HHS Public Access

Author manuscript

Pharmacol Biochem Behav. Author manuscript; available in PMC 2024 October 14.

Published in final edited form as:

Pharmacol Biochem Behav. 2023 March ; 224: 173543. doi:10.1016/j.pbb.2023.173543.

A small molecule ligand for the novel pain target, GPR171, produces minimal reward in mice

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Abstract

ProSAAS is one of the most abundant proteins in the brain and is processed into several smaller peptides. One of which, BigLEN, is an endogenous ligand for the G protein-coupled receptor, GPR171. Recent work in rodent models has shown that a small-molecule ligand for GPR171, MS15203, increases morphine antinociception and is effective in lessening chronic pain. While these studies provide evidence for GPR171 as a possible pain target, its abuse liability has not yet been assessed and was evaluated in the current study. We first mapped the distribution of GPR171 and ProSAAS throughout the reward circuit of the brain using immunohistochemistry and showed that GPR171 and ProSAAS are localized in the hippocampus, basolateral amygdala, nucleus accumbens, prefrontal cortex. In the major dopaminergic structure, the ventral tegmental area (VTA), GPR171 appeared to be primarily localized in dopamine neurons while ProSAAS is outside of dopamine neurons. Next, MS15203 was administered to mice with or without morphine, and VTA slices were stained for the immediate early gene c-Fos as a marker of neuronal activation. Quantification of c-Fos-positive cells revealed no statistical difference between MS15203 and saline, suggesting that MS15203 does not increase VTA activation and dopamine release. The results of a conditioned place preference experiment showed that treatment with MS15203 produced no place preference indicating a lack of reward-related behavior. Taken together this data provides evidence that the novel pain therapeutic, MS15203, has minimal reward liability. Therefore, GPR171 deserves further exploration as a pain target.

Introduction

Opioids are among the most effective pain medications available, but their addictive liability (Fields and Margolis, 2015), strong overdose potential (Pattinson, 2008; Rudd et al., 2016; Scholl et al., 2018), and limited effectiveness in the treatment of chronic pain (Volkow

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Contribution

Participating in research design: McDermott, Ram, Bobeck,

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et al., 2018; Glare et al., 2019) necessitates the development of new pain medications (Skolnick, 2018). The opioid epidemic in the U.S.A. and its continued severity (Friedman et al., 2020; Silva and Kelly, 2020; Sterling and Platt, 2022), highlights the urgent need for more effective and less addictive alternatives. One area of active research addressing this need is G protein-coupled receptors (GPCR), which are the most "druggable" targets in the human body, accounting for approximately 35% of all FDA approved drugs (Wacker et al., 2017; Insel et al., 2019). However, recently deorphanized GPCRs remain an underexplored target for pain modulation. One such target is the deorphanized GPCR, GPR171. GPR171 is coupled to inhibitory $G\alpha_{i/o}$ proteins and activation of the receptor results in decreased intracellular cAMP signaling and reduced neuron excitability (Jonathan H. Wardman et al., 2016). GPR171 was discovered in 2001 (Wittenberger et al., 2001), and was deorphanized when its endogenous ligand was found to be the small peptide, BigLEN (Gomes et al., 2013). BigLEN is derived from the large propeptide, ProSAAS, an abundantly expressed protein in the brain (Fricker, 2010), a putative inhibitor of prohormone convertase 1/3 (Fricker et al., 2000), and a protein implicated in anxiety, feeding, and circadian rhythms, among other behaviors (Wardman et al., 2011; Aryal et al., 2022). ProSAAS is post-translationally cleaved into several smaller peptides including PEN, the endogenous ligand for GPR83 (Gomes et al., 2016), and BigLEN, the endogenous ligand for GPR171.

Previously our lab and others have shown GPR171 to be a promising antinociceptive target (McDermott et al., 2019; Cho et al., 2021; Ram et al., 2021). GPR171 is localized in the ventrolateral periaqueductal gray (McDermott et al., 2019) a structure essential for pain modulation and opioid action. An agonist for GPR171, MS15203, enhances morphine-mediated antinociception (McDermott et al., 2019), suggesting that in a clinical setting a GPR171 agonist could potentially enhance opioid analgesia, thus necessitating a lower dosage of opioid. Our lab has also shown that MS15203 is effective in reducing both inflammatory and paclitaxel-induced neuropathic pain in male but not female mice (Ram et al., 2021). Other work has shown MS15203 to effectively attenuate nociceptor mediated acute pain, inflammatory pain, and chronic constriction injury neuropathic pain (Cho et al., 2021). Evidence points towards GPR171 and MS15203 as a promising target and ligand for treating a wide range of pain states. Before this receptor is further explored as a novel pain therapy, it is essential that its action on reward be assessed. It is crucial that receptor activation does not enhance behavioral morphine reward or cause behavioral reward on its own. Additionally, due to the essential role of mesolimbic dopamine release in reward, it is important that MS15203 does not activate the dopaminergic center, the ventral tegmental area (VTA). Pain-induced alterations in VTA functioning (Trang et al., 2015) warrant an exploration of this new pain therapeutic's effect on VTA activation. As of yet, the role of GPR171 in reward, and more specifically opioid-induced reward at both the behavioral and circuitry levels is unknown.

Here, we explored GPR171 in rodent reward-related neural circuitry and behavior. To better understand GPR171's role in reward we undertook the following three experiments. First, we mapped GPR171 and ProSAAS throughout the reward structures of the brain; our targeted regions of interest included the hippocampus (HPC), basolateral amygdala (BLA), nucleus accumbens (NAc), prefrontal cortex (PFC), and ventral tegmental area. Next, we measured the effect of a GPR171 agonist on VTA activation and morphine-induced

activation. Lastly, we assessed the effects of GPR171 activation in an *in vivo* conditioned place preference (CPP) paradigm. Taken together, this study sought to determine GPR171's role in reward and opioid-mediated reward for the purpose of exploring this novel receptor as a novel pain target.

