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Activation of TRPV1 in the VTA Excites Dopaminergic Neurons and Increases Chemical- and Noxious-Induced Dopamine Release in the Nucleus Accumbens

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Dopamine (DA)-containing neurons of the ventral tegmental area (VTA) provide dopaminergic input to the nucleus accumbens and to the prefrontal cortex within the mesolimbic pathway. In the present study, we combined electrophysiological recordings and microdialysis techniques to investigate the function of transient receptor potential vanilloid 1 (TRPV1) channel in the VTA. In brain slices, application of the TRPV1 receptor agonist capsaicin increased the firing rate of rat dopamine neurons and in a proportion of tested cells (44%) it also induced a bursting behavior. The effects of capsaicin were concentration dependent. The increase in neuronal firing was dependent on enhanced glutamatergic transmission since it was blocked by the superfusion of the ionotropic glutamate antagonists, CNQX and AP5. Interestingly, microinjection of capsaicin into the VTA and noxious tail stimulation transiently enhanced dopamine release into the nucleus accumbens. Both the *in vitro* and *in vivo* effects were mediated by TRPV1 activation in the VTA since they were reduced by co-perfusion of the selective TRPV1 receptor antagonist iodoresineferatoxin. Our data suggest a novel role for TRPV1 channels in the mesencephalon of rat, namely activation of the DA system following a peripheral noxious stimulation.
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Keywords: mesolimbic area; noxious stimulation; dopamine; capsaicin; patch-clamp recordings; microdialysis

INTRODUCTION

The dopaminergic (DA) neurons of the ventral tegmental area (VTA), together with its afferents to the nucleus accumbens (NAc) and to the prefrontal cortex (PFC), constitute a crucial neuronal system that processes natural rewards (Hollerman and Schultz, 1998; Schultz, 1986; Di Chiara *et al*, 1993; Schmidt, 1998; Wise, 2002) and drug-seeking behavior (Phillips *et al*, 2003; Bonci *et al*, 2003). However, several studies have shown that VTA DA neurons can also be involved in acute stress responses (Maeda and Mogenson, 1982; Mantz *et al*, 1989; Gao *et al*, 1990), which cause an increase of DA efflux into the output areas (Cabib and Puglisi-Allegra, 1994; Horger and Roth, 1996; Finlay and Zigmond, 1997; Guarraci and Kapp, 1999). These studies suggest that changes in the activity of DA neurons are potential indicators of the responses of these cells either to pleasant, or to noxious stimuli (Moore *et al*, 2001), and are strictly controlled by excitatory and inhibitory inputs (Overton and Clark, 1997; Grillner and Mercuri, 2002; Prisco *et al*, 2002; Floresco *et al*, 2003).

Recently, we have shown that the vanilloid receptor (TRPV1), which belongs to the transient receptor potential channel family, increases glutamatergic transmission onto midbrain DA neurons (Marinelli *et al*, 2003), suggesting that TRPV1 activation can increase cellular excitation of these cells.

Besides the control of synaptic transmission in the brain (Sasamura *et al*, 1998; Doyle *et al*, 2002; Marinelli *et al*, 2002, 2003), the TRPV1 channels are also known to be involved in mediating nociceptive and stressful processes in the peripheral nervous system (O'Neil and Brown, 2003).

Therefore, we performed *in vitro* and *in vivo* experiments aimed to investigate the role of TRPV1 channels in modulating DA release in the mesolimbic system, either in response to direct activation of TRPV1 by local capsaicin or by peripheral noxious stimulation of the tail in freely moving rats.

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METHODS

Slice Preparation

Wistar rats, 2–3-weeks old, were anesthetized by halothane and killed by decapitation. The brain was rapidly removed from the skull and horizontal brain stem slices (300 μM) were cut in cold (8–12°C) artificial cerebrospinal fluid (ACSF), using a vibratome, and left to recover at 33°C for at least 1 h. Slices were separately placed in a recording chamber and submerged in a continuously flowing (3 ml/min, 34°C) ACSF. ACSF composition was (in mM): NaCl 126; KCl 2.5; $MgCl_2$ 1.2; $CaCl_2$ 2.4; NaH_2PO_4 1.2; $NaHCO_3$ 24; glucose 10.

