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RUNNING HEAD: Neurotensin reduces 22-kHz USVs

Neurotensin NTS$_1$ and NTS$_2$ receptor agonists produce anxiolytic-like effects in the 22-kHz ultrasonic vocalization model in rats

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Abbreviations: ultrasonic vocalizations (USV); basolateral amygdala (BLA); central nucleus of the amygdala (CeA)

ABSTRACT

Neurotensin is a neuropeptide neurotransmitter that interacts with multiple

neurotransmitter systems, including those regulating amygdalar function, via NTS_1 and NTS_2

receptors. Both receptors are expressed in the amygdala and agonists for NTS_1 or NTS_2 receptors

have exhibited anxiolytic effects in animal models. Systemic adminstration of NTS_1 receptor

agonist PD149163 was recently shown to reduce footshock conditioned 22-kHz ultrasonic

vocalizations in rats, suggesting that PD149163 produced an anxiolytic effect. The effects that

neurotensin may have or a selective NTS_2 receptor agonist may have on 22-kHz vocalizations

has yet to be examined. The current study evaluated the effects of intracerebroventricularly

administered neurotensin (0.1 - 10.0μg), PD149163 (0.1-10.0 ng), or the NTS_2 receptor agonist

JMV-431 (0.1 - 1.0 μg) on footshock conditioned 22-kHz vocalizations in male Wistar rats.

Neurotensin, PD149163, and JMV-431 all significantly reduced the number 22-kHz calls. No

changes in call duration were found, suggesting that non-specific drug effects do not account for

the reductions in 22-kHz calls. These data support anxiolytic effects produced by activation of

NTS_1 or NTS_2 receptors, and suggest that neurotensin plays a natural role in the expression of

conditioned USVs. These data suggest that both receptor subtypes are putative pharmacologic

targets.

Keywords: anxiety; ultrasonic vocalization; neurotensin; NTS_1 receptor; NTS_2 receptor

1. Introduction

Neurotensin is a neuropeptide neurotransmitter distributed throughout much of the CNS and that interacts with a number of neurotransmitter systems through NTS_1 or NTS_2 receptors (Kleczkowska and Lipkowski, 2013; Vincent et al., 1999). Soon after its discovery by Carraway and Leeman (Carraway and Leeman, 1973), studies found that intracranial administration of neurotensin produced some physiological and behavioral effects similar to those produced by antipsychotic drugs (Jolicoeur et al., 1993; Nemeroff, 1980). These findings formed the basis for developing a number of synthetic neurotensin analogs capable of crossing the blood-brain barrier, such as NT69L (Tyler et al., 1999) and PD149163 (Wustrow et al., 1995).

There is an emerging line of research examining the role of neurotensin in anxiety and the potential of neurotensin receptor agonists to treat anxiety disorders. In post traumatic stress disorder, Maes et al. (1999) reported high levels of prolyl endopeptidase, a degradation enzyme of neurotensin, in blood plasma, suggesting lower levels of neurotensin in these patients (Maes et al., 1999). Corresponding with these findings, Ruiz et al. (1992) found lower levels of neurotensin in blood plasma of those with either an anxiety disorder or depression, which were raised upon recovery. Both NTS_1 and NTS_2 receptors are also found in the amygdala, including basolateral amygdala and central nucleus (Boudin et al., 1996; Lantos et al., 1996; Sarret et al., 2003).

An early suggestion that neurotensin might exhibit anxiolytic-like effects was demonstrated by Elliot et al. (1986) who found that intracranial neurotensin administration led rats to spend more time in the center versus the perimeter of an open field. Shilling & Feifel (2008) found that the highly selective NTS_1 receptor agonist PD149163 (Petrie et al., 2004;

Table 1) attenuated fear-potentiated startle in rats. The peptide ß-lactotensin, which has a preferential affinity as an agonist for NTS_2 receptors over NTS_1 receptors (Yamauchi et al., 2003), has also been shown to weaken fear conditioning and attenuate the effects of restraint stress measured during a hole-board test (Yamauchi et al., 2006), increase the time spent in open elevated plus maze arms, and time spent in the center of an open field (Hou et al., 2011).