Material and Methods

Subjects:

For all three experiments, Male C57BL/6 mice (Charles Rivers Laboratories, CA), age 6–10 weeks, were used. Animals weighed 20g-35g at the start of each experiment and were given unlimited access to food and water when not undergoing experimentation. Mice were housed in groups of five in a temperature-controlled room with a 12:12 hour light:dark cycle (on at 07:00). All procedures were approved by Utah State University Institutional Care and Use Committee (Protocol #10038) and performed in accordance with the *Guide for the Care and Use of Laboratory Animals* adopted by the National Institutes of Health.

Mapping GPR171 & ProSAAS in the reward structures of the brain

Immunohistochemistry: Drug naïve male C57BL/6 mice (n=5) were deeply anesthetized using isoflurane, and then transcardially perfused with 4% paraformaldehyde. Perfused brains were post-fixed for 24 hours in 4% paraformaldehyde and transferred to 1XPBS for storage and subsequent immunohistochemistry. Fixed brains were dissected for the following brain region: HPC, BLA, NAc, PFC, and VTA. Coronal sections were sliced at 50 microns with a vibrating blade microtome (Leica, VT1000S) and refrigerated in 1X PBS for storage.

Immunohistochemistry was performed as described previously (McDermott et al., 2019) with slices washed in 1X PBS between all steps. Briefly, tissue slices were incubated for 30 minutes in 1% sodium borohydride, and subsequently placed in 5% normal goat serum blocking buffer (0.3% Triton X-100, 1X PBS) for one hour. After blocking, NAc, HPC, BLA, PFC slices were incubated and lightly shaken overnight at 4°C in 1:400 dilution of GPR171 (anti-rabbit, GeneTex, GTX108131) or 1:500 ProSAAS (anti-rabbit, MilliporeSigma ABN2268) in a solution of 0.3% Triton X-100 and 1% BSA. VTA slices were incubated in ProSAAS and GPR171 primary antibodies and a 1:500 dilution of Tyrosine Hydroxylase (TH, mouse; Invitrogen; MA1–24654). All brain region slices were then incubated for 2 hours in a 1:1000 dilution of secondary antibody: goat anti-rabbit (Alexa Flour 594, Life Technologies; A11037). VTA slices also received goat anti-mouse (Alexa Flour 488, Life Technologies; A11001). Lastly, slices were mounted and cover slipped onto glass microscope slides using ProLong Diamond Antifade (Invitrogen) mounting media. Images of fluorescent staining were captured using a Keyence BZ-X800 fluorescent microscope 4x and 20x magnification. Images were post-processed using Keyence image-analyzer software for haze reduction and lookup table contrast adjustment.

Quantifying VTA activation after MS15203 and morphine challenge

Group designation and drug preparation: Animals (n=30) were randomized into four groups: Morphine (10 mg/Kg, i.p.), MS15203 (10 mg/Kg, i.p.), Morphine + MS15203 (10

mg/Kg; 10 mg/Kg, i.p.), and Saline (0.9%, i.p.). Morphine was prepared by adding stock morphine sulfate (West-Ward (Hikma) Pharmaceuticals, Eatontown, NJ) to 0.9% saline. For MS15203 (Gift from Sanjai Pathak, Queens College), solid powdered drug was dissolved in 0.9% saline. For Morphine + MS15203, powdered MS15203 was dissolved in morphine sulfate and 0.9% saline solution.

Experimental Design: Animals were gently restrained and interperitoneally injected with their designated drug. Ninety minutes after injection (Campos-Jurado et al., 2019), animals were deeply anesthetized with isoflurane, and then transcardially perfused with 4% paraformaldehyde. Perfused brains were post fixed for 24 hours in 4% paraformaldehyde and transferred to 1X PBS for storage and subsequent immunohistochemistry.

Immunohistochemistry: Fixed brains were dissected for VTA. Midbrain was sliced coronally at 50 microns with a vibratome and refrigerated in 1X PBS for storage. Immunohistochemistry was performed as described above. Slices were incubated overnight at 4°C in a 1:500 dilution of primary antibody: Tyrosine Hydroxylase (TH, mouse, Invitrogen; MA1–24654) and the immediate early gene protein c-Fos (c-Fos, rabbit, Abcam; ab190289). Slices were then incubated in a 1:1000 dilution of secondary antibody: goat anti-mouse (Alexa Flour 488, Life Technologies; A11001) and goat anti-rabbit (Alexa Flour 594, Life Technologies; A11037). Lastly, slices were mounted and coverslipped onto glass microscope slides using Cytoseal 60 or ProLong Diamond Antifade (Invitrogen) mounting media.

Microscopy and Statistical Analysis: Animals with high quality perfusion and intact VTA were selected for microscopy and statistical analysis (N=24, n= 4–8 animals per group, 2–6 slices per animal). Images of fluorescently stained VTA were obtained using a Keyence microscope at 20x magnification. 300×300 micron regions of the VTA were captured, and images were post-processed with haze reduction and contrast adjustment (lookup table settings). Red channel c-Fos activated cells were hand-counted by an experimenter blinded to experimental conditions. The experimenter also counted double-labeled cells with c-Fos and TH to determine the proportion of VTA activated cells that were dopaminergic. Statistical analysis of experimental group averages were analyzed using a one-way independent groups ANOVA and subsequent Tukey's multiple comparisons test with GraphPad Prism 9 software.

Assessment of reward of GPR171 ligands *in vivo* using conditioned place preference

Drug designations: Animals (n=48, 10–14 animals per group) were randomly divided into four groups as above: Morphine (10mg/Kg), Morphine + MS15203 (10mg/Kg + 10mg/Kg), MS15203 (10mg/Kg), or Saline. Animals were gently restrained and administered their designated treatment interperitoneally with a 27-gauge hypodermic needle at a volume of 10mL/Kg. All drugs were prepared as described above.