Electrophysiology

The neurons were visualized using infrared Nomarski optics mounted on an upright microscope (Olympus BX50WI) and equipped with infrared-DIC enhancement. Patch-clamp recordings in whole-cell and cell-attached mode were obtained with glass electrodes (3.7–4 MΩ) filled with (in mM): K-gluconate 145; $MgCl_2$ 2; $CaCl_2$ 0.1; EGTA 0.75; HEPES 10; MgATP 2 mM; Na_3GTP 0.3 (pH 7.3, with KOH) and using an Axopatch 1D (Axon Instruments) ($I=$ clamp and $I=0$ in whole-cell and cell-attached mode, respectively). Acquisition of spikes was filtered at 1 kHz, digitized at 10 kHz, and acquired on a PC by means of Axoscope 9 software (Axon Instruments).

Whole-cell patch-clamp recordings were obtained from 21 VTA DA neurons, which were identified using well-established criteria, such as spontaneous single-pacemaker firing at 1–3 Hz, hyperpolarization of the membrane potential in response to exogenous application of dopamine and the presence of a hyperpolarization-activated inward current (I_h) in response to voltage steps to −100 mV, from a holding potential of −60 mV (Grace and Onn, 1989; Johnson and North, 1992; Mercuri *et al*, 1995).

Firing Analyses

The firing activity and burst events were detected and analyzed with Clampfit 9 software (Axon Instruments). The mean firing frequency (in Hz) of each neuron was determined over a time period during which cell firing had stabilized (usually 2–3 min). For the evaluation of capsaicin effect, the last two minutes of capsaicin application and first two minutes of wash out were analysed.

Changes of membrane potential in spontaneously active DA cells were detected by measuring membrane potential values between spikes (see the asterisk on the vertical and horizontal dashed lines in Figure 1a, left panel).

For burst analyses, we chose an appropriate number of action potential (3–4 at least), together with a burst delimiting interval of 300 ms (Prisco *et al*, 2002). A burst of spikes was superimposed on a depolarizing phase (envelope) of the membrane potential that advances and terminates when the membrane potential is driven into a temporary state of depolarization block (see the inset of Figure 1a, Shepard and Bunney, 1991).

The data in the text are given as mean ± SEM and as percentage of control. Statistical differences were determined on the raw data by using paired Student's *t*-test.

