TRPV1 Receptor Mediates Glutamatergic Synaptic Input to Dorsolateral Periaqueductal Gray (dl-PAG) Neurons

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Running Head: Capsaicin and Neuronal Activity within Midbrain PAG

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ABSTRACT

The purpose of this study was to determine the role of TRPV1 receptor in modulating neuronal activity of the dorsolateral periaqueductal gray (dl-PAG) through excitatory and inhibitory synaptic inputs. First, whole cell voltage-clamp recording was performed to obtain the spontaneous miniature excitatory postsynaptic currents (mEPSCs) and inhibitory postsynaptic currents (mIPSCs) of the dl-PAG neurons. As 1 μM of capsaicin was applied into the perfusion chamber, the frequency of mEPSCs was increased from 3.21±0.49 to 5.64±0.64 Hz (P<0.05, n=12) without altering the amplitude and the decay time constant of mEPSCs. In contrast, capsaicin had no distinct effect on mIPSCs. A specific TRPV1 receptor antagonist, iodo-resiniferatoxin (i-RTX, 300 nM) decreased the frequency of mEPSCs from 3.51±0.29 to 2.01±0.2 Hz (P<0.05, n=8) but did not alter the amplitude and decay time. In addition, i-RTX applied into the chamber abolished the effect of capsaicin on mEPSC of the dl-PAG. In another experiment, spontaneous action potential of the dl-PAG neurons was recorded using whole cell current-clamp methods. Capsaicin significantly elevated the discharge rate of the dl-PAG neurons from 3.03±0.38 to 5.96±0.87 Hz (n=8). The increased firing activity was abolished in the presence of glutamate NMDA and non-NMDA antagonists, 2-amino-5-phosphonopentanoic acid and 6-cyano-7-nitroquinoxaline-2,3-dione. The results from the current study provide the first evidence indicating that activation of TRPV1 receptors increases the neuronal activity of the dl-PAG via selective potentiation of glutamatergic synaptic inputs.

Key Words: synaptic transmission; glutamate; patch-clamp; midbrain PAG.
INTRODUCTION

The midbrain periaqueductal gray (PAG) is an important neural site for a number of physiological functions related to behavioral reactions, cardiovascular regulation as well as pain modulation (Bandler et al. 1991; Behbehani 1995; Lovick 1996). Among regions of the PAG, the dorsolateral (dl) region receives abundant somatic afferent inputs from the dorsal horn of the spinal cord (Craig 1995; Keay et al. 1997) and also sends descending neuronal projections to the medulla in regulating cardiovascular activity and pain (McGaraughty et al. 2003; Tjen-A-Looi et al. 2006; Verberne and Guyenet 1992). Activation of the dl-PAG contributes to an increase in arterial blood pressure and antinociception (Bandler et al. 1991; Behbehani 1995).

Glutamate, the major excitatory neurotransmitter, appears in the dl-PAG region (Beitz and Williams 1991). The dl-PAG also has the high density of excitatory amino acid binding sites (glutamate receptor subtypes) including a-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)/kainate, N-methyl-d-aspartate (NMDA) and metabotropic receptors (Albin et al. 1990; Cotman et al. 1987).

Transient receptor potential vanilloid type 1 (TRPV1) is widely found on small- and medium-size primary afferent neurons (Guo et al. 1999; Ma 2002) and mediates numerous sensory afferent activations (Caterina et al. 1997; Nault et al. 1999; Smith and McQueen 2001; Zahner et al. 2003). Recent studies have also shown that TRPV1 receptors are located in several regions of the CNS including hypothalamus, midbrain PAG, substantia nigra and locus coeruleus (McGaraughty et al. 2003; Mezey et al. 2000; Toth et al. 2005). Moreover, activation of TRPV1 receptors induces hypoalgesia, similar to the effect of glutamate, and enhances glutamatergic synaptic transmission in the substantia nigra, locus coeruleus and hypothalamus (Marinelli et al. 2003; Marinelli et al. 2002; Sasamura et al. 1998). Although capsaicin microinjected into the
PAG produces antinociception by increasing glutamate release (Palazzo et al. 2002), the effect of TRPV1 receptors on glutamatergic synaptic inputs to the dl-PAG neurons has not specifically been studied using electrophysiological methods.

In this report, therefore, we used an in vitro whole cell recording technique in the midbrain slice to determine the role of TRPV1 in modulating the firing activity of the dl-PAG neurons through the excitatory glutamatergic inputs. We hypothesized that TRPV1 activation would increase discharge of the dl-PAG neurons via potentiation of glutamatergic synaptic inputs.