A model becoming increasingly used to screen anxiolytic drugs is the footshock conditioned 22-kHz ultrasonic vocalization (USV) model in rats (Molewijk et al., 1995; Sánchez, 2003; Simola, 2015). Calls at this frequency are produced when rats are confronted with natural predators (Brudzynski, 2015) and increase as the distance to a predator decreases (Litvin, Blanchard, & Blanchard, 2007). Audio playback of rat 22-kHz calls to non-conditioned rats produces increased c-fos activity in brain regions associated with fear conditioning, such as the amygdala and periaqueductal grey area. These calls also correlate with defensive behaviors such as conditioned freezing and staying in the closed arms of an elevated plus maze (Borta et al., 2006). In a previous study from this laboratory, Prus et al. (2014) demonstrated that PD149163 significantly reduced footshock conditioned 22-kHz USVs in rats, which was a result typical of anxiolytic drugs such as the $5\text{-}HT_{1A}$ partial agonist buspirone (Molewijk et al., 1995; Prus et al., 2014) and some benzodiazepines (Molewijk et al., 1995). Chou et al. (2015) measured 22-kHz USVs in rats performing a conditioned avoidance response task, and in these animals, PD149163 also reduced 22-kHz USVs.

The aim of the current study was to add to past findings on the effects of neurotensin ligands on anxiety by testing the CNS effects of PD149163, neurotensin and the NTS_2 receptor agonist Boc-[Ψ11,12]NT(8-13) (JMV-431) (Dubuc et al., 1999; Richard et al., 2001; Table 1) in the conditioned foot-shock induced 22-kHz USV model. Past findings have shown PD149163 to

reduce USVs in this model, but an assessment of neurotensin has not been evaluated. Studies

have yet to be conducted using a selective NTS_2 receptor agonist in this model. In order to

closely compare the effects of these selective ligands with neurotensin, which does not cross the

blood-brain barrier, all compounds were microinjected into the lateral ventricle.

2. Results

Animals that did not emit at least 80 vocalizations during a test after conditioning were

eliminated prior to surgery and data were removed for rats with incorrect cannula placements.

Based on this, data were analyzed from 19 rats [PD149169 (N=7), JMV-431 (N=7) and NT

(N=5)]. There was not a statistically significant difference (p > 0.05) between the first and

second vehicle assessments on the number of USV calls for either neurotensin [t(4)=0.95, p >

0.05], PD149163 [t(6)=0.12, p > 0.05], or JMV-431[t(6)=0.02, p > 0.05]; moreover, the vehicle

data for neurotensin, PD149163 or JMV-431 did not differ significantly between these groups,

F(2, 16) = 1.13, p > 0.05. Neurotensin (0.1, 1.0, and 10.0µg) significantly reduced the number of

USV calls, F(3,12) = 6.69, p < 0.05, and post hoc testing found significantly fewer calls for only

10 µg of neurotensin compared to vehicle (figure 1). PD149163 (0.1, 1.0, & 10.0 ng) also

significantly reduced USV calls, F(3,18) = 17.33, p < 0.001, which occurred as a reduction in

calls following administration of 10 ng of PD149163 compared to vehicle (figure 2, top panel).

JMV-431 (0.1 and 1.0µg) significantly reduced the number of calls F(2,12) = 19.08, p < 0.001,

following administration of 1.0µg compared to vehicle (figure 2, bottom panel).

There was not a significant difference between the USV call durations of the first and

second vehicle tests for any of the drugs evaluated (data not shown), nor did USV call durations

for vehicle differ between the treatment groups. None of the compounds tested produced

statistical differences in USV call duration (all p values > 0.05, see Table 2).