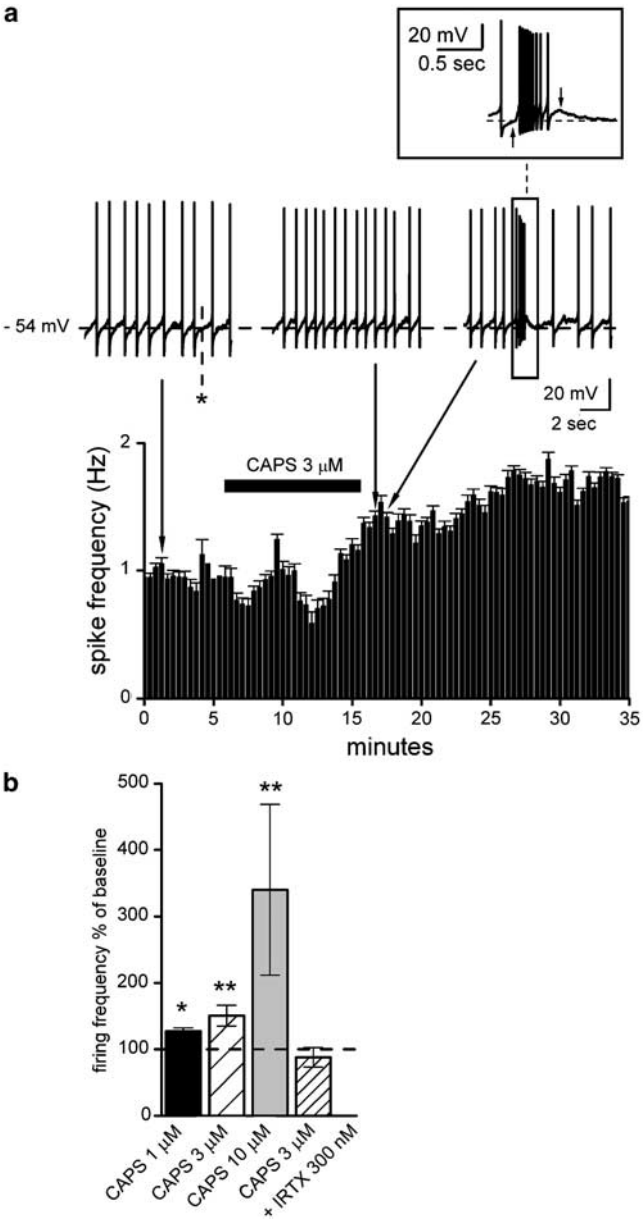

Figure 1 Excitatory effects of capsaicin on the firing activity of a VTA dopamine neuron. (a) Top, raw traces of whole-cell current-clamp recordings from a single neuron in control condition (left panel), during bath application of CAPS (middle panel) and CAPS-induced burst discharge (right panel). The trace in the inset shows at a larger view the action potentials superimposed in the depolarizing envelope delimiting the depolarization. Note that the arrows indicate the initiation and peak of the depolarization. The dotted line between spikes indicated an extrapolated value of potential below spike threshold—54 mV. Bottom, running spike frequency histogram of the same neuron shown at the points indicated by the arrows. Note the irreversible effect of CAPS on firing activity. (b) Histogram summarizing CAPS effects (1–3–10 μM) as percentage change of firing activity and the antagonism of CAPS (3 μM) effects in the presence of IRTX ($p>0.05$). *$p<0.05$, **$p<0.01$.

Animals

Male Wistar rats (175–200 g; Charles River, Italy) were housed in groups of three per cage and maintained in an air-conditioned room at 21 ± 1°C, on a 12-h light–dark cycle

(0700–1900 h) with food and water *ad libitum* for at least 10 days before use.

All animals were tested between 1000 and 1700 h and were exposed individually to one experiment only.

The procedures used in this study were in strict accordance with the European legislation (EEC no. 86/609), the Italian national legislation (DL no. 116/92) governing the use of animals for research, and with the guidelines of the National Institutes of Health on the use and care of laboratory animals reported.

Microdialysis

Animals were anesthetized with an intraperitoneal injection of ketamine/xylazine (80:10 mg/kg), mounted in a stereotaxic frame (David Kopf Instrument, Tujunga, CA) and implanted unilaterally with a guide cannula (stainless steel; shaft OD, 0.38 mm; length 6.5 mm Metalant AB, Stockholm, Sweden) in NAc and VTA. The length of the guide cannula was 6 mm for NAc and 7.5 mm for VTA. The guide cannula was fixed with epoxy glue and dental cement was added for additional stabilization. The coordinates from the bregma (measured according to the atlas of Paxinos and Watson (1982)) were (in mm): +1.7 AP, −0.9 L for NAc and −5 AP, −0.7 L for VTA. At the end of surgery, animals were housed individually into a new home cage adapted to avoid the breaking of the implantation.

The following day, rats were lightly anesthetized to facilitate insertion of the microdialysis probe (dialysis membrane length 2 mm; OD of 0.24 mm, MAB4 cuprophane microdialysis probe, Metalant AB) into the guide cannula of the NAc.