Conditioned place preference experimental densig: The rodent behavioral assay, conditioned place preference (CPP) was used to assess the *in vivo* propensity of MS15203 to

invoke reward or aversion alone, and with morphine. The CPP apparatus (Maze Engineers) consisted of two distinct chambers (34 × 25 cm) separated by a neutral middle compartment (6 × 25 cm). The two chambers, one grey-walled and one striped-walled, were distinct in smell and floor texture (see Figure 6). Between trials the grey chamber was cleaned with a mixture of water and dish soap while the striped chamber was cleaned with ethanol. The neutral middle compartment was white-walled and free of smell. The experimental paradigm lasted 10 days, with conditioning days on Days 2–9, and a pretest on Day 1 and posttest on Day 10. For conditioning days, mice were given their designated drug or saline and placed in the grey or striped chamber, with the inability to access other compartments, for 30 minutes. On odd days (e.g., 3, 5, 7, 9) animals received their designated drug while on alternate even days (e.g., 2, 4, 6, 8) all animals received saline. The chamber that animals received saline or drug were randomly counterbalanced to account for innate preference for an experimental chamber. The control group, Saline, received saline in both chambers on alternate days. On Day 1 (pretest) and 10 (posttest) mice were placed in the middle compartment and allowed 15 minutes to freely move between all compartments. Percent of time spent in each compartment was quantified using the following equation (time in drug paired chamber)/(time in saline paired chamber + time in drug paired chamber)X100.

Data collection and analysis: Time spent in each chamber of the CPP apparatus was visualized and measured using an ANY-maze video camera and motion tracking software. A two-way ANOVA with drug treatment as one factor and the pretest vs posttest as the second factor. For the immunohistochemistry experiment, group means were compared using an omnibus one-way independent groups ANOVA followed by a Tukey's *post hoc* multiple comparisons. All analysis was done using GraphPad Prism 9 software.

Results

GPR171 and ProSAAS are localized throughout reward-related structures

Immunohistochemistry results show expression of GPR171 throughout four reward-related structures of the mouse brain: HPC, BLA, NAc, PFC (Figure 1). Similarly, ProSAAS is found in all four structures: HPC, BLA, NAc, and PFC (Figure 2), but there was markedly less localization of ProSAAS in the BLA than surrounding tissue. GPR171 appeared to be localized in dopamine neurons of the VTA as observed by yellow-orange staining in the GPR171-TH overlay (Figure 3b). Notably GPR171 punctae appeared to be localized largely in the cell bodies of dopamine neurons (Figure 3c). In contrast, ProSAAS appeared to be primarily localized outside of dopamine neurons of the VTA (Figure 4b) and appeared to show little colocalization between the two channels. In total, these results suggest the presence, but differential expression of, GPR171 and ProSAAS within the VTA.

MS15203 does not increases c-Fos expression in the VTA

IHC was performed on midbrain sections stained for TH and c-Fos for four experimental groups: Saline, MS15203, Morphine, and Morphine + MS15203 (Figure 5). First, c-Fos activated cells in the VTA were quantified and group differences were analyzed using a one-way independent groups ANOVA. An overall main effect was statistically significant [$F(3,55)=11.37$, $p<.0001$]. Since there was an overall main effect, a Tukey's *post hoc*

multiple comparisons was used to compare group means to Saline. Unsurprisingly, Morphine and Saline showed a significant difference (Tukey's, p=0.0292). Morphine + MS15203 and Saline were significantly different from each other (Tukey's, $p<0.0001$). Importantly, there was no significant difference between MS15203 and Saline (Tukey's, $p=0.9984$). Crucially, MS15203 + Morphine and Morphine were not significantly different from one another, suggesting that MS15203 did not alter morphine reward (Tukey's, $p=0.0601$).

Next, the number of activated cells that were dopaminergic (c-Fos positive cells colabeled with TH) were counted, and the number of dopamine activated cells were divided by the total number of activated cells to obtain a percentage. A one-way independent groups ANOVA was run on these percentages. Group averages showed an overall omnibus effect [$F(3,55)=3.083$, $p=0.0347$], and a Tukey's *post hoc* multiple comparisons test was used. There was no significant difference between Morphine (M=55.8%, $p=0.8725$), Morphine + MS15203 (M=66.8%, $p=0.0851$), or MS15203 (M=47.6%, $p=0.9092$) when compared to saline (M=51.5%). The only groups that showed a significant difference were MS15203 and MS15203+Morphine (p=0.0275).

MS15203 fails to induce place preference or increase morphine place preference

The behavioral conditioned place preference (CPP) paradigm included the groups: Saline, Morphine, MS15203, and MS15203 + Morphine (Figure 6). A two-way repeated measures ANOVA on group means showed an overall statistically significant drug effect [$F(3,44)=3.412$, $p=0.0255$] and across between pretest and posttest [$F(3,44)=7.303$, $p=0.0097$]. Since there was an overall main effect, a Šidák's multiple comparisons *post hoc* test was used to compare group means between pretest and posttest. Unsurprisingly, morphine-treated mice produced a significant greater percentage of time in the drug-paired chamber on the posttest compared to pretest (Šidák's, $p=0.0313$). There was also a significant difference in the MS15203 + Morphine group (Šidák's $p=0.0262$). While both groups that received morphine showed an increase percentage of time in the drug-paired chamber, those mice that received MS15203+Morphine spent less time (58.9%) compared to morphine (63.2%). Importantly, there was no statistical difference in the MS15203 group (Šidák's $p=0.9997$).

Discussion

In this study we showed that ProSAAS and GPR171 are found in important reward related brain structures: hippocampus, basolateral amygdala, nucleus accumbens, prefrontal cortex, and ventral tegmental area. While GPR171 is localized in a subset of dopamine neurons within the VTA, minimal localization of ProSAAS is found within dopamine neurons of the VTA. Also using fluorescent IHC we observed that MS15203 does not alter c-Fos activation in the VTA. Similarly, behavioral data in our conditioned place preference experiment showed that MS15203 administration failed to induce place preference alone. Taken together, these studies lend credence for the continued exploration of MS15203 as a novel pain.