3. Discussion

In the present study, intracerebroventricular administration of the neuropeptide neurotensin, the selective NTS_1 receptor agonist PD149163, and the selective NTS_2 receptor agonist JMV-431 produced a significant reduction in footshock conditioned 22-kHz USV calls in rats, an effect considered predictive of anxiolytic efficacy (e.g., Molewijk et al., 1995; Simola, 2015). These reductions were shown for the "long" type of 22-kHz USVs (approx. 1s), which are the length of calls emitted from rats presented with threatening stimuli (Simola, 2015). As addressed in the introduction, several past studies suggest an anxiolytic profile for neurotensin and a role for anxiolytic effects via activation of NTS_1 or NTS_2 receptors. In particular, a past study in this laboratory found that systemic administration of PD149163 significantly reduced 22-kHz USVs using procedures similar to those in the present study (Prus et al., 2014). In the present study, PD149163 was 1,000 fold more potent than neurotensin (0.01 vs 10.0 ug, respectively) and 100 fold more potent than JMV-431 (1.0 ug) in reducing the number of 22-kHz calls emitted. Graded dose response curves were not shown for these compounds, likely due to using 10-fold differences between each dose.

Brain neurotensin systems may have specific relationships with mechanisms mediating conditioned 22-kHz USV production, but it seems more likely that neurotensin and neurotensin receptor agonists reduce 22-kHz USVs by acting to reduce anxiety. Numerous pharmacological studies link cholinergic activity and cholinergic muscarinic receptor activation to the production of 22-kHz USVs (for review see Brudzynski, 2015); neurotensin's interactions with cholinergic systems (Szigethy et al., 1988) enhances the activity of cholinergic neurons (Alonso et al., 1994; Gully et al., 1997) leading to acetylcholine efflux (e.g., Gully et al., 1997; Kitabgi and Freychet, 1979; Lapchak et al., 1991; Prus et al., 2007; Rakovska et al., 1998). Thus, from these actions

alone, neurotensin might be predicted to enhance 22-kHZ USVs, which is the opposite of what is found.

Likely, the ability of neurotensin and its agonists to reduce the emission of 22-kHz USVs occurs through processing and conditioning of fear responses, as has been demonstrated by neurotensin, NTS_1 receptor agonists and NTS_2 receptor agonists in past studies noted earlier. Dense neurotensin-containing fibers terminate in the central nucleus of the amygdala (CeA) (Jennes et al., 1982), and the basolateral amygdala (BLA) and CeA contain high levels of NTS_1 and NTS_2 receptors (Boudin et al., 1996; Lantos et al., 1996; Sarret et al., 2003). Neurotensin receptors may alter glutamatergic neurotransmission in the amygdala, which may alter fear conditioning processes. Neurotensin has been shown to facilitate NMDA-elicited increases in glutamate (Ferraro et al., 2011) and NTS_1 receptor activation may disinhibit BLA pyramidal neurons, thereby promoting long-term potentiation (Kroner et al., 2005). Beyond glutamate interactions, there is evidence that neurotensin receptors interact with neurotransmitters with terminals in the amygdala, including dopamine (Asan, 1998) and serotonin (Boudin et al., 1996; Jolas and Aghajanian, 1996; Sarret et al., 2003).

The present study provided further evidence that neurotensin receptor mechanisms appear involved in fear conditioning processes and that agonists for neurotensin receptors may be pursued as novel anxiolytic drugs. Neurotensin, like the NTS_1 receptor agonist PD148163 in a previous study (Prus et al., 2014), exhibited an attenuation on footshock-conditioned USVs, suggesting that neurotensin plays a natural role in reducing anxiety. The findings with JMV-431 also support anxiolytic effects ocurring through NTS_2 receptor activation. These receptors, in particular, are worth pursuing as a novel pharmacologic strategy for treating anxiety as NTS_1, but not NTS_2, receptors have been associated with malignant tumor growth in human cancer cells

(Dal Farra et al., 2001). JMV-431 does not readily cross the blood-brain barrier (unpublished findings), and therefore, there is a need for highly selective and brain penetrant NTS_2 receptor agonists in order to assess the clinical utility of such compounds in animal models.