Immediately after implantation, microdialysis probe was connected via polyethylene-20 tubing and a dual-channel swivel (model 375/D/22QM; Instech Laboratories, Plymouth Meeting, PA) to a syringe of a CMA/100 pump (Carnegie Medicine, Stockholm, Sweden) and perfused with artificial CSF (in mM: 147 NaCl, 2.2 $CaCl_2$, and 4 KCl) (Pontieri *et al*, 1995) at a constant flow rate of 2 μl/min for at least 2 h. Experiments were performed 24 h after probe placement. During experiment, each rat was left in its home cage and microdialysis probe was connected to the pump. After the start of dialysis perfusion, rats were left undisturbed for 2 h before collection of baseline samples. Four baseline samples before treatment were collected. The dialysate was collected every 8 min for 120 min post-treatment, and a volume of 10 μl was analyzed by HPLC system to quantify the dopamine levels, as described previously (Ventura *et al*, 2003). Briefly, the HPLC system consisted of an Alliance (Waters Corporation, Milford, MA) and a coulometric detector (model 5200A Coulochem II; ESA, Chelmsford, MA). A conditioning cell (+400 mV; ESA; model 5021) was placed immediately after a C18 column (3.9 × 150 mm; Waters Corporation), and followed by an analytical cell (electrode 1 at +200 mV, and electrode 2 at −250; ESA; model 5011). The column was maintained at 33°C; the flow rat was 1.2 ml/min. The mobile phase was as described previously (Westerink *et al*, 1998). Peak height produced by reduction of DA was compared with that produced by a standard. The detection limit of assay was 0.1 pg.

Vehicle (CSF plus ethanol) or iodoresineferatoxin 1 μM (IRTX, 0.2 μl/2 min) was injected into VTA through a stainless-steel cannula (0.15 mm OD; 8 mm lenght; UNIMED, Switzerland) connected to a 1 μl syringe by a polyethylene tube and driven by a CMA/100 pump. The cannula was left in place for an additional 2 min after the end of infusion. At 15 min after the first injection, rats that were injected by vehicle received another injection of vehicle (veh + veh group) or capsaicin (CAPS) 50 μM (veh + CAPS group) by the same system; while rats injected by IRTX received CAPS 50 μM (IRTX + CAPS group) or again IRTX. Half of the rats in the veh + veh group and rats injected twice by IRTX were exposed to tail-shock stress (veh + shock and IRTX + shock groups) immediately after the second injection. Tail shock (3 s, 0.50 mA) was administered through stainless steel electrodes applied to the tail.

Statistical Analysis

The effects of CAPS and IRTX + CAPS on extracellular DA levels in the NAc were analyzed by repeated-measures ANOVA with one between factor (treatment, three levels: veh, CAPS and IRTX + CAPS) and one within factor (time, six levels: 0, 8, 16, 24, 32, 40 min from drug injection, $n = 15$).

The effects of shock and IRTX + shock on accumbal DA release were analyzed by repeated-measures ANOVA with one between factor (treatment, two levels: shock and IRTX + shock) and one within factor (time, eight levels: 0, 8, 16, 24, 32, 40, 48, 56 min ($n = 10$)). Simple effects were assessed by one-way ANOVA for each time point. Individual between-group comparisons, when appropriate, were performed by *post hoc* test (Duncan's multiple range test).

Drug Application

For the electrophysiological experiments, drugs were bath applied at the following concentration: CAPS, 1–3–10 μM, IRTX, 300 nM, D-(−)-2-Amino-5-phosphonopentanoic acid (D-AP5, 50 μM), and 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX, 10 μM). CAPS and IRTX were dissolved in DMSO and the final concentration of the vehicle was less than 0.05%, which was devoid of any effects. For these reasons, we considered control conditions as those with and without vehicle. Each slice received only a single exposure to CAPS. All these drugs were purchased from Tocris (UK), except for dopamine that was obtained from Sigma.