In addition, GABA-mediated neuronal elements constituting ~50% of the total population of neurons play a crucial role in the intrinsic neuronal circuitry of the PAG (Mugnaini and Oertel 1985; Reichling 1991). The GABA synaptic inputs make up ~50% of the synaptic innervation of the PAG neurons and the majority of GABAergic neurons are tonic active interneurons (Strack et al. 1989). The release of GABA from those neurons may play a role in modulation of the synaptic inputs to the PAG neurons. Studies have further shown that GABA$_A$ receptors are dense within the PAG (Bowery et al. 1987; Chu et al. 1990). Thus the effect TRPV1 activation on the inhibitory GABAergic inputs to the dl-PAG neurons was also examined in this study.

**METHODS**

*Brain Slice Preparations*

All procedures outlined in this study were approved by the Animal Care Committee of Penn State College of Medicine. Sprague Dawley rats of either gender (4-6 weeks old) were anesthetized by inhalation of isoflurane oxygen mixture (5% isoflurane in 100% oxygen), and then were decapitated. Briefly, the brain was quickly removed and placed in ice-cold artificial
cerebral spinal fluid (aCSF) perfusion solution. A tissue block containing the midbrain PAG was cut from the brain and glued onto the stage of the vibratome (Technical Product International, St. Louis, MO). Coronal slices (300 μm) containing the midbrain PAG were dissected from the tissue block in ice-cold aCSF solution. The slices were incubated in the aCSF at 34°C for an equilibrium period of 60 min. The slices were then transferred to the recording chamber. During the procedures described as above, aCSF were saturated with 95% O₂ - 5% CO₂. The aCSF perfusion solution contained (in mM) 124.0 NaCl, 3.0 KCl, 1.3 MgSO₄, 2.4 CaCl₂, 1.4 NaH₂PO₄, 10.0 glucose, and 26.0 NaHCO₃ (Li et al. 2002; Li et al. 2004).

**Electrophysiological Recordings**

**Postsynaptic currents of dl-PAG neurons:** A whole cell voltage-clamp technique was used to record postsynaptic currents in the dl-PAG neurons. Borosilicate glass capillaries (1.2 mm OD, 0.86 mm ID; World Precision Instruments, Sarasota, FL) were pulled to make the recording pipettes using a puller (Sutter Instrument, Novato, CA). The resistance of the pipette was 4–6 MΩ when it was filled with the internal solution (contained in mM: 130.0 potassium gluconate, 1.0 MgCl₂, 10.0 HEPES, 10.0 EGTA, 1.0 CaCl₂, and 4.0 ATP-Mg) (Li et al. 2002; Li et al. 2004). The solution was adjusted to pH 7.25 with 1 M of KOH and osmolarity of 280–300 mOsm. The slice was placed in a recording chamber (Warner Instruments, Hamden, CT) and fixed with a grid of parallel nylon threads supported by a U-shaped stainless steel weight. The aCSF saturated with 95% O₂ - 5% CO₂ was perfused into the chamber at 3.0 ml/min. The temperature of the perfusion solution was maintained at 34°C by an in-line solution heater with a temperature controller (Model TC-324; Warner Instruments). Whole cell recordings from the dl-PAG neurons were performed visually using differential interference contrast (DIC) optics on an upright microscope (BX50WI, Olympus, Tokyo, Japan). The tissue image was captured and
enhanced through a camera and displayed on a video monitor. A tight giga-ohm seal was subsequently obtained in the dl-PAG neuron viewed using DIC optics. A 5-10 min equilibration period was allowed after whole cell access was established and the recording reached a steady state. The recording was abandoned if the monitored input resistance changed >15%.

Postsynaptic currents were then recorded.

The miniature excitatory postsynaptic currents (mEPSCs) were recorded in the presence of 1 μM of TTX and 20 μM of bicuculline (a GABA<sub>A</sub> receptor antagonist) at a holding potential of -70 mV. The miniature inhibitory postsynaptic currents (mIPSCs) were recorded in the presence of 1 μM of tetrodotoxin (TTX) and 20 μM of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, a non-NMDA receptor antagonist) at a holding potential of 0 mV.

**Spontaneous action potentials of dl-PAG neurons:** A whole cell current-clamp technique was used to record the spontaneous firing activity of the dl-PAG neurons. The recording procedures were described as above. It is noted that TTX was not used in this experiment. Recordings of the firing activity from the dl-PAG neurons began 5-10 min after the whole cell access was established and the firing activity reached a steady state.