We have previously shown that MS15203 increases morphine antinociception, and that it is likely mediated through the actions of the periaqueductal grey (McDermott et al., 2019). The

current study is the first to evaluate the role of GPR171 in drug reward. Peripherally related work has also focused on the topic of ProSAAS and reward. For example, it has been shown that in response to chronic cocaine administration, ProSAAS becomes down-regulated in the VTA and the NAc, though ProSAAS knockouts still show place preference to cocaine (Berezniuk et al., 2017). Another peptide derived from ProSAAS, PEN, the endogenous ligand for the deorphanized receptor, GPR83, has been explored in reward (Fakira et al., 2019). GPR83 is expressed in the VTA, and GPR83 knockdown in the NAc results in decreased morphine place preference in male rodents. The NAc is also important for motivation for non-drug stimuli. BigLEN reduces NAc transmission, and antagonizing the GPR171 receptor results in decreased persistent food seeking (Smith et al., 2022). These studies leave open the question as to whether GPR171 activation mediates reward and VTA engagement.

Behaviorally, the current study showed that MS15203 failed to induce reward, as measured by the conditioned place preference assay. Previous studies have linked alterations in morphine CPP to actions through dopamine receptors. One study has shown that a D1 receptor antagonist blocks morphine place preference in rats (Grenier et al., 2022). Additionally, the atypical antipsychotic quetiapine, which also has antagonistic properties at the dopamine receptors, and at the serotonin receptors, attenuates morphine CPP in rats (Khezri et al., 2022). Similar to GPR171, Neuropeptide S, the endogenous ligand for the previously orphaned GPCR, GPR154, has been shown to be neutral alone, but decrease morphine place preference in mice (Li et al., 2009). Additionally, it has been shown to cause antinociception when administered nasally (Medina et al., 2014) and intracerebroventricularly (Peng et al., 2010).

In large part, this study focused on the relationship between GPR171 and the ventral tegmental area. The VTA is a major mesolimbic dopaminergic center of the brain, and release of dopamine from this area into the NAc is a crucial mechanism for opioid-induced reward (Fields and Margolis, 2015; Kim et al., 2016) and reward generally. Opioid agonists disinhibit GABA interneurons that tonically suppress dopamine release in the VTA resulting in activation of dopamine neurons (Johnson and North, 1992; Trang et al., 2015). Though the mechanism of VTA activation varies depending on the drug involved, reward is largely mediated by dopamine release in structures like the NAc, regardless of substance (Fujita et al., 2019). Mesolimbic dopamine functioning becomes impaired during chronic pain (Taylor et al., 2014) in part due to microglia activation the VTA (Taylor et al., 2015). Due to the importance of the VTA in reward behaviors, as well as its impairment in chronic pain, we explored the activation of this region after MS15203 administration. IHC staining for c-Fos was used as an indirect indicator of neuronal activation in the VTA as has been done previously (Dela Cruz et al., 2016; Dehkordi et al., 2017; Campos-Jurado et al., 2019). Indeed, the rapid transcription from neuronal activation has made c-Fos one of the most frequently utilized immediate early genes for locating neuronal activation in addiction research (Cruz et al., 2015).

We showed that Morphine + MS15203 did not significantly alter c-Fos activation in the VTA compared to Morphine alone. Additionally, the average number of c-Fos activated cells was not significantly different between MS15203 and Saline, suggesting that MS15203

does not significantly activate the VTA. Congruent with this observation we show that, for MS15203, 47% of total neurons activated are dopaminergic and this is not significantly different than the total percentage of dopamine cells activated in the Saline group. And indeed all experimental groups show that between 47.6–66.7% of VTA neurons activated were dopaminergic, and there is no statistical difference between these groups with Saline.

GPR171 is localized on dopamine neurons, and is coupled to inhibitory Gαi/o proteins, activation with MS15203 would result in diminished neuronal excitability. However, there was no statistical difference in c-Fos activation or place preference. The VTA is a heterogenous region (Fields and Margolis, 2015) that contains both GABA and glutamate neurons. Therefore, direct effects of MS15203 activation in the VTA could be a result of a complex network of multiple different cell types. Further exploration to determine the complex activation of circuitry is needed. Nonetheless, the c-Fos activation data shown here, paired with MS15203 failure to induce place preference suggests that when administered alone, MS15203 produces no significant difference in reward or aversion compared to Saline. It should be noted that the GPR171 antibody used in this experiment has been evaluated using western blot analysis of hypothalamic shRNA knockdown tissue (Jonathan H Wardman et al., 2016) and in shRNA knockdown tissue of the basolateral amgydala (Bobeck et al., 2017), while the ProSAAS antibody has not been validated in tissue. However, a high and distinct staining of ProSAAS in the arcuate nucleus (See supplementary Figure 1) lends some credibility to the specificity of this antibody as this area has been shown to have high ProSAAS mRNA expression (Fricker et al., 2000).

This study assessed the reward liability of MS15203 using a dose of 10 mg/Kg of both morphine and MS15203. Our previous studies used 5 mg/Kg of morphine with a dose of 10 mg/Kg of MS15203 (McDermott et al., 2019). A reduced morphine dose was decided upon after identifying a ceiling effect observed with a higher dose of morphine on the tail-flick test. This ceiling effect limited our ability to observe MS15203 enhancement of morphine antinociception. We used 10 mg/Kg of morphine in the current study due to its use as a standard dose (Morón et al., 2010; Fakira et al., 2019) that ensures robust place preference. Due to this discrepancy in dosing, we are limited in drawing direct comparisons between the effects of MS15203 in the current study and the previous one.

The presence of both ProSAAS and GPR171 in the ventral tegmental area suggests an endogenous role for the BigLEN-GPR171 neuropeptide system in this brain structure. Interestingly, IHC and fluorescent microscopy demonstrated that GPR171 and ProSAAS are differentially dispersed throughout the VTAthough GPR171 appeared to be located primarily in the cell bodies of dopamine neurons, and ProSAAS was distinctly located outside of these structures. ProSAAS-containing neurons could be secreting BigLEN presynaptically onto the dopamine neurons containing GPR171. It is also possible that the high ProSAAS expression points towards a larger role being played by the other small ProSAAS-derived peptides, like PEN, the endogenous ligand for GPR83. However, since ProSAAS is one of the most highly expressed proteins in the brain (Fricker, 2010) It’s possible that ProSAAS is simply acting in this region similar to its theorized house-keeping role as a chaperone protein (Hoshino et al., 2014).