For microdialysis experiments, both IRTX (1 μM) and CAPS (50 μM) were dissolved in 100% ethanol and later diluted in artificial CSF (in mM: 147 NaCl, 2.2 $CaCl_2$, and 4 KCl). The CAPS and IRTX doses were selected on the basis of preliminary experiments. For CAPS we found that 50 μM was a threshold dose in a range going from 25 to 200 μM, while for IRTX we observed an antagonist effect at 1 μM, a threshold dose in a range going from 300 nM to 10 μM.

RESULTS

Capsaicin Increases the Firing Activity of VTA Dopamine Neurons

Since we have previously shown that CAPS increases glutamatergic synaptic transmission onto midbrain DA

neurones (Marinelli *et al*, 2003), in this study we tested whether the activation of TRPV1 could affect the firing of spontaneously active DA cells in the VTA.

In whole-cell current-clamp recordings, bath application of the TRPV1 agonist CAPS (1 μM) caused a significant increase of the firing rate of DA neurons from 1.12 ± 0.46 to 1.44 ± 0.59 Hz (27.50 ± 4%, $p < 0.05$, $n = 4$, Figure 1b) without affecting the membrane potential. The effect of CAPS was dose-dependent. CAPS 3 μM increased the firing frequency by 46% of control, from 1.30 ± 0.59 to 1.76 ± 0.41 Hz ($n = 5$, $p < 0.01$, Figure 1a, b) and 10 μM raised the firing rate to 240% (from 1.83 ± 0.72 to 3.84 ± 0.65 Hz; $n = 4$, $p < 0.01$, Figure 1b). The interspike membrane potential was slightly depolarized by CAPS 3 and 10 μM (3.20 ± 1.63 and 3.83 ± 1 mV, respectively). The increase in firing had a slow onset and fully developed at the end of perfusion (5–10 min).

In four out of nine neurons, superfusion of 3–10 μM CAPS caused a depolarizing envelope (9.76 ± 0.79 mV) and bursts of action potentials (12.80 ± 1.28 spikes in a burst, 126 ± 70 ms intraburst interval, 1441.46 ± 773 ms burst duration). The interburst interval was irregular and could vary from 11 to 59 s (30.82 ± 9.75 s) (Figure 1a).

The effect of CAPS on the firing activity of dopamine neurons has been also investigated by using single-unit recordings. Application of CAPS 3 μM caused a significant increase in the spike frequency by 21.90 ± 8.68%, from 3.33 ± 1.01 to 4.05 ± 1.03 Hz ($n = 5$, $p < 0.05$) and in two out of five DA neurons we observed bursts of spikes (data not shown).

