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Intra-ventral tegmental area microinjections of urotensin II modulate the effects of cocaine

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Abstract

Although the peptide urotensin II (UII) has well studied direct actions on the cardiovascular system, the UII receptor (UIIR) is expressed by neurons of the hindbrain. Specifically, the UIIR is expressed by the cholinergic neurons of the laterodorsal tegmentum (LDTg) and the pedunculopontine tegmentum (PPTg). These neurons send axons to the ventral tegmental area (VTA), for which the PPTg and LDTg are the sole source of acetylcholine. Therefore, it was hypothesized that UIIR activation within the VTA would modulate reward-related behaviors, such as cocaine-induced drug seeking. Intra-VTA microinjections of UII at high concentrations (1 nmole) established conditioned place preference (CPP), but also blocked cocaine-mediated CPP (10 mg/kg). When rats received systemic sub-effectual doses of cocaine (7.5mg/kg) with intra-VTA injections of 1 or 10 pmole of UII CPP was formed. Furthermore, the second endogenous ligand for the UIIR, urotensin II-related peptide, had the same effect at the 10 pmole dose. The effects of low doses of UII were blocked by pretreatment with the UIIR antagonist SB657510. Furthermore, it was found that intra-VTA UII (10 pmole) further increased cocaine-mediated (7.5 mg/kg) rises in electrically evoked dopamine in the nucleus accumbens.

Our study has found that activation of VTA-resident UIIR produces observable behavioral changes in rats, and that UIIR is able to modulate the effects of cocaine. In addition, it was found that UIIR activation within the VTA can potentiate cocaine-mediated neurochemical effects. Therefore, the coincident activation of the UII-system and cocaine administration may increase the liability for drug taking behavior.

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Keywords

urotensin II; pedunculopontine tegmentum; laterodorsal tegmentum; conditioned place preference; cocaine

1. Introduction

In the last decade the discovery of a number of neuropeptides has greatly increased our understanding of brain function. Moreover, a number of these novel neuropeptides have been implicated in addiction- and reward-related behaviors (e.g. melanin-concentrating hormone, neuropeptide S, hypocretin (Chung *et al.*, 2009; Li *et al.*, 2009; Mochizuki *et al.*, 2010; Smith *et al.*, 2009; Smith *et al.*, 2010)). Another neuropeptide which holds promise in modulating reward-related behaviors is urotensin II (UII). The UII-system is comprised of one receptor (a G protein-coupled receptor (GPCR)) and two ligands (UII and UII-related peptide (URP)). In the mammalian brain the urotensin II receptor (UIIR) is selectively expressed in the mesopontine tegmentum by cholinergic neurons of the laterodorsal tegmentum (LDTg) and the pedunculopontine tegmentum (PPTg) (Clark *et al.*, 2001). These brain structures are widely interconnected with the basal ganglia (Mena-Segovia *et al.*, 2004), and their involvement in motivated and reward-related behavior (Alderson *et al.*, 2004; Alderson *et al.*, 2006; Bechara *et al.*, 1992a; Bechara *et al.*, 1992b; Bechara *et al.*, 1989; Diederich *et al.*, 2005; Olmstead *et al.*, 1997; Olmstead *et al.*, 1993; Olmstead *et al.*, 1994; Olmstead *et al.*, 1998; Wilson *et al.*, 2009; Yeomans *et al.*, 1985) is thought to be primarily mediated through connections to midbrain DA systems.

The ventral tegmental area (VTA) is a key component of the so called “Reward Pathway” and it is believed that dysregulation of the VTA-accumbens-prefrontal cortex (PFC) circuitry is a critical neuronal mechanism of addiction. The LDTg is a major excitatory input to the VTA, with the caudal PPTg also innervating the VTA (Holmstrand *et al.*, 2011; Oakman *et al.*, 1995). Moreover, the PPTg and LDTg are the sole cholinergic input to the VTA (Maskos, 2008) and recently have been shown to directly innervate the nucleus accumbens (NAc; Dautan *et al.*, 2014). The inactivation of the LDTg or the PPTg reduces the phasic firing of VTA dopaminergic neurons (Lodge *et al.*, 2006; Pan *et al.*, 2005), which is thought to be sufficient for behavioral conditioning (Tsai *et al.*, 2009). In addition, previous to the LDTg studies, it was found that PPTg activation drives phasic firing of dopaminergic VTA neurons (Floresco *et al.*, 2003). However, this effect was abolished when the LDTg is inactivated (Lodge *et al.*, 2006).

The present understanding is that input from the LDTg and PPTg is important for both the tonic to phasic firing of the VTA dopaminergic neurons and it is this phasic firing that is important in the learning of reward-related behaviors. As for the effects of cholinergic agents within the VTA, LDTg electrically evoked dopamine release in the NAc is blocked by intra-VTA cholinergic antagonists (Forster *et al.*, 2000) and intra-VTA carbochol can produce conditioned place preference (Ikemoto *et al.*, 2002; Yeomans *et al.*, 1985). Moreover, cholinergic input has been implicated in the control of the firing pattern of dopaminergic VTA neurons (Mameli-Engvall *et al.*, 2006). Therefore, the evidence suggests

that the direct modulation of the cholinergic terminals within the VTA would modulate reward-related behaviors.