Intriguingly, we have also demonstrated differential expression of ProSAAS and GPR171 in the basolateral amygdala. Our lab has shown similar high expression of GPR171 in this region in the past, and knockdown of GPR171 in this region lowers anxiety (Bobeck et al., 2017), but to date, no one has looked at ProSAAS expression in the BLA. While GPR171 had high expression in the BLA, ProSAAS showed markedly low expression compared to surrounding amygdala regions, such as the central amygdala. Future studies will look at the behavioral effect of knocking down ProSAAS in this region and the naïve colocalization between ProSAAS and GPR171 to better understand the actions of BigLEN-GPR171 system in the amygdala.

In conclusion, we have shown that MS15203 does not increase c-Fos VTA activation, and it fails to induce place preference in mice *in vivo*. The current data lends evidence and confidence towards the continued exploration of GPR171 as a novel pain therapeutic, capable of enhancing opioid analgesia without increasing its reward. Future studies should probe the exact mechanism regulating GPR171 actions in the VTA and further explore the relationship between ProSAAS and GPR171 in the mesolimbic pathway. Further assessment on the safety profile of MS15203 should be investigated including side effects such as withdrawal, tolerance and opioid-induced respiratory depression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to thank Dr. Sanjai Pathak from Queens college, NY for his generous gift of MS15203. And to Dr. Megen Kepas for her constructive editorial feedback.

This work was funded on startup funds from Utah State University (to ENB) and a Pharmacology & Toxicology Startup grant from the PhRMA Foundation (to ENB) and NIH grant (TR003667-01).

List of abbreviations

BLA	Basolateral Amygdala
CPP	Conditioned Place Preference
HPC	Hippocampus
NAc	Nucleus Accumbens
PFC	Prefrontal Cortex
TH	Tyrosine Hydroxylase
VTA	Ventral Tegmental Area

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Highlights

- ProSAAS and GPR171 are expressed within the reward circuitry
- GPR171 small molecule ligand does not produce conditioned place preference
- GPR171 small molecule ligand does not activate cells in ventral tegmental area

Significance Statement

MS15203, a drug that activates the receptor GPR171, was previously shown to increase morphine analgesia. The authors use *in vivo* and histological techniques to show that it fails to activate the rodent reward circuitry, providing support for the continued exploration of MS15203 as a novel pain drug, and GPR171 a novel pain target.

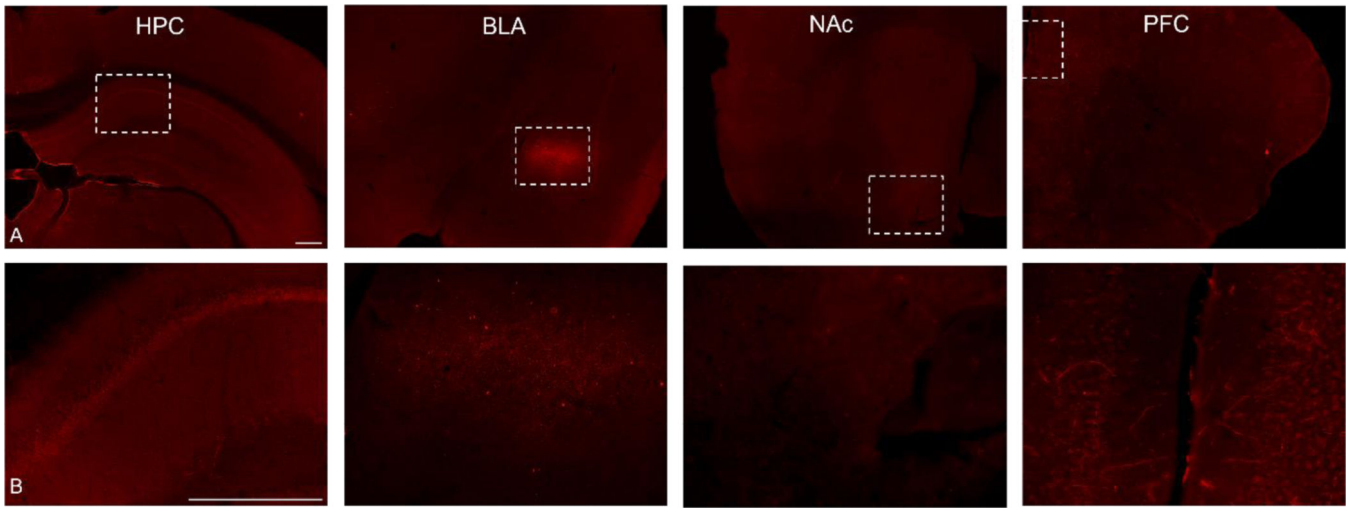

Figure 1. Localization of GPR171 in reward and addiction-related structures of the brain.

4x (A) and 20x (B) images of the hippocampus (HPC), basolateral amygdala (BLA), nucleus accumbens (NAc), and prefrontal cortex (PFC). All four regions show GPR171 expression. In particular, GPR171 is distinctly localized in the BLA. Scale bars = 300μm