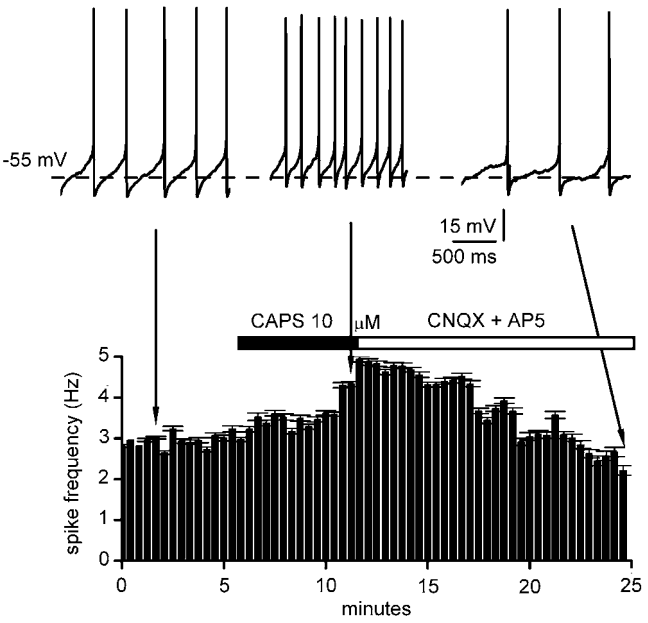

Figure 2 Capsaicin augments the discharge of DA neurons by activating glutamatergic neurotransmission. Top, traces: current-clamp recordings of a single neuron in control (left panel), under CAPS perfusion (middle panel), and in the presence of the ionotropic glutamate receptor antagonist CNQX (10 μM) and AP5 (50 μM) (right panel). Bottom, running frequency histogram showing the time-course of effects of CAPS on the same neuron shown on the top. Note that the excitatory effects of CAPS rapidly reversed in the presence of CNQX and AP5. This effect was significantly different from the irreversible effect of CAPS perfused alone (χ^2 two-population $p < 0.05$).

The Increase in Firing Rate was TRPV1-Mediated and Glutamate-Dependent

To confirm that the increased frequency of firing induced by CAPS was mediated by the activation of TRPV1, we performed experiments in the presence of the TRPV1 antagonist IRTX (300 nM). Application of IRTX reduced *per se* by 15 ± 5.70% the frequency of spikes from 2.12 ± 0.27 to 1.77 ± 0.22 Hz ($n = 7$, $p < 0.05$). Subsequent perfusion of CAPS 3 μM did not significantly change the firing frequency in all tested neurons (1.77 ± 0.22 Hz control in IRTX *vs* 1.79 ± 0.30 Hz added CAPS; $p = 0.789$, $n = 7$, Figure 1b).

The role of glutamatergic transmission in the CAPS-induced excitation was evaluated in eight neurons. Four out of eight DA cells were perfused with CAPS 3 μM and then exposed to ionotropic glutamate antagonists (Figure 2). Under these conditions, CAPS induced an increase in firing that readily reversed in 6.74 ± 0.16*n* min of superfusion with CNQX and AP5 ($n = 4$, Figure 2), whereas, in the absence of these ionotropic glutamate antagonists, the CAPS-induced effects did not reverse even after 20–30 min of wash. In the remnant 4 DA neurons, incubated for 8–10 min with CNQX and AP5, the subsequent application of CAPS 3 μM did not significantly modify the firing frequency (from 0.75 ± 0.17 to 0.71 ± 0.22 Hz, $p = 0.929$).

Capsaicin and Noxious Stimuli Increase Dopamine Efflux

DA response in CAPS and IRTX + CAPS groups. The effects of CAPS and IRTX + CAPS injection on DA release in NAc are shown in Figure 3. Statistical analyses revealed a significant interaction between treatment and time ($F_{(10,60)} = 2.77$; $p < 0.01$). A simple effect analysis revealed a significant effect of time only for CAPS at 8, 16, and 24 min, and a maximal increase of DA compared with veh at 16 min (~60%). No significant differences between veh and CAPS + IRTX were evident.

Thus, CAPS injection in VTA produces a significant increase of DA release in the NAc, while simultaneous IRTX injection counteracts the CAPS excitatory effect.

DA response in shock and IRTX + shock groups. The effects of shock and IRTX + shock on DA release in the NAc are shown in Figure 4. Statistical analyses revealed a significant treatment × time interaction ($F_{(7,56)} = 3.67$, $p < 0.01$). A simple effect analysis revealed a significant effect of time only for shock-induced DA release. Veh + shock group showed significant, time-dependent increase of DA outflow, reaching maximal DA increase 32 min (~50%) after shock administration. No significant increase in DA outflow was evident in the IRTX + shock group.

These results show that the increase in DA outflow in NAc that resulted by tail-shock administration has been completely prevented by IRTX injection in the VTA.