Evidence for a role of the mesopontine tegmentum in reward-related behaviors comes from studies demonstrating that non-selective (ibotenic acid) lesioning of the PPTg blocks the acquisition of morphine conditioned place preference (CPP), but not cocaine CPP (Bechara *et al.*, 1992a; Olmstead *et al.*, 1997; Olmstead *et al.*, 1994)), reduces the breakpoint for heroin in the progressive ratio schedule (Olmstead *et al.*, 1998), impairs the acquisition of amphetamine self-administration (Alderson *et al.*, 2004) and lesioning the posterior PPTg increases nicotine self-administration (Alderson *et al.*, 2006). In addition, non-selective inactivation of the PPTg (e.g. by muscimol) blocks the ability to form new associations between actions and outcomes (Maclaren *et al.*, 2013). The accumulated evidence suggests that the LDTg/PPTg play an important role in reward-related behaviors.

The expression of the UIIR by cholinergic LDTg/PPTg neurons may allow for the specific pharmacological modulation of this brain region. We previously found that activation of UIIR in the VTA (expressed by presynaptic PPTg/LDTg neurons (Blaha *et al.*, 2010)) produces a sustained release of dopamine in the NAc (Clark *et al.*, 2005). To investigate whether our neurochemical findings translate to measurable behavioral changes we used the CPP paradigm. In addition, lower doses (10 and 1 pmole) not previously tested for their effect on NAc dopamine levels were investigated for their abilities to modulate cocaine-mediated CPP and VTA-targeted electrically evoked dopamine release in the NAc. The present study is supportive of UIIR activation having behavioral effects that are of key interest to the fields of drugs of abuse and reward-related behaviors.

2. Experimental Procedures

2.1 Conditioned Place Preference

2.1.1 Animals—Male Sprague-Dawley rats (290-340g) (Charles River, Wilmington, MA) were used for all experiments. Animals were housed in an environmentally controlled vivarium with a 12 hour light/dark cycle (lights on at 07:00) with an ambient temperature of ~21°C. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University at Buffalo, and were in compliance with guidelines set by the National Institute of Health (NIH). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.1.2 Stereotaxic Surgery—Bilateral cannulation of the ventral tegmental area (VTA) using a stereotaxic apparatus (Steolting Co, Wood Dale, IL) was performed under Ketamine HCl (Ketaset, Fort Dodge, IA) /Xylazine (Anased, Lloyd, Shenandoah, IA) (65mg/5mg/kg) anesthesia. Stereotaxic coordinates of subjects' bregma were recorded, and drill sites for guide cannula (Plastics One, Roanoke, VA) were calculated [anteroposterior (AP), -6.5 mm; mediolateral (ML), ± 3.2 mm]. Cannula sites were drilled, and three additional holes were made for the placement of anchor screws (Plastics One, Roanoke, VA), one on each skull plate just posterior to bregma, and one posterior to lambda. Anchor screws were secured to the skull, and stereotaxic arms (20°) lowered cannula into holes until dura. From dura, cannula were slowly lowered [dorsoventral (DV), -6.9 mm] and held in place for the

application of methyl methacrylate (Co-Oral-It Dental Mfg. Co., Diamond Springs, CA). Using this technique the desired placement of the cannula was 1mm above VTA with final cannula coordinates of (AP - 6.5 mm, ML 0.6 mm, DV 7 mm, from bregma (Paxinos *et al.*, 2007); the placement was based on estimates from the in situ radioligand binding to UIIR (Clark *et al.*, 2001)). Methyl methacrylate head caps were allowed to harden, and screw caps (Plastics One, Roanoke, VA) were inserted into cannula. Following surgery, buprenorphine hydrochloride (0.05 mg/kg; Reckitt Benckiser Pharmaceuticals, Richmond, VA) was administered subcutaneously. Subjects were also treated with a 6.0 mL injection (SC) of lactated ringers (Hospira, Lake Forest, IL) for hydration, and an injection (SC) of enrofloxacin (Bayer, Leverkusen, Germany) to prevent infection.

Post-operatively, subjects were individually housed to recover for 5 to 6 days, and handled daily in preparation for the behavioral testing.

2.1.3 Behavioral Assay—Conditioned place preference (CPP) boxes (39 cm × 39 cm) were designed and constructed inhouse to suit the experimental environment. Boxes were constructed with plexiglass so that two equivalent compartments, one with black and white horizontally stripped walls with metal grid flooring and lemon scent, and the other with black and white dotted walls with metal bar flooring and banana scent. Boxes were placed in infrared locomotor capture apparatus (OMNITECH Electronics, Columbus, OH) controlled by Fusion software. All 30 minute sessions were run during the day (09:00-15:00). Dose groups were randomized throughout the day to mitigate any time of day effects.