4. Experimental Procedure

4.1 Subjects

Thirty male Wistar rats (Charles River Laboratories, Portage, MI) were used in this study and housed in a temperature and humidity controlled vivarium set on a 12 hr light/dark cycle. Rats were 10-12 weeks old when experiments began. All procedures were approved by the Institutional Animal Care and Use Committee at Northern Michigan University and followed the Guide for the Care and Use of Laboratory Animals (National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals et al., 2011).

4.2 Pharmacological compounds

The test compounds consisted of neurotensin (0.1, 1.0, & 10.0μg doses, AnaSpec, Fremont, CA, USA), PD149163 HCl (0.1, 1.0, & 10ng doses) and JMV-431 (0.1 & 1.0μg) (both generously provided by the NIMH Drug Repository, Bethesda, MD, USA). All compounds were dissolved in a phosphate buffering solution (Sigma-Aldrich, St. Louis, MO) with 1.2 mM CaCl added. The salt form of PD149163 was used.

An Equithesin solution was used for as an injectable (i.p.) surgical anesthesia for the majority of the animals; later in the study, during experiments to test JMV-431, an isoflurane vaporizer became available for gas surgical anesthesia and was used instead. An injection (s.c.) of Penicillin G (Butler Schein Animal Health, Dublin, OH, USA) was given after an animal was sedated and prior to beginning surgery. Buprenorphine (0.05 mg/kg, s.c.; Sigma Aldrich) was

given twice a day as an analgesic for two days following surgery. Animals were given one week to recover before testing began.

4.3 Apparatus and materials

Each test compound for this study was injected into the right lateral ventricle via a 26 ga. guide cannulae (C315G-SPC; PlasticsOne, Roanoke, VA, USA) that was surgically installed using a rodent stereotaxic device (Stoelting Co., Wood Dale, IL, USA). A matching 33 ga. dummy cannula (C315DC-SPC, PlasticsOne) was implanted into the guide immediately following surgery and the dummy cannula was replaced by a 33 ga. injection cannula (C315I-SPC, PlasticsOne) for drug injection prior to a test session. A syringe pump (MD-1001, Bioanalytical Systems, Inc., Lafayette, IN, USA) injected a drug solution through an injection cannula at a rate of 2μL per minute.

Conditioning and testing sessions were conducted using an ultrasonic vocalization chamber equipped with a shock grid floor and USV detector (Med Associates, St. Albans, VT, USA). Two key lights positioned at the front of the chamber were used for illumination during experimental sessions. All experimental events and data recording were conducted using MED PC version 4 software (Med Associates).

4.4 Surgical and behavioral procedures

Prior to the cannula implantation surgery, rats were first footshock conditioned to the USV chamber and then pre-tested to determine if conditioning produced 22-kHz USVs. The pre-training/testing procedures were modified from those described previously by this laboratory (Prus et al., 2014). Conditioning was conducted during two consecutive daily sessions. During these sessions, each rat was exposed to 6 floor grid shocks (0.8 mA, 8s duration) pseudo-randomly distributed during a 7 min session. Inter-shock intervals of 27, 40, 50, 75, and 100

seconds were randomly selected after a shock was presented 60 seconds after the beginning of the training session. A pre-test session was conducted on the following day.

The pre-test session consisted of two trials. The first trial was 2 min long and one shock (0.8 mA, 8 s duration) was given approximately halfway through the trial. The rat was removed from the chamber at the end of this trial and then returned to the chamber 30 min later for the second trial. The second trial was 10 min long. During this trial 22-kHz USVs were recorded and no shocks were administered. Rats that failed to emit at least 80 USVs were excluded from the study prior to surgery. This training requirement was used in Molewijk et al. (1995).