DISCUSSION

This is the first study examining the excitatory effect of TRPV1 receptor activation on the firing activity of DA cells

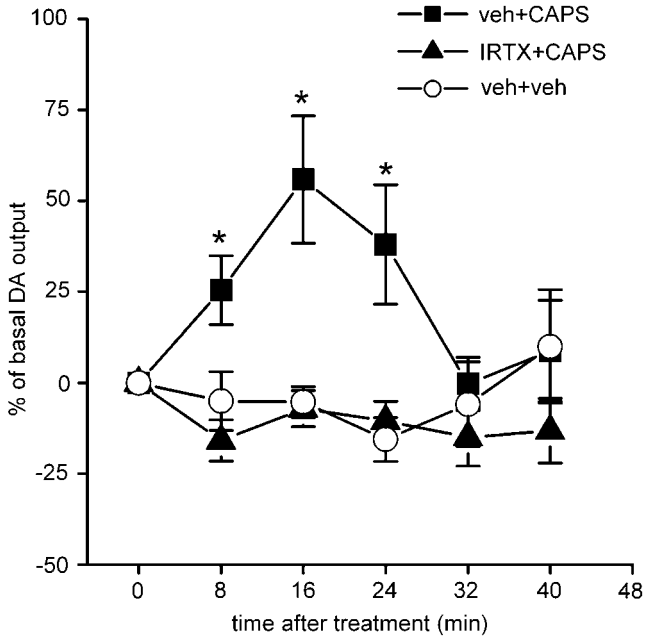

Figure 3 The effects of veh (0.01% of ethanol, $n=5$), CAPS 50 μM ($n=5$), and IRTX 1 μM + CAPS 50 μM ($n=5$) injection on DA release in NAc. Results are expressed as percentage changes (means ± SE) from basal values (3.26 ± 0.33 pg/20 μl). Statistical analyses were performed on raw data. *$p<0.005$ compared with vehicle.

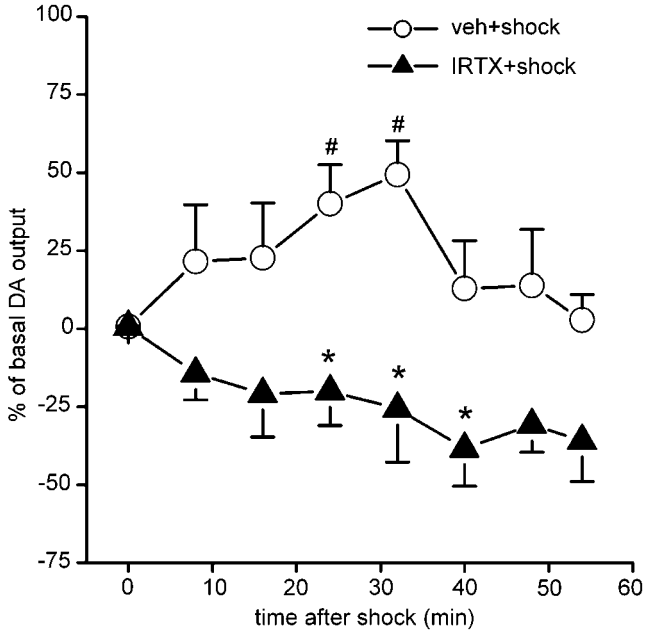

Figure 4 The effects of shock ($n=5$) and IRTX 1 μM + shock (n = 5) on DA release in NAc. Results are expressed as percentage changes (means ± SE) from basal values (3.65 ± 0.43 pg/20 μl). Statistical analyses were performed on raw data. *$p<0.05$ compared with the shock + veh group; #$p<0.05$ compare with the basal values.

within the VTA and on the release of dopamine within the NAc following a noxious stimulus.

It appears that the increased firing rate caused by the TRPV1 agonist, CAPS, is due to an indirect mechanism onto DA neurons. Specifically, it is caused by an enhanced glutamatergic transmission onto these neurons, since the glutamatergic ionotropic antagonists reduced the capsaicin-induced excitatory effect. Interestingly, we also observed a TRPV1-mediated burst-firing pattern in a significant amount of spontaneously active DA neurons (44% of cells) treated with CAPS 3 and 10 μM.