On day 1 (pretest), partitions with cutout doorways were inserted into boxes allowing subjects to freely explore both compartments; amount of time spent in each compartment was calculated (subjects displaying greater than 20% side preference were removed from the study). On days 2-9 (conditioning days), full plexiglass partitions confined subjects to a particular compartment (4 pairings each of vehicle or test compounds, with 30 min exposures). Immediately prior to placement into the apparatus, each subject was first given an intracranial microinjection of aCSF, UII or URP (0.001 mL at 0.001mL/min) followed by a intraperitoneal (i.p.) injections of 0.9% saline or cocaine (NIDA Drug Supply Program) as described in results. For antagonist studies, the UIIR antagonist SB657510 (10uM, 0.001 mL at 0.001mL/min; NIMH Chemical Synthesis and Drug Supply Program) or aCSF, was delivered and the subject was placed back into their home cage for five minutes before delivery of test compounds. On day 10 (test day), partitions with cutout doorways were reinserted into boxes allowing subjects to freely move about both compartments. Amount of time spent on each side was recorded and used to calculate CPP preference score. Preference scores were calculated by subtracting amount of time the subject spent on the unpaired-side from the time spent on paired-side for both pretest and test days. Data was analyzed using GraphPad Software (San Diego, CA, USA), one-way ANOVA with Bonferroni post-hoc analysis. Locomotor activity was also quantified using the same software.

2.1.4 Histology—Upon completion of behavioral testing, rats were euthanized, followed by the injection of fast green dye through the cannula. The rats were then decapitated and brains extracted. Brains were immediately frozen by submersion in -16°C isopentane and stored at -80°C until used. Fifty micron and twenty micron slices were serially sectioned, so

that any two adjacent sections were a pair of twenty micron and fifty micron sections, using a cryostat (Leica, Buffalo Grove, IL), and mounted onto Super Frost slides. Mounted tissue was fixed using 4% paraformaldehyde in 0.1M phosphate buffer (PB) at 22°C for 1h, rinsed in 0.1M PB, air dried, and stored with desiccant at -20°C until processed. All chemicals were procured through Thermo Fisher Scientific (Pittsburgh, PA).

The fifty-micron slices were incubated in cresyl violet (Acros Organics, Beel, Belgium) at 22°C for 20 minutes, dehydrated through graded ethanol, and submerged into CitriSolv Hybrid (Thermo Fisher Scientific, Pittsburgh, PA). Slides were immediately cover slipped using DPX mountant (VWR, Radnor, PA) and examined using a microscope (Olympus, Center Valley, PA).

The twenty micron sections were processed by in situ hybridization for the presence of tyrosine hydroxylase (TH) mRNA. The cRNA probe for TH was a 280 bp fragment (subcloned into pGEM with PstI/KpnI (Xu *et al.*, 2007)) synthesized using digoxigenin-11 labeled UTP (Roche Diagnostics, Indianapolis, IN) as per manufacturer's instructions. A dot blot was performed to quantify probe concentration. The procedure was done as described previously (Clark *et al.*, 2001; Xu *et al.*, 2007; Xu *et al.*, 2004). Briefly, TH antisense probe (0.025ng/uL) was added to hybridization solution (50% formamide, 50% dextran sulfate, 5M NaCl, 50X Denhardt's solution (G-Biosciences, St. Louis, MO), 1M Tris (pH 8.0), 0.5M EDTA (pH 8.0), DEPC H₂O, tRNA (10mg/mL), 1M DTT (Amresco, Solon, OH), applied directly to tissue, and incubated at 60°C for 16 hours. Slides were incubated in an RNase A digestion solution (0.5M EDTA (pH 8.0), 1M Tris (pH 8.0), 5M NaCl, RNase A (10mg/mL, Thermo Fisher Scientific, Pittsburgh, PA)) at 37°C for 30 minutes. Sections were gradually desalted by incubation in SSC of decreasing salinity at 22°C, with a final 30 min wash (0.1X SSC) at 68°C. Slides were then incubated in Genius Buffer 1 (100mM Tris (pH 7.5), 150mM NaCl), and subsequently washed in Genius Buffer 2 (Genius Buffer 1, 5% skim powdered milk (Village Farm, Manawa, WI), 0.25% Triton-X 100 (Alfa Aesar, Ward Hill, MA) at 22°C for 30 min. Anti-digoxigenin alkaline phosphatase conjugated Fab antibody (Roche Diagnostics, Indianapolis, IN) solution was prepared at a 1:5000 dilution with Genius buffer 2 then applied to slides by drop technique and incubated at 37°C for 3h. Three Genius buffer 2 washes (1, 5, 10 min) followed by color reagent solution (NBT and BCIP) (Roche Diagnostics, Indianapolis, IN) in Genius Buffer 3 (100mM Tris (pH 9.5), 100mM NaCl, 50 mM MgCl₂) and incubated in the dark at 22°C overnight. Slides were washed twice in Genius Buffer 4 (10mM Tris (pH 8.0), 1mM EDTA (pH 8.0)), rinsed in deionized distilled water, dehydrated in graded ethanol and cover slipped.

2.2 Voltammetric Recording

2.2.1 Surgery—Sprague-Dawley rats were anesthetized with urethane (1.5 g/kg, i.p.) and placed in a stereotaxic apparatus (Kopf, Tujunga, CA). According to procedures described in earlier studies (Park *et al.*, 2010; Park *et al.*, 2009), small holes were drilled in the left hemisphere of the skull for a reference electrode and the right hemisphere of the skull for the working and stimulating electrodes. The dura mater was punctured, carefully removed, and a carbon-fiber microelectrode was lowered into the NAc (AP + 1.2 mm, ML +2.0 mm, DV from -6.0 to -8.0 mm), coordinates from a stereotaxic atlas (Paxinos *et al.*, 2007). An Ag/

AgCl reference electrode was employed for all measurements and it was secured on the skull with dental cement. Electrical stimulation was accomplished with a bipolar, stainless-steel electrode (0.2 mm diameter, Plastics One, Roanoke, VA). The stimulating electrode was insulated to the tip. It was placed into the VTA (VTA) (AP -6.5 mm, ML +1.2 mm, DV from -8.0 to -9.0 mm).