All the drugs including TTX, bicuculline, CNQX, 2-amino-5-phosphonopentanoic acid (AP-5), capsaicin and iodo-resiniferatoxin (i-RTX, a specific TRPV1 antagonist) were obtained from Sigma Co, and dissolved in the aCSF solution immediately before they were used. According to experimental protocol, the drugs were delivered into the recording chamber at final concentrations using syringe pumps during the experiment (Li et al. 2004).

**Data Acquisition and Analysis**

Signals were recorded with a MultiClamp 700B amplifier (Axon Instruments, Foster City, CA), digitized at 10 kHz with a DigiData 1322, and filtered at 1-2 kHz and saved in a PC-
based computer using pClamp 9.0 software (Axon Instruments). A liquid junction potential of -15.0 mV (for the potassium gluconate pipette solution) was corrected during off-line analysis (Li et al. 2002; Li et al. 2004). The mEPSCs, mIPSCs, and firing activities of the PAG neurons were analyzed off-line with a peak detection program (MiniAnalysis, Synaptosoft, Leonia, NJ). Detection of events was accomplished by setting a threshold above the noise level. The distribution of cumulative probability of the inter-event interval and amplitude of mEPSCs and mIPSCs was estimated using the Komogorov–Smirnov test (Li et al. 2002; Li et al. 2004). Experimental data (frequency, amplitude and decay time of mEPSCs and mIPSCs, and the firing rate of dl-PAG neurons) were analyzed with one-way ANOVA. Tukey’s post hoc analyses were utilized to determine differences between groups, as appropriate. All values were expressed mean ± SE. For all analyses, differences were considered significant at \( P < 0.05 \). All statistical analyses were performed using SPSS for windows version 13.0.

**RESULTS**

At the end of each experiment, the recording site in the PAG tissue was examined. It was confirmed that all the cells included for data analysis in this experiment located in the dl-PAG (anterior-posterior coordinates from AP: -7.1 to -7.9) (Swanson 1998). Whole cell patch-clamp experiments were performed and experimental data were collected from 55 dl-PAG neurons. The resting membrane potential was -66.7±2.9 mV (from -78.5 to -60.8 mV) and amplitude of action potential was >60 mV. The input resistance was 511.7±11.2 MΩ (from 320.1 to 678.6 MΩ).

*Effect of Capsaicin on Glutamatergic mEPSCs*
The spontaneous mEPSCs were recorded in the dl-PAG in order to determine the effect of TRPV1 activation on synaptic glutamate release onto the neurons. The mEPSCs were recorded in the presence of 1 μM TTX and 20 μM bicuculline. Capsaicin (1μM) was perfused into the recording chamber (n=12). This significantly increased the frequency of mEPSCs from 3.21±0.49 to 5.64±0.64 Hz (P<0.05), but did not alter the amplitude and the decay time constant of mEPSCs in all neurons tested (Fig. 1). The mEPSCs recovered during washout of the perfusion solution and were completely abolished by blocking non-NMDA glutamate receptors after bath application of 20 μM CNQX (Fig. 1A).

The effect of capsaicin on mEPSCs was analyzed by measuring the time constant of the decay phase of mEPSCs. The decay time constant of mEPSCs after application of capsaicin (3.32±0.50 ms) was not different from the control (3.56±0.42 ms, P>0.05 vs capsaicin). As average of 100 consecutive mEPSCs was superimposed during control and capsaicin application, the decay phase of mEPSCs was best fitted by a single exponential function (Fig. 1B). The cumulative probability analysis of mEPSCs shows that the distribution pattern of the inter-event interval of mEPSCs shifted toward the left but the distribution pattern of the amplitude was not altered as capsaicin was applied (Fig. 1C&D). Average data further show the effect of capsaicin on the frequency and amplitude of mEPSC of the dl-PAG neurons (Fig. 1E&F).

**Effect of i-RTX on Glutamatergic mEPSCs**

To determine tonic effect of endogenous TRPV1 activation on glutamatergic inputs to the dl-PAG neurons, 300 nM of i-RTX was applied into the recording chamber and mEPSCs were then examined (n=8). i-RTX alone decreased the frequency of mEPSCs from 3.51±0.29 to 2.01±0.2 Hz (P <0.05) without affecting the amplitude and decay time constant (Fig. 2A-F). The inhibitory effect of i-RTX on the frequency of mEPSCs was no longer present after washout.
In order to confirm that the effect of capsaicin on mEPSCs was mediated via TRPV1 receptor, i-RTX was perfused prior to capsaicin application (n=10). Our results show that capsaicin failed to increase the frequency of mEPSCs of the dlPAG neurons in the presence of 300 nM of i-RTX (Fig. 3A-E).