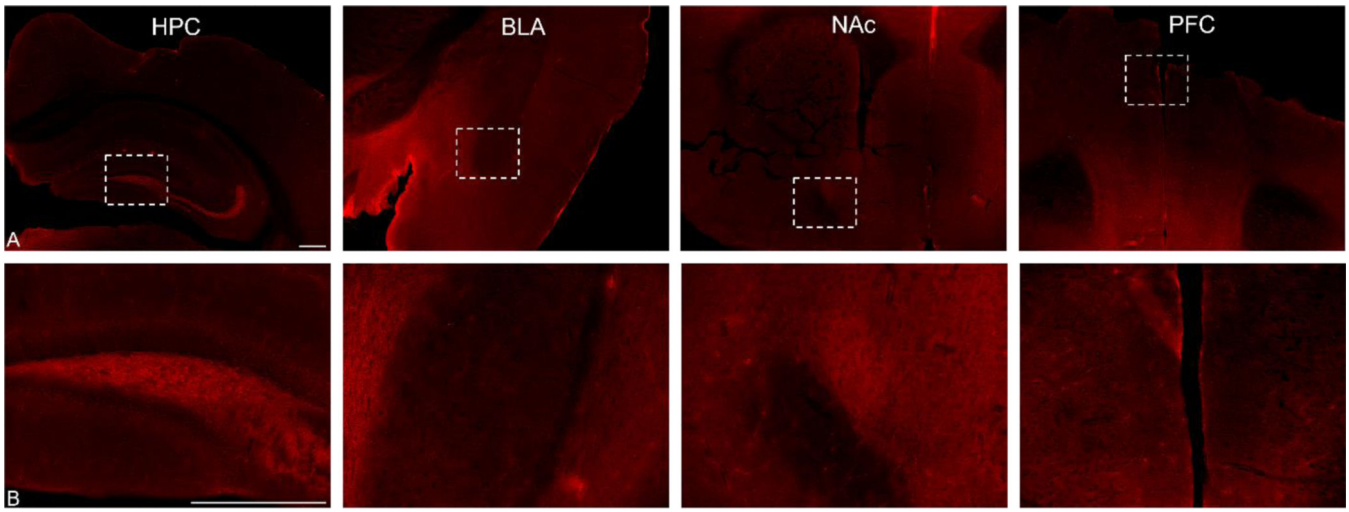

Figure 2. Localization of ProSAAS in reward and addiction-related structures of the brain.
4x (A) and 20x (B) images of the hippocampus (HPC), nucleus accumbens (NAc), and prefrontal cortex (PFC). All regions show ProSAAS localization. In particular, ProSAAS shows less expression in the BLA than its surrounding tissue. Scale bars = 300μm

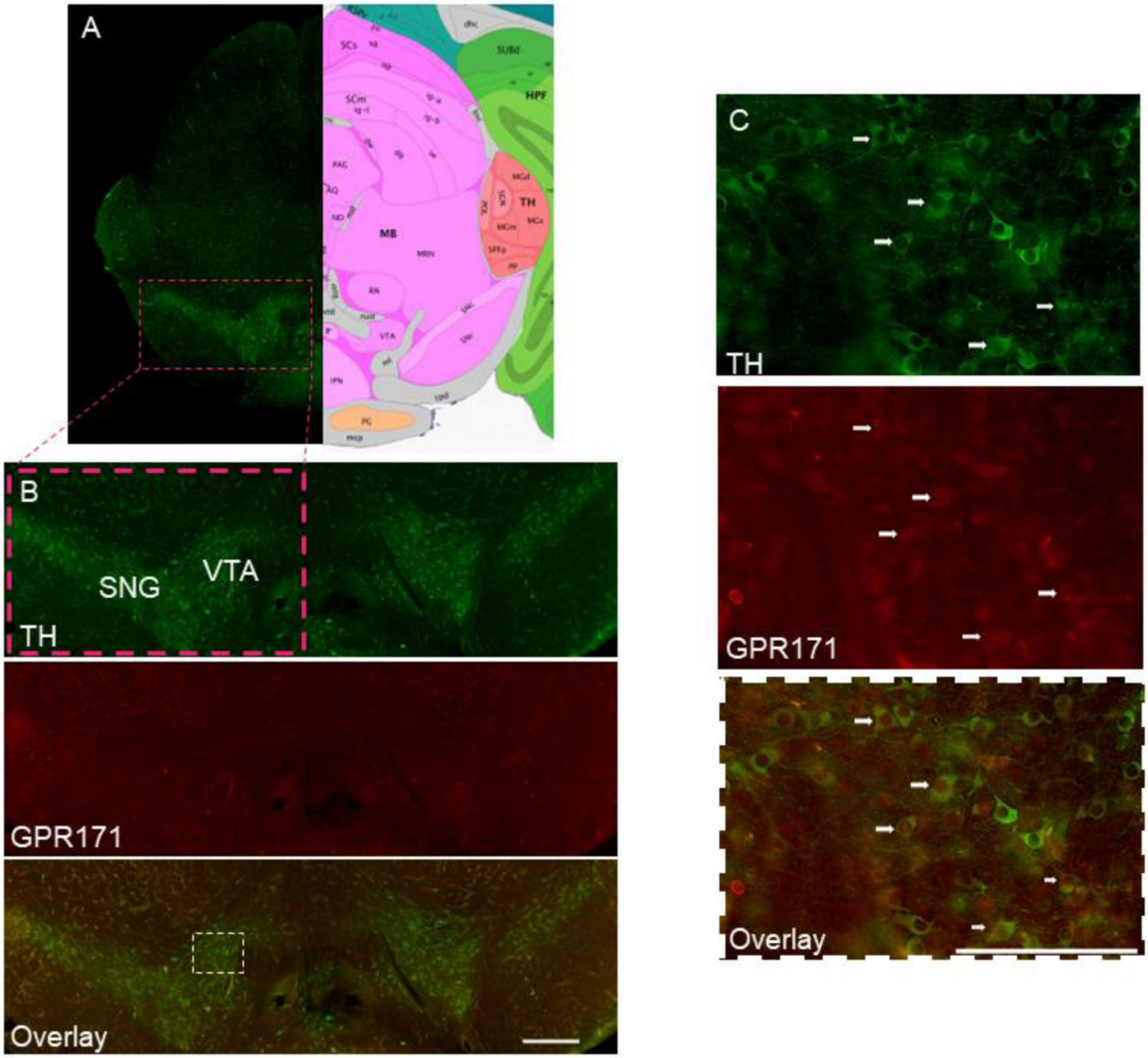

Figure 3. Expression of GPR171 in dopamine neurons of the ventral tegmental area

(A) A midbrain coronal section showing dopamine neurons of the left ventral tegmental area (VTA) and substantia nigra (SNG), and a schematic adapted from the Allen Brain Atlas' Mouse Brain Reference Atlas (Mouse P56 Coronal) showing, among other regions, right VTA and SNG. (B) GPR171 expression in the VTA and SNG left and right VTA and SNG stained for TH, GPR171, and an overlay of the two channels show GPR171 colocalized in subset of dopamine neurons. (C) a 20x capture of left VTA from white box in 3B showing GPR171 staining. Arrows on the TH-GPR171 overlay show regions of colocalization, mainly in the cell body of TH neurons. Scale bars = 300μm