2.2.2 VTA microinjections of UII—Evoked NAc dopamine overflow was monitored while the electrical stimulation (60 Hz, 24 pulses, 300 μ A) was delivered at different depths, 200-300 μ m increments. After optimizing the placements of the carbon-fiber electrode in the NAc and the stimulating electrode in the VTA to record maximal dopamine release, the stimulating electrode combined with an infusion cannula was secured on the skull with dental cement. After evoked dopamine overflow during control experiments with aCSF (vehicle) was stable and reproducible for at least 30 min one of two experiments was performed: 1) UII (10 pmole), and 2) systemic injection of cocaine (7.5 mg/kg, i.p.) followed by UII (10 pmole). UII was administered via an infusion cannula (33 gauge) that was combined with the bipolar stimulating electrodes and inserted into the implanted guide cannula (26 gauge, Plastics One Inc., VA, USA). VTA microinfusions of UII (10 pmole, 1 μ L) were made over 60 s with a syringe pump. The dopaminergic cell bodies in the VTA were electrically stimulated (biphasic square wave pulses (300 μ A and 2 ms each phase) with 60 Hz stimulation frequency). The number of stimulus pulses was held constant at 24 and stimulations were repeated every 2 or 3 min.

2.2.3 Voltammetric procedures—Glass-sealed T-650 untreated carbon fibers (7 μ m in nominal diameter, Thornel, Amoco Corp., Greenville, SC) with an exposed length of 75 - 100 μ m was lowered into the NAc. FSCV was computer-controlled and has been described in detail previously (Heien *et al.*, 2003). A triangular scan (-0.4 to +1.3 V, 400V/s) was repeated every 100 ms. Data was digitized and stored on a computer using software written in LABVIEW (National Instruments). Temporal responses were determined by monitoring the current at the peak oxidation potential for dopamine in successive voltammograms. The current was converted to concentration based on calibration obtained after the *in vivo* experiment with known concentrations of dopamine *in vitro*. As the carbon-fiber microelectrode was used to lesion (see below), an average postcalibration factor, 7.5 ± 0.7 pA/(μ M $\cdot\mu$ m²) for dopamine, based on the average response obtained multiple electrodes as described in previous study (Park *et al.*, 2011). Data are represented as mean $\pm$ SE and 'n' values indicate the number of rats. Statistical analysis was done by using one-way ANNOVA with Bonferroni post-hoc (GraphPad Software, San Diego, CA, USA).

2.2.4 Histology—Electrode placements were verified by electrolytic lesions made with the carbon-fiber microelectrodes at the end of the experiment. Rats were euthanized with an overdose of urethane (2.0 g/kg) and a lesion was made at the recording site by applying constant current (20 μ A for 10s) to the carbon-fiber microelectrodes (Park *et al.*, 2010). Brains were removed and stored in 10 % formalin solution for a week before being sectioned into 40~50 μ m coronal slices. The sections were mounted on slides and viewed with an optical microscope.

3. Results

3.1 Cannula Placement was within the Posterior Ventral Tegmental Area

Verification of cannula placement was performed by postmortem injection of fast green dye. Animals were excluded from the study if the average position of the two cannula was more posterior than -6.7 mm (AP), or if both exhibited midline wider than 1.6mm, or if both were located above -6.6 mm (DV), or if either cannula was more anterior than -6.0 mm (AP). These criteria resulted in cannula placements above the parainterfascicular nucleus and the paranigral nucleus of the VTA (Paxinos *et al.*, 2007) (Fig 1).

3.2 Conditioned place preference - High doses of VTA administered UII mediates CPP

On test day, rats that received 1 nmole UII microinfusions during paired conditioning days spent more time on the paired side of the box (Fig 2), resulting in a significant preference score ($n=13$, $p < 0.05$ as compared to pretest day). Previously, intra-VTA injection of 1 nmole UII was shown to produce dopamine efflux in the NAc (Clark *et al.*, 2005). Although in the same study we show that higher doses of UII (10 and 100 nmole) also drive dopamine release in the NAc (Clark *et al.*, 2005) those doses may activate other receptors (e.g. somatostatin receptor (Nothacker *et al.*, 1999)) and may be supra-physiological, and so were not tested.

3.3 Conditioned place preference - High doses of VTA administered UII block cocaine induced CPP

Pilot studies using rats without cannula established 10mg/kg of cocaine (i.p.) to be rewarding by virtue of mediating CPP in our chambers (cocaine dose response not shown). As expected, when coincidentally given with intra-VTA microinjections of aCSF the administration of 10 mg/kg of cocaine produced significant CPP (Fig 3A; $p < 0.05$). This demonstrates that the vehicle used for all of the experiments does not disrupt cocaine-mediated CPP. Rats who received 1 nmole UII at the same time as 10 mg/kg cocaine did not show a preference, suggesting this combination is not rewarding (Fig 3A). However, a lower dose (10 pmole) had no such effect. The distance traveled on test day across dose groups (aCSF, UII 10 pmole, UII 1 nmole; Fig 3B) was not significantly different. In addition, distance traveled per 30 minute session across all treatment days was unaffected (days in which they received co-administration of cocaine and UII; data not shown).