A single guide cannula was installed in the right lateral ventricle at anterior/posterior = -1.0 mm, medial/lateral = +2.0 mm relative to bregma and at a depth of 2 mm from the skull surface according to stereotaxic coordinates (Paxinos and Watson, 2005) and fixed using cranioplastic cement. The injection cannula, when implanted prior to a test session, extended 2 mm beyond the tip of the guide cannula to reach the lateral ventricle. Rats were given one week to recover from surgery before testing sessions were conducted.

The testing procedure was conducted over the course of consecutive daily sessions. The first session consisted of a conditioned session as described above. The remaining sessions were test sessions as described earlier, except that the dummy cannula was replaced with an injection cannula immediately after the 2 min trial. After implanting the injection cannula, 2μL of drug or vehicle solution (i.e., the phosphate buffering solution) was perfused through the cannula for one minute. The syringe pump was then deactivated and remained connected to the injection cannula for an additional minute before disconnecting it. The dummy cannula was replaced after the injection cannula was removed. The 10 min test trial was conducted 30 min after the injection. For each test compound, the phosphate buffering solution vehicle was injected on the first test

day and drug doses were then escalated by 10 fold for each subsequent daily test session. The

final test day for each compound consisted of a second administration of phosphate buffering

solution to assess if changes in the number of USVs occurred to baseline responding after drug

testing. Daily doses were increased instead of counterbalanced due to evidence of PD149163

producing behavioral tolerance in the USV preparation (Prus, Hillhouse, & LaCrosse, 2014).

Following testing, the probe placements were verified using histology and data were excluded

for incorrect placements.

Data Analysis

The number and duration (s) of 22-kHz calls were reported as means (+/- standard error

of the mean [SEM]). As noted earlier, a vehicle test was conducted before drug testing and after

drug testing; a paired samples t test was subsequently conducted to determine if these before and

after vehicle data differed significantly. Means for each animal of the before and after tests were

calculated and used as the vehicle condition for subsequent analyses. A one-way repeated

measures analysis of variance was used to assess differences across doses and vehicle for each

compound. Dunnett's post hoc tests were used to compare doses to vehicle, when appropriate, at

the $p < 0.05$ level. Statistical analyses were conducted using Graph Pad Prism 6 software

(GraphPad Software, Inc., La Jolla, CA).

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Figure Captions

Figure 1. The effects of the neuropeptide neurotensin on USV calls in male Wistar rats

(N=5). USV calls (means +/- SEM) refer to 22-kHz vocalizations. The abscissa refers to amounts

of the compounds administered icv. * $P < 0.05$ compared to 0.0 μg (i.e., vehicle).

Figure 2. The effects of the NTS_1 receptor agonist PD149163 (N=7) (top) and the NTS_2

receptor agonist JMV-431 (N=7) (bottom) on USV calls. ***$P < 0.001$ compared to 0.0 μg or

0.0 ng (i.e., vehicle). See figure 1 for further details.

