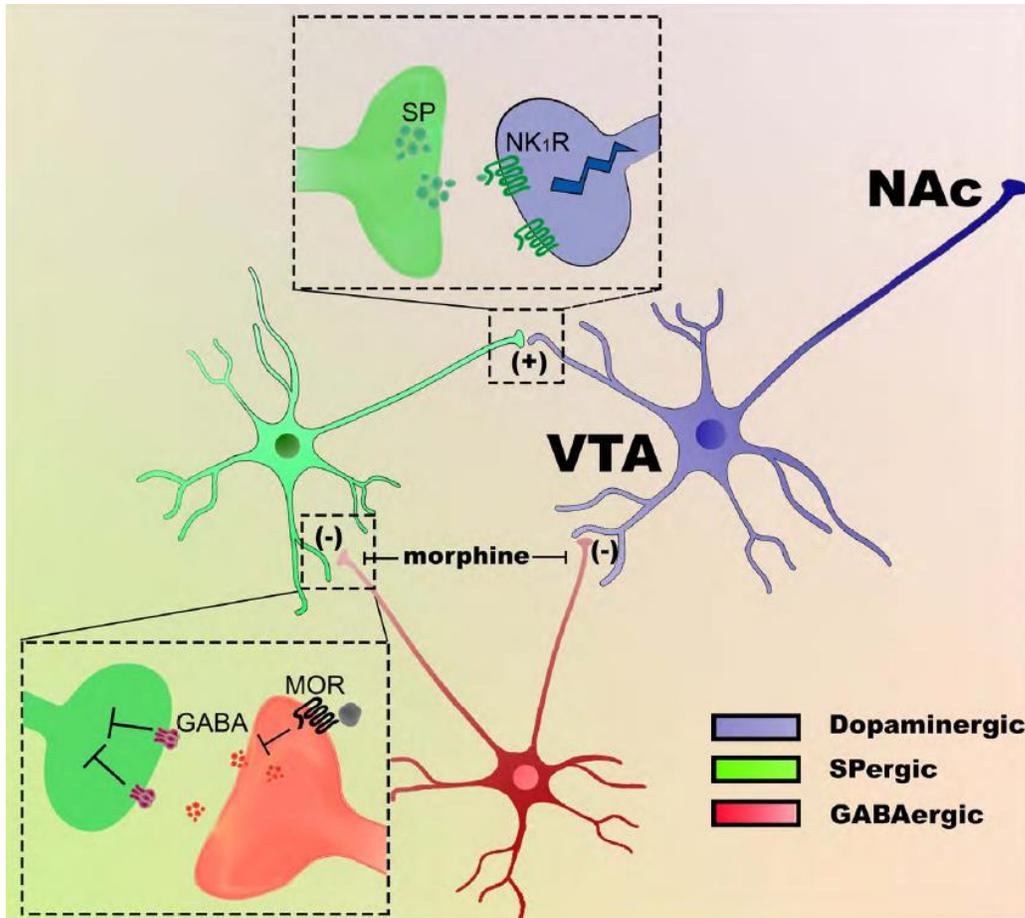


Figure 5: A model of VTA neuronal signaling in the reward pathway. It is known that inhibitory GABAergic neurons (*red*) maintain tonic control over DA cells (*blue*) in the VTA of the midbrain, normally preventing their release of DA in the NAc. Opiates such as morphine inhibit GABAergic neurons, thus preventing the release of GABA onto DA neurons. This removal of inhibition allows DA cells to fire and release their contents into the NAc, leading to feeling of euphoria. We hypothesize that GABAergic neurons also maintain inhibitory control over SP cells (*green*). This would mean that when morphine inhibits GABAergic neurons, SPergic neurons fire and release their contents onto DA cells, acting as an enhancer of DA release.

The results presented in this paper importantly correspond to findings presented in Sandweiss et al (2017), which, in addition to presenting the chemical

correlates of the experiment, offer behavioral data necessary to conclude that our compound offers a combination of anti-nociceptive and non-rewarding benefits in a single compound. In Sandweiss et al (2017) it is shown that the CRISPR control animals trained with morphine show significantly higher CPP scores as compared to CRISPR KO animals. Intrathecal administration of TY032 was shown to reduce SP induced flinching and scratching. TY032 also increased tail withdrawal and hind paw withdrawal latency compared to vehicle animals. Finally, it was also demonstrated that TY032 does not produce a significantly different CPP score compared to the controls administered.

Together, these results are promising and suggest the creation of a multi-functional drug that blocks pain at the level of the spinal cord through a mu opioid agonist and NK1 receptor antagonist which attenuates the addictive side effects observed with traditional opioids. This research has also furthered our current understanding of the reward system and provided important insight upon which the field can continue to build.

Methods- methods same as those described in Sandweiss et al (2017)

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 the animal was given prophylactic subcutaneous antibiotic (gentamycin, 1 mg/kg). Animals were allowed to recover for 3 days before microdialysis experiments.

For nucleus accumbens (NAc) microdialysis experiments, NAc microdialysis and VTA microinjection guide cannulas were installed. 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 and VTA guide cannulas were fixed in place with dental cement, and the animal was 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 cannular 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 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 tube was snap frozen in liquid nitrogen and subsequently stored at -80 °C until the dialysate was analyzed for SP content. VTA dialysate was analyzed for SP 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 $\mu\text{L}/\text{min}$, 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 $\mu\text{L}/\text{sec}$) 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 $-80\text{ }^{\circ}\text{C}$ 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; and VTA/NAc blocks were sectioned in 40- μm increments on a cryostat (Microm HM 525) and mounted. Slides were viewed microscopically for visual identification of NAc and VTA cannula placements. Terminations of the guide cannulas were plotted on a rat brain atlas²⁶ 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 which 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>).²⁷ 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 the exon 2 (on the reverse strand: GATGACCACTTTGGTAGCCG, score 89; **Figure 4a**). As a control, we chose a gRNA targeting the same gene but in a non-coding region located in the 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)²⁸.

Cloning of gRNAs

We used the pSpCas9(BB)-2A-GFP plasmid (PX458, Cat#48138, Addgene, Cambridge, MA)²⁹ 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 to²⁹ 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 µM 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* DH5α 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 DH5α *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 µg/µl 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, 2 µL of the plasmid complexes were injected 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.

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 UV microscope and merged.

Statistical analysis

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 $p < 0.05$.

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Thank you, Tally, for being my go-to guide, always believing in my abilities, and pushing me to do my best. I have learned so much under your guidance. Thank you, Alex, for being a great mentor, friend, and never-ending fountain of knowledge. I will miss you constantly quizzing me and testing my knowledge. I would also like to thank the NIH for funding this research project: *NIH/NIDA DA018717, NIH/NIDA DA013449.*

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