3.4 Conditioned place preference - Low doses of UII do not mediate CPP

The dose of UII (1 nmole) used to induced CPP (Fig 2) and that blocked cocaine-mediated CPP (Fig 3) is likely supra-physiological and likely with a large sphere of diffusion. Therefore, it cannot be ruled out that observed behavioral changes resulted from UII activation of other receptors (eg. somatostatin receptor (Nothacker *et al.*, 1999)) in the VTA or immediately adjacent to the VTA. Three studies using rats have described radioligand binding to somatostatin receptors in the interpeduncular nucleus, substantia nigra, (Martin *et al.*, 1991; Sato *et al.*, 1991; Uhl *et al.*, 1985) and one of these studies reports binding in the VTA (Sato *et al.*, 1991). In addition, through diffusion the microinjection of high doses of UII could activate UIIRs expressed in adjacent brain structures (e.g. interpeduncular nucleus

(Clark *et al.*, 2001)). To address this issue, low doses of UII that more closely match known pharmacological characteristics of the UIIR were used. In vitro the EC50 for UIIR (calcium mobilization in CHO cells) is 100 pM (Nothacker *et al.*, 1999). The microinjection of 10 pmole of UII/URP, assuming a diffusion radius of 0.3cm would be a diffusion volume of ~0.1 mL, would result in a final concentration (assuming even distribution) of 100nM. This concentration would produce maximal response but is well below the 2,000 nM that is reported as the EC50 of UII at the subtype 2A somatostatin receptor (Nothacker *et al.*, 1999). Therefore, 1 pmole and 10 pmole were tested in the remainder of our experiments.

Co-administration of a saline i.p. injection, with either an aCSF, UII 1pmole, or UII 10pmole microinfusion was not able to produce CPP in rats, and did not significantly affect time spent on the paired side (Fig 4A). The animals who received intra-VTA aCSF with saline i.p. injections produced no preference (Fig 4; animals in this group were randomly assigned a “paired” side) and so this demonstrates the unbiased nature of our CPP apparatus. The distance traveled on test day across dose groups (aCSF, UII 10 pmole, UII 1 nmole; Fig 4B) was not significantly different. The lower doses of UII which have no significant effects on CPP (Fig 4) were used in subsequent studies.

3.5 Conditioned place preference - Physiologically relevant doses of UII potentiate sub- efficacious doses of cocaine to produce CPP

When 7.5 mg/kg of systemic cocaine was co-administered with aCSF there was no change in the amount of time spent on the paired side, indicating that 7.5 mg/kg of cocaine is a sub-
efficacious dose within our paradigm. However, when paired with sub-effectual doses of UII (from Fig 4A), 7.5 mg/kg of cocaine did produce significant CPP (Fig 5A; UII 1 pmole, $n = 9$; UII 10 pmole, $n = 7$; $p < 0.05$ (compared to pretest)). The UIIR antagonist SB657510 blocked the effects of 10 pmole UII, and did not produce significant effects when co-administered with 7.5 mg/kg of cocaine (Fig 5A). The high variation within the group treated with only SB657510 (and cocaine) is due to a bimodal distribution of scores with some very negative and some very positive. SB657510 treated animals did not exhibit a score with a magnitude of less than 206 seconds, and so should be interpreted as a group these animals have no preference.

The distance traveled remained the same for all treatment groups over all days, suggesting that the locomotor activity was not affected by the combination of cocaine and UII (data not shown). Distance traveled on test day for UII treated groups was not significantly different than controls ($p > 0.05$). However, there was a significant increase in the distance traveled on test day of the groups with had received the combination of 10 pmole UII and SB657510, as compared to aCSF treated (Fig 5B; $p = 0.0217$). This is curious as all animals on test day are drug-free and that the difference is only when compared to the aCSF treated group, not the 10 pmole or SB657510 treated groups.

3.6 Conditioned place preference - Urotensin II-Related Peptide modulates cocaine- mediated CPP

In vitro URP and UII have equal EC50 and binding affinities (Chatenet *et al.*, 2004), and so they are believed to have equivalent pharmacological effects. Therefore, to test whether

these pharmacological similarities hold true in vivo we tested URP for its ability to potentiate the effects of cocaine in CPP. When administered at the same dose at which UII was able to potentiate the effects of cocaine (Fig 4), intra-VTA URP is able to induce CPP (Fig 6) when co-administered with sub-efficacious doses of cocaine (cocaine, 7.5 mg/kg; URP, 10 pmole; $n = 15$, $p < 0.01$). As shown for the equivalent dose of UII (Fig 4), 10 pmole of URP when co-administered with systemic saline (i.p.) did not produce CPP as compared to pretest day (Fig 6; $n = 9$).