Figure 1
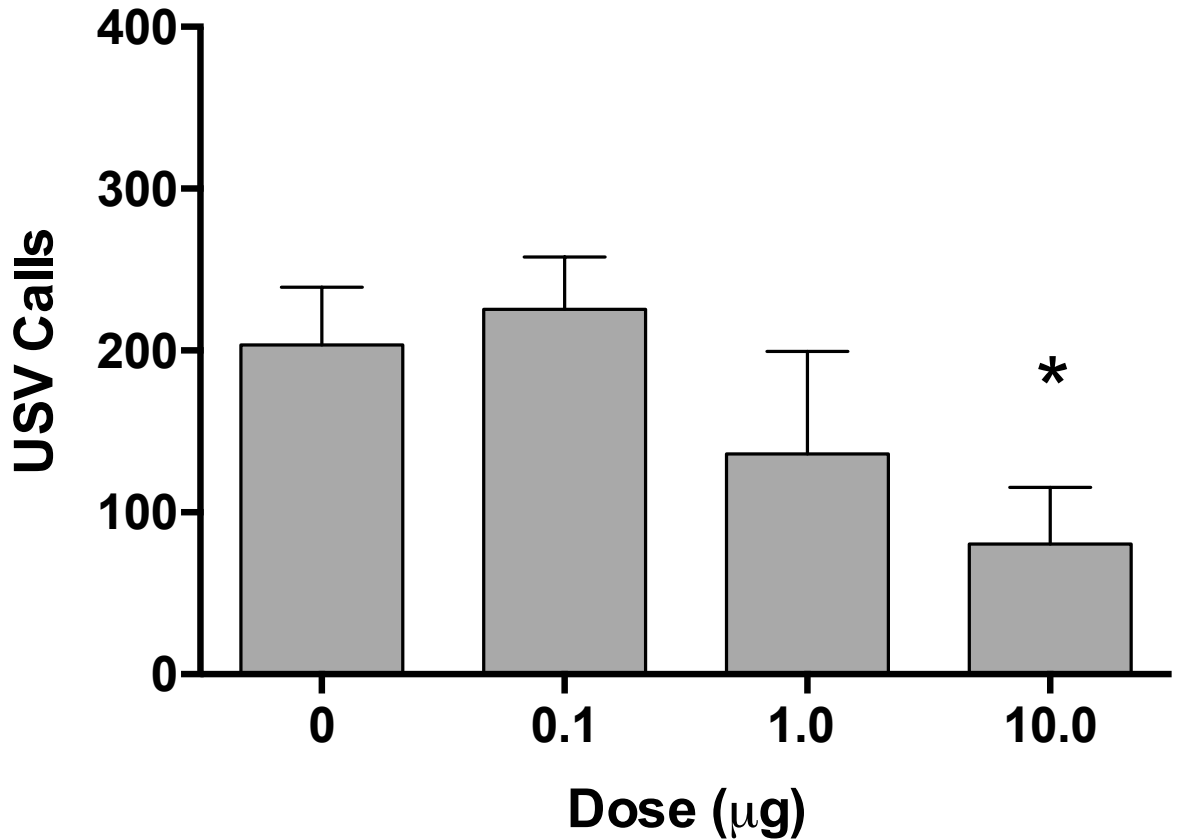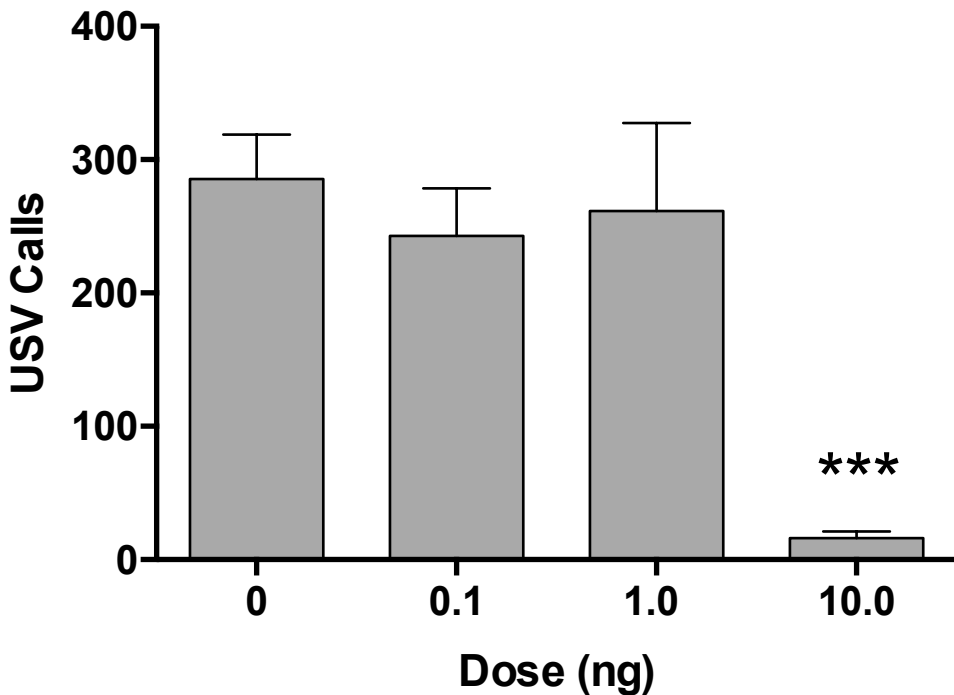[Click here to download Figure: Figure 1.pdf](#)