The increase in neuronal firing caused by CAPS has been previously reported in an *in vivo* study in the locus coeruleus (Hajos *et al*, 1987), confirming the increase of adrenergic neurotransmission induced by CAPS (Watanabe *et al*, 2001; Marinelli *et al*, 2002). Collectively, these studies support the idea that stimulation of central TRPV1 can alter the discharge of aminergic neurons in order to modulate more efficiently the release of neurotransmitters into target areas (Manley *et al*, 1992; Suaud-Chagny *et al*, 1992). Moreover, it is generally accepted that bursting events can lead to a much larger DA efflux than a regular spontaneous activity in dopaminergic cells (Gonon, 1988; Garris *et al*, 1994) and, in this regard, we observed that CAPS promotes a transition from single to burst firing. However, enhanced activity in the single-spike mode could also be important in releasing DA, as observed in a previous study (Floresco *et al*, 2003).

The increase in DA efflux within the NAc produced by injection of CAPS into the VTA is consistent with the CAPS-induced changes in firing rate observed in the *in vitro* experiments. It is interesting to note that previous studies have also reported that intranigral injection of CAPS enhances locomotor activity and this could result from an augmented release of DA (Dawbarn *et al*, 1981).

In agreement with a previous report (Yalid *et al*, 2001), a short-lasting tail shock produced a delayed increase of DA release in the NAc. In the present study, this increase was prevented by concomitant intra-VTA administration of the TRPV1 antagonist IRTX, indicating that acute stressful stimuli could impose changes in dopaminergic functions through TRPV1 activation. Noticeably, both the increased firing frequency of DA neurons following superfusion of CAPS and the release of DA caused either by local application of CAPS, or by tail shock, were abolished by the co-application of IRTX. Consistent with previous findings (Marinelli *et al*, 2003), IRTX decreased *per se* the release of DA into the NAc (as shown in Figure 4) and the firing frequency of DA neurons, suggesting a tonic control of the discharge activity by TRPV1. Thus, the pharmacology of the *in vivo* and *in vitro* experiments is consistent with TRPV1-mediated actions.

Our data provide evidence that a peripheral aversive stimulus can activate TRPV1 receptors located in the VTA, where they cause neuronal activation and DA release.

Although the mesolimbic dopamine system is known to play a crucial role in the detection of reward salience and/or the incentive motivation that precedes the earning of reward (Wise, 2004), several reports have demonstrated DA release in response to aversive stimuli in animals and recently also in humans, suggesting an involvement of this neuronal circuit in both appetitive and aversive conditions (Berridge and Robinson, 1998; Pruessner *et al*, 2004). The present observations suggest that the responses to painful stimulation depend upon the activation of TRPV1 receptors within the VTA.

These observations are in agreement with prior studies, which have reported that noxious stimuli applied to the tail increase spike firing (Maeda and Mogenson, 1982; Mantz *et al*, 1989; Gao *et al*, 1990) and *c-fos* proto-oncogene expression in midbrain dopaminergic cells (Ma *et al*, 1993). One of the mechanisms responsible for these changes of firing rate could be an activation of excitatory inputs on DA cells caused by TRPV1 stimulation (Marinelli *et al*, 2003).

It is known that the glutamatergic inputs to the VTA that cause neuronal excitation could arise from the PFC, several subdivisions of amygdaloid complex, and the peduncolopontine nucleus (Jackson and Crossman, 1983; Christie *et al*, 1985; McDonald, 1996). Interestingly, the PFC and the amygdaloid nuclei express TRPV1 (Mezey *et al*, 2000; Szabo *et al*, 2002; Roberts *et al*, 2004) and both areas can be activated by stressful and noxious events (Herman *et al*, 1982; Abercrombie *et al*, 1989; Coco *et al*, 1992), producing changes in the mesolimbic dopamine system that are required for aversive responses (Kim *et al*, 2004).

In conclusion, we have presented for the first time that TRPV1 activation in the brain is related to the dopamine release, following a painful stimulus.

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