3.7 FSCV - UII further increases cocaine-mediated electrically evoked dopamine NAc levels

Dopamine is a major catecholamine in the NAc (Park *et al.*, 2010) and the dense dopaminergic innervation of the NAc is primarily from the cell bodies that originated in the VTA. To study the effect of UII VTA microinfusion on systemic cocaine-mediated dopamine release in the brain, dopamine release was measured by FSCV in the NAc during electrical stimulation of the VTA. Electrically evoked DA release was assayed due to 10 pmole UII having no behavioral effect in the absence of cocaine (Fig 4). Furthermore, that 10 pmole of UII is a 100-fold lower dose than had been previously shown to have elicited dopamine efflux (Clark *et al.*, 2005), it was hypothesized that UII acts as a neuromodulator not a transmitter. Therefore, if UII only modulates the circuitry under study (not directly producing activity changes), passive recording of dopamine levels may not reveal the true nature of this neuromodulation. Moreover, it is known that while cocaine increases phasic firing of VTA neurons in awake animals, this is not reliable in anaesthetized animals, perhaps due to reduced sensory inputs (Koulchitsky *et al.*, 2012). While it is understood that under anesthesia neither passive or electrically evoked measurement of DA are a particularly close reflection of what happens in the VTA in awake animals, the use of electrical stimulation does provide a level of activity to modulate.

After evoked dopamine overflow during control experiments with aCSF (vehicle) was stable and reproducible for at least 30 min one of two experiments was performed: 1) UII (10 pmole), and 2) systemic injection of cocaine (7.5 mg/kg, i.p.) followed by UII (10 pmole). In the absence of cocaine, UII infusion did not alter electrically evoked DA levels in the NAc (Fig 7). After systemic cocaine injections the maximal evoked dopamine concentration ($[DA]_{max}$) was significantly increased (Fig. 7). The effect of cocaine was apparent within 10 min of administration and reached a maximum value at ~20-30 min. These maximum responses were further increased when UII (10 pmole) was microinfused onto the electrical stimulation tips located in the VTA (Fig 7). UII increased stimulated release within 5 min after microinfusion and returned to pre-UII levels after 15-20 minutes.

4. Discussion

CPP was conducted to determine whether the previous findings that intra-VTA UII elicited neurochemical changes in VTA (Clark *et al.*, 2005) translated into behavioral effects in rats. This study found that rats treated with 1 nmole of UII formed CPP (Fig 2), and when 1 nmole UII was co-administered with cocaine (10 mg/kg i.p.) it blocked cocaine-mediated CPP (Fig 3). It was also found when rats receiving systemic sub-effectual doses of cocaine (7.5 mg/kg i.p.) were at the same time treated with low doses of UII or URP they formed CPP (Fig 5&6). Corroborating the behavioral findings, the FSCV results showed that intra-

VTA administration of UII can potentiate cocaine-mediated NAc dopamine efflux, at concentrations of UII and cocaine used during CPP (Fig 7). Therefore, the UII-system can influence the effects of cocaine through a VTA-mediated mechanism.

The finding that a high dose of UII microinjected into the VTA produced CPP (Fig 2) is in line with our previous findings that there is an increase in DA efflux in the NAc at the same concentration of UII (Clark *et al.*, 2005). However, surprisingly it was found that a high dose of UII (1 nmole) blocked CPP mediated by 10 mg/kg cocaine (Fig 3). A possible reason for this could be that in rats robust UIIR activation produces subjective adverse effects when in combination with higher doses of cocaine. Cocaine has been repeatedly shown to exhibit an inverted U-shaped dose-response relationship in paradigms designed to measure the rewarding effects of drugs (Uhl *et al.*, 2013). Simply, there is a dose range in which cocaine is found to have rewarding effects. Higher or lower doses are either aversive (due to secondary effects) or not rewarding, respectively. It may be that the combination of high doses of both cocaine and UII results in a shift in the cocaine dose-response to the downward slope of the U-shaped curve, which translates to non-preferred side effects. An additional explanation is the heightened drive induced by the cocaine in combination with the high dose of UII could put the dopaminergic neurons into depolarization inactivation. As examples, the acute administration of high concentrations of excitatory substances, including peptides, has been shown to produce depolarization inactivation (Grace *et al.*, 1986; Muschamp *et al.*, 2007). It would appear from our previous studies that high doses of intra-VTA UII (up to 100 nmoles) do not put these neurons into block (Clark *et al.*, 2005), however, that was not in combination with cocaine. Thus, thorough examination of intra-VTA UII/URP effects on dopamine efflux in the NAc will need to be performed.

The VTA and surrounding structures (e.g. interpeduncular nucleus and substantia nigra) express somatostatin receptors (Martin *et al.*, 1991; Sato *et al.*, 1991; Uhl *et al.*, 1985) that are known to be activated by high concentrations of UII (Nothacker *et al.*, 1999). In addition, the interpeduncular nucleus expresses the UIIR (Clark *et al.*, 2001). Therefore, to test whether doses of UII/URP in a more physiological range and at doses that are more likely to be restricted within a discreet anatomical region, lower doses of UII were tested. A sub-efficacious dose of cocaine (7.5 mg/kg) was potentiated by sub-efficacious doses of UII (Fig 5) and URP (Fig 6) to produce CPP. This is a UIIR specific effect, as pretreatment with a UIIR antagonist blocks the effects of 10 pmole intra-VTA UII (Fig 5). The most straightforward explanation is that although the dose of cocaine used is unable to produce CPP, there is still an increase in NAc dopamine and that the co-administration of UIIR agonists potentiates this dopamine efflux, thereby, increases the “rewarding” properties of cocaine.