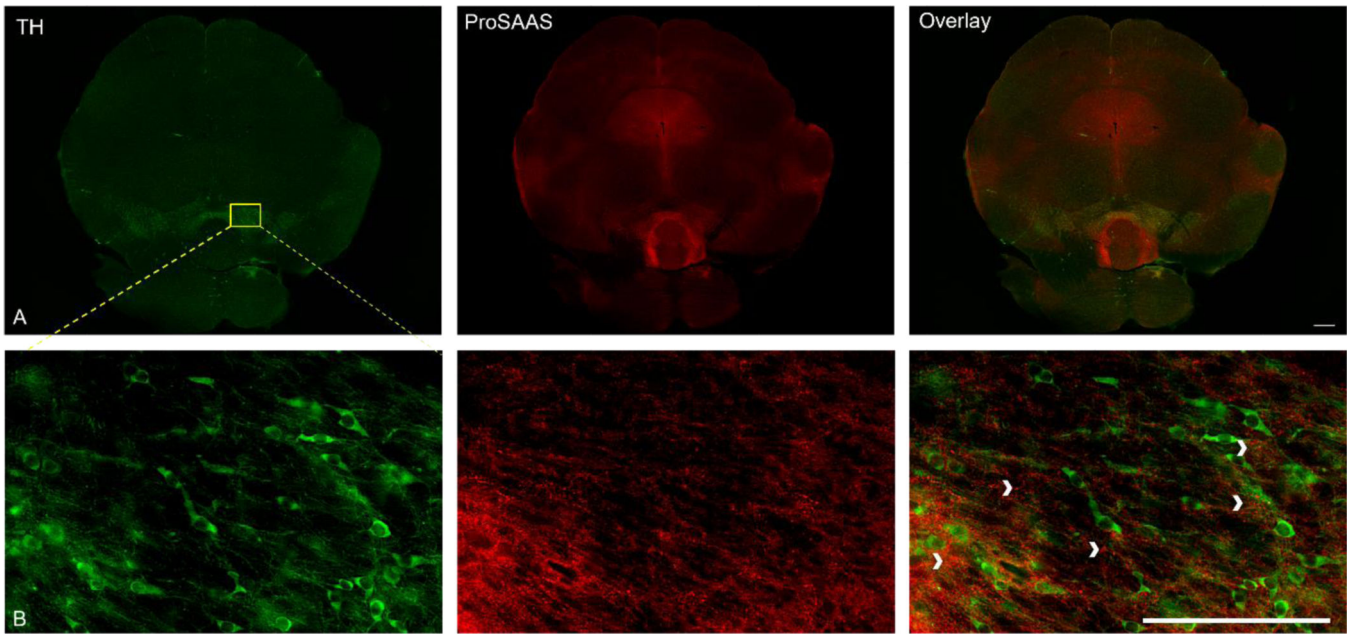

Figure 4. ProSAAS localization in the VTA

(A) A stitched 4x coronal midbrain section displaying TH and ProSAAS staining (B) 20x image of right VTA showing TH and ProSAAS staining. Chevrons display ProSAAS punctae located outside of dopamine neurons. Scale bars = 300μm