Neurotensin

Figure 2

PD149163
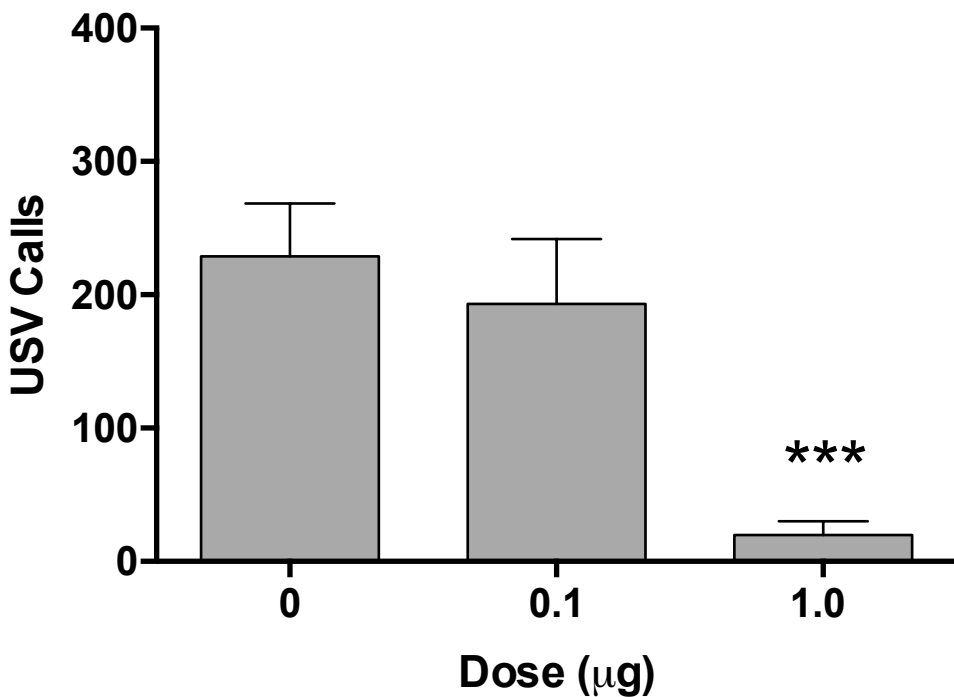
JMV-431

Table 1

Table 1. Binding affinities for NTS_1 and NTS_2 receptors

Compound	NTS_1	NTS_2	Reference
Neurotensin	0.2 nM (Kd)	4nM (Kd)	Vincent et al. 1999
PD149163	159 nM (Ki)	No affinity	Petrie et al. 2004
JMV-431	3315 nM (Ki)	38 nM (Ki)	Dubuc et al. 1999

Table 2

Table 2. USV call durations

Compound	Dose	Mean (SEM) USV Call Duration (s)
Neurotensin	Vehicle	1.10 (0.12)
	0.1 µg	1.08 (0.14)
	1.0 µg	0.88 (0.15)
	10.0 µg	1.08 (0.05)
PD149163	Vehicle	1.14 (0.08)
	0.1 ng	1.15 (0.12)
	1.0 ng	1.09 (0.09)
	10.0 ng	1.02 (0.05)
JMV-431	Vehicle	1.12 (0.10)
	1.0 µg	1.09 (0.09)
	10.0 µg	0.95 (0.05)