To directly test the hypothesis that UII potentiates cocaine-mediated NAc DA levels, electrically evoked dopamine release in the NAc was measured in urethane-anesthetized animals. As previously demonstrated, administration of 7.5 mg/kg cocaine (i.p.) increases the electrically evoked dopamine release in the NAc (stimulation within the VTA; (Park *et al.*, 2010)). After intra-VTA UII (10 pmole) there was a potentiation of the cocaine-mediated increase (Fig. 7). However, there was no such increase in NAc DA in the absence of cocaine, suggesting the UIIR activation at the doses used is purely modulatory. In

conclusion, UIIR activation within the VTA is able to interact with systemic injections of cocaine to increase the levels of VTA-electrically evoked dopamine in the NAc.

The evidence that is available suggests that UIIR within the VTA is not expressed postsynaptically, but rather by the cholinergic presynapses (Blaha *et al.*, 2010; Clark *et al.*, 2001). Therefore, the obvious hypothesis is that the presented UIIR-mediated behavioral effects are through the facilitation of acetylcholine release from LDTg and/or PPTg terminals. Theoretically, the testing of this hypothesis is straightforward; microinfuse anti-cholinergic agents into the VTA before the administration of UIIR agonists. However, multiple labs have found anti-muscarinic agents, predominately scopolamine, infused intra-VTA reduces the levels of dopamine in the NAc under basal conditions (Forster *et al.*, 2000; Lester *et al.*, 2010; Miller *et al.*, 2005; Steidl *et al.*, 2011). This finding precludes a definitive conclusion in the event that anti-muscarinic pretreatment blocks UII-mediated effects. Likewise nicotinic antagonists do not seem to be benign (Solecki *et al.*, 2013; Wickham *et al.*, 2013; Zanetti *et al.*, 2007) and therefore not ideally suited to testing the acetylcholine hypothesis of UIIR-mediated actions. This is not to say the previous findings are in error, but rather strongly supports that acetylcholine within the VTA has important functions not only when strong/salient stimuli (chemical or otherwise) are present. In addition, similar VTA microinjection approaches of cholinergic agents have been used to show the importance of acetylcholine within the VTA to reward-related behaviors (Laviolette *et al.*, 2003; Shinohara *et al.*, 2014). However, it is difficult to resolve whether a hypothetical blockade of UII-mediated effects after anti-cholinergic treatment would be due to a direct blockade of UIIR actions or due to another competing mechanism. Before we are able to test the hypothesis that UIIR-mediated effects in the VTA are due to acetylcholine, doses of anticholinergic drugs that do not impact the basal functioning of this circuit need to be found. Alternatively, microdialysis within the VTA during UII agonist infusion may at least indicate that UIIR activation directly or indirectly can facilitate the release of acetylcholine. It is more likely that well executed electrophysiological studies utilizing circuit specific technologies will be the means by which the mechanism of action of UII within the VTA will be resolved.

The voltammetric studies suggest UII/URP-mediated effects could be due to NAc DA potentiation. This is palatable and is an intuitively easy explanation, however, it relies on observations from an acute study to explain a behavior which is the result of repeated treatments. To produce CPP UII/URP and cocaine are injected multiple times over the course of a number of days. Therefore, the UII/URP-mediated effects in CPP could be due to changes in synaptic plasticity induced directly by UIIR activation. G protein-coupled receptors are well known for their actions on cell proliferation and cytoskeletal rearrangements. Specifically, the UIIR has been shown to modulate cell migration and the small GTPase RhoA in vascular smooth muscle cells (Matsusaka *et al.*, 2006; Sauzeau *et al.*, 2001). Therefore, the ability of UIIR activation to alter cocaine-mediated increases in NAc dopamine may be secondary to long-term changes mediated by repeated UIIR activation within the VTA. Future studies, which utilize intra-NAc dopamine receptor antagonists in the context of CPP and that assess transcriptional changes within the VTA, PPTg, LDTg, and NAc due to UIIR activation will need to be completed.

Currently, it is not known why or when URP or UII are released in the brain. However, our results suggest that in the event that the UII-system is coincidentally activated during cocaine administration there would be a potentiation of the effects of cocaine. If the UII-system is moderately active and lower doses of cocaine are administered there may be a greater liability to form drug-associations. We did see a potential “protective” effect of UII at high doses. However, the low dose scenario is more likely in vivo because the high doses of UII used in the preliminary studies are likely supra-physiological. Moreover, the interaction of the UII-system and cocaine is of particular interest as some of the URP expressing neurons in the hypothalamus co-express gonadotropin-releasing hormone (GnRH; (Egginger *et al.*, 2005)). It is hypothesized that there is co-release of URP and GnRH, and it is known these neurons become activated during stress and by circulating sex hormones (Clarke, 2011; Maeda *et al.*, 2010). It is well accepted that stress is a risk factor for both the development of addiction and relapse of addiction (Sinha, 2008). In addition, it has been suggested by some that cocaine may be more addictive in adolescents than in adults (Estroff *et al.*, 1989). Therefore, the UII-system could potentially contribute to addiction liability in adolescents or in those under stressful conditions.