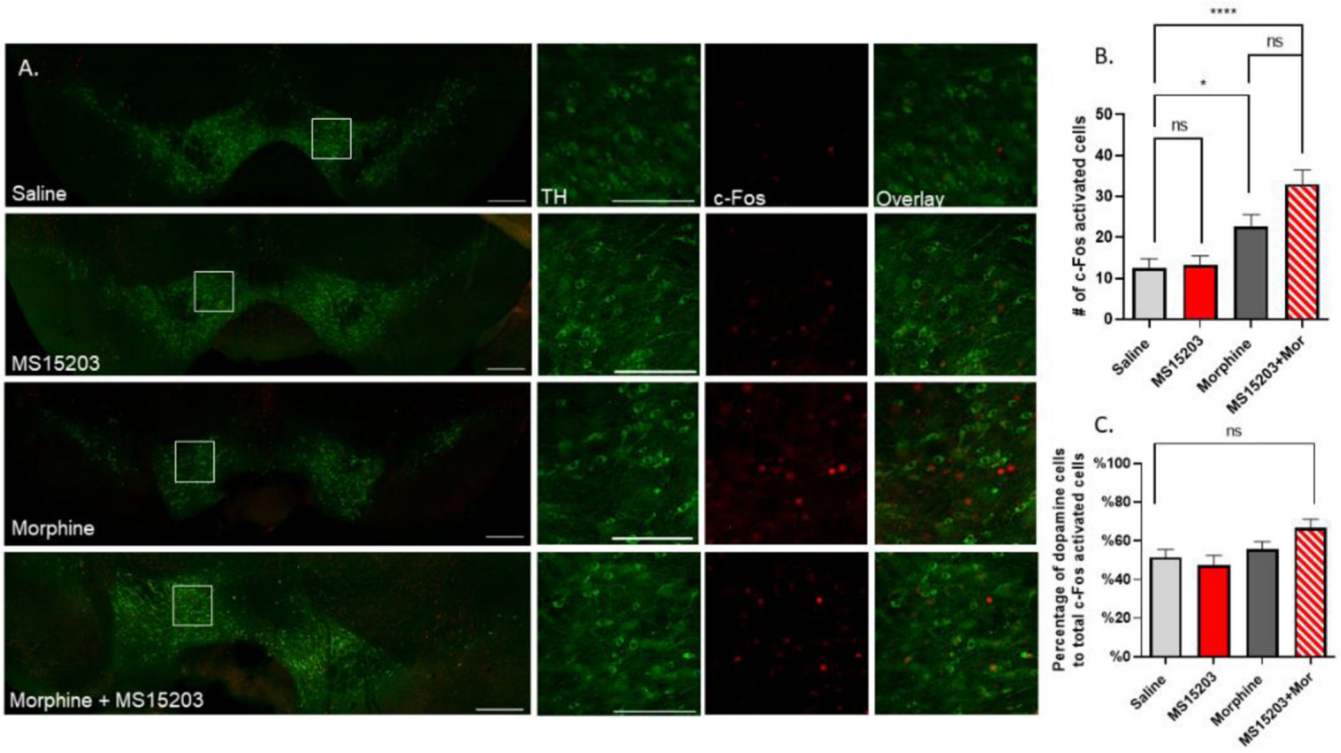

Figure 5. Quantification of c-Fos activated cells in the VTA following drug treatment.

Saline, MS15203, Morphine, and Morphine + MS15203 show the overlay of c-Fos and TH captured at 20x and stitched 8×3. White boxes delineate the 300×300 region of VTA used for c-Fos quantification and which are respectively showcased to the right of each stitched image. 300×300 images show TH, c-Fos and overlay for their respective group. Ninety minutes after designated drug treatment (Saline; 0.9%, MS15203; 10 mg/Kg, Morphine; 10 mg/Kg, Morphine + MS15203; 10 mg/Kg, 10 mg/Kg) animals were transcardially perfused with paraformaldehyde. Figure 5a. Number of c-Fos activated cells in the VTA were quantified. A one-way independent groups ANOVA with Dunnet's multiple comparisons was run to analyze group averages. MS15203 (n=5, slices =14) was compared to Saline (n=8, slices =18) (p=0.997). Morphine (n=7, slices =16) and Morphine + MS15203 (n=4, slices=11) were also compared to Saline (p = 0.0165, p <.0001, respectively). Figure 5c. The number of dopamine c-Fos activated cells was quantified. A one-way independent groups ANOVA with Tukey's multiple comparisons showed no significant difference between the experimental groups: Morphine ($p = 0.8015$); Morphine + MS15203 ($p = 0.0510$); MS15203 ($p = 0.8542$) and saline. Scale bars = 300μms *p<0.05, ****p<0.0001

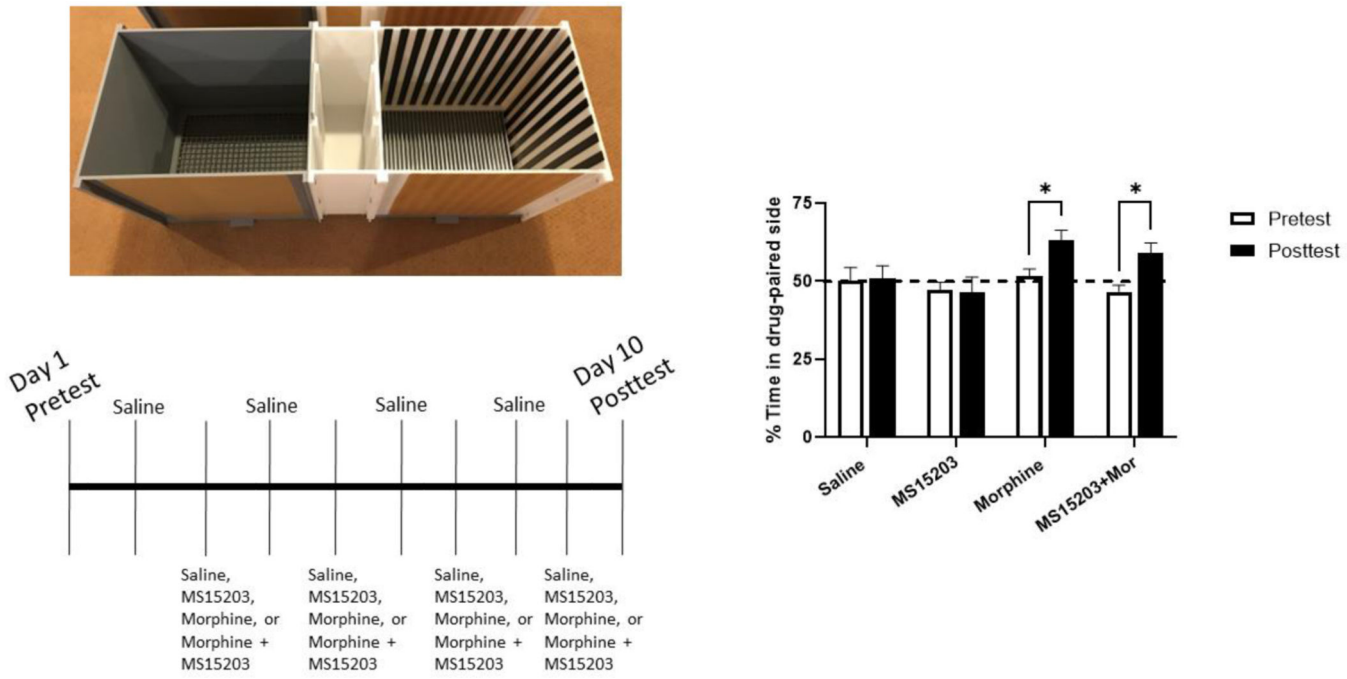

Figure 6. Assessment of Conditioned place preference.

Percent of time spent in drug paired chamber on Day 1 and 10 for each experimental group were compared using a repeated measures two-way ANOVA. Saline (n =10) and MS1520 (n=11) show no preference on the posttest compared to pretest (Šidák's p >0.05). Morphine +MS15203 (n=13) and Morphine (n=14) were significantly increased on the posttest compared to pretest (p=0.0262, p=0.0313, respectively). *p<0.05