Our future studies will focus on elucidating the intra-VTA mechanism of UIIR-mediated potentiation of cocaine-induced CPP and NAc dopamine release. In addition, the interaction of the UII-system with other drugs of abuse will be determined. Specifically, the ability to modulate self-administration of drugs of abuse will be explored. Our results suggest that the UII-system has a role in the acquisition and/or maintenance of reward-related behaviors.

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Abbreviations

aCSF	artificial cerebral spinal fluid
CPP	conditioned place preference
DA	dopamine
GnRH	gonadotropin-releasing hormone
i.p.	Intraperitoneal injection
LDTg	laterodorsal tegmentum
NAc	nucleus accumbens
PPTg	pedunculopontine tegmentum
SN	substantia nigra
Ull	urotensin II
UllR	urotensin II receptor
URP	urotensin II-related peptide
VTa	ventral tegmental area

Highlights

Urotensin II (UII) is a neuropeptide not previously linked to reward behaviors
high concentrations of UII in the VTA produces conditioned place preference (CPP)
low concentrations of UII potentiates sub-threshold doses of cocaine to produce CPP
UII potentiates cocaine-mediated release of dopamine in the nucleus accumbens
the endogenous UII-system may play a role in modulating reward systems

Figure 1. Verification of Cannula Placement

Semi-transparent dots denote the placement of the injection sites from all animals' cannula (A). Overlap of the dots results in a darker shade, with heavy overlap appearing black. Cresyl violet stain was used to determine the extent of the cannula tracks and the termination point of the cannula (B). Verification of TH positive neurons in the area of injection was performed by in situ hybridization (C). Figure 1A was adapted from Paxinos et al. (Paxinos *et al.*, 2007). Figures 1B and 1C are from the same animal, and represent adjacent sections.

Figure 2. Microinfusion of Urotensin II induces CPP at high dose

The pairing of 1 nmole of UII (1 μ l microinfusion into the VTA) to a specific compartment resulted in a significant preference for the side paired with UII ($n = 13$; $p < 0.05$, compared to pretest). All error bars are SEM.

Figure 3. High doses of UII block cocaine induced CPP

(A) Coincident administration of 1 nmole of UII (VTA) blocked the effects of cocaine (i.p.; 10 mg/kg) to mediate CPP (aCSF, $n = 9$, $p < 0.05$ (compared to pretest); UII 1 nmole, $n = 8$). A lower doses of UII (10 pmole) was unable to significantly change the effect of cocaine (UII 10 pmole, $n = 7$, $p < 0.05$ (compared to pretest)). (B) The distance traveled during the 20 minute test session was not significantly different, as compared between groups ($p > 0.05$). All error bars are SEM.

Figure 4. Low doses of UII do not produce CPP

(A) Pairings of low doses of UII resulted in no change in preference (aCSF, $n = 18$; UII 1 pmole, $n = 16$; UII 10 pmole, $n = 17$). (B) The distance traveled during the 20 minute test session was not significantly different, as compared between groups ($p > 0.05$). (B) The distance traveled during the 20 minute test session was not significantly different, as compared between groups ($p > 0.05$). All error bars are SEM.

Figure 5. Low doses of cocaine and UII interact to mediate CPP

(A) Cocaine at the dose of 7.5 mg/kg with intra-VTA injections of aCSF was found not to induce CPP ($n = 11$). However, when low doses of UII are injected intra-VTA coincidentally with cocaine (7.5 mg/kg) CPP is induced (UII 1 pmole, $n = 9$; UII 10 pmole, $n = 7$; $p < 0.05$ (compared to pretest)). The effect of 10 pmole UII is blocked when the UIIR antagonist SB657510 is injected intra-VTA five minutes prior to the UII injection ($n = 7$), while the antagonist alone does not produce significant CPP ($n = 10$). (B) The distance traveled during the 20 minute test session was significantly different in the group treated with the combination of 10 pmole UII and SB657510, as compared to the aCSF treated group ($p = 0.0217$). All error bars are SEM.

Figure 6. URP enhances effects of sub-efficacious cocaine

URP at the dose of 10 pmole (intra-VTA) does not mediate CPP ($n = 9$). However, when coincidentally administered with cocaine at a dose that normally does not produce CPP (7.5 mg/kg), there is significant CPP ($n = 10$, $p < 0.05$ (compared to pretest)). All error bars are SEM.

Figure 7. UII enhances cocaine-mediated dopamine levels in the NAc

Intra-VTA administration of UII (10 pmole) without prior treatment with cocaine did not produce a change in evoked DA levels ($n = 4$), as compared to aCSF treatment. Cocaine (7.5 mg/kg, i.p.) increased electrically evoked DA levels in the NAc ($n = 4$). Intra-VTA administration of UII (10 pmole) further increased evoked DA levels ($n = 4$). (error bars represent SE; ** $p < 0.01$ as compared to basal aCSF treated; * $p < 0.05$ cocaine alone compared to subsequent UII intra-VTA infusion)