Effect of Capsaicin on GABAergic mIPSCs

The spontaneous mIPSCs were recorded in the dl-PAG neurons in the presence of 1 μM TTX and 20 μM CNQX (n=8). Capsaicin, in a concentration of 1μM, did not produce a significant effect on the frequency and amplitude of mIPSCs in the dl-PAG neurons (Fig. 4). The mIPSCs were completely eliminated after bath application of 20 μM of bicuculline, blocking GABA_\text{A} receptors (Fig. 4A).

The effect of capsaicin on mIPSCs was analyzed by measuring the time constant of the decay phase of mIPSCs. As average of 100 consecutive mIPSCs was superimposed during control and capsaicin application, the decay time constant of mIPSCs was best fitted by a double exponential function (Fig. 4B). The decay time constant was similar during control (16.38±0.91 ms) and during capsaicin application (17.02±1.09 ms, P>0.05 vs. control). The cumulative probability analysis also shows that capsaicin did not alter the distribution pattern of either the inter-event interval or the amplitude of the mIPSCs (Fig. 4C&D). Average data further show capsaicin had no effect on the frequency and amplitude of mIPSCs of the dl-PAG neurons (Fig. 4E&F).

Effect of Capsaicin on Discharge of dl-PAG Neurons

It was likely that capsaicin enhanced the activity of the dl-PAG neurons because capsaicin increased the excitatory glutamatergic inputs to the dl-PAG neurons without altering
the inhibitory GABAergic synaptic activity. To test this hypothesis, the effect of capsaicin on
the discharge of the dl-PAG neurons was examined using whole cell current-clamp recordings
(n=8). Capsaicin (1 μM) increased the discharge rate of the dl-PAG neurons from 3.03±0.38 to
5.96±0.87 Hz (P<0.05). Application of capsaicin did not significantly alter the resting
membrane potential of the dl-PAG neurons. The effect of capsaicin is shown in Fig. 5A-C.

In addition, the role of the glutamatergic synaptic inputs and glutamate receptors in
capsaicin activation of the dl-PAG neurons was determined (n=9). The firing activities of the dl-
PAG neurons were examined in the presence of glutamate NMDA and non-NMDA antagonists,
AP5 and CNQX, following application of capsaicin (Fig. 5D-F). The spontaneous discharge
activities of the PAG neurons were slightly decreased following perfusion of 20 μM of CNQX
and 50 μM of AP-5 (4.24±0.61 vs. 3.46±0.67 Hz, P>0.05). Subsequent application of 1 μM of
capsaicin failed to increase the spontaneous neuronal activities of in the presence of CNQX and
AP-5.

DISCUSSION

In the present study, in vitro PAG slice preparation allowed us to determine regulatory
actions of TRPV1 activation on excitatory glutamatergic and inhibitory GABAergic synaptic
activity in the dl-PAG (Kabashima et al. 1997; Ozaki et al. 2000; Sulzer and Pothos 2000). Our
results have demonstrated that bath application of capsaicin significantly increased the frequency
of mEPSCs of the dl-PAG neurons, but had no distinct effect on the amplitude of mEPSCs (Fig.
1). These data suggest that TRPV1 activation increased the synaptic glutamate release in the
PAG and the site of the action was likely at the presynaptic glutamatergic terminals (Sulzer and
Pothos 2000). Furthermore, the effect of capsaicin on mEPSCs of the dl-PAG was completely
eliminated by the specific TRPV1 receptor antagonist, i-RTX (Fig. 3), suggesting that mEPSC enhancement of capsaicin was due to TRPV1 receptors. In addition, i-RTX alone decreased the frequency of mEPSCs of the dl-PAG neurons (Fig. 2). This indicates that endogenous TRPV1 receptors tonically influenced glutamatergic inputs to the dl-PAG neurons.

In contrast to its actions on glutamatergic mEPSCs, capsaicin had no distinct effect on the frequency and amplitude of GABAergic mIPSCs recorded from the dl-PAG neurons (Fig. 4). This suggests the lack of TRPV1 effect on the synaptic GABAergic terminals. The similar action of TRPV1 on glutamatergic and GABAergic neurotransmission has been reported in the substantia nigra, locus coeruleus and paraventricular nucleus in perfused brain slices (Li et al. 2004; Marinelli et al. 2003; Marinelli et al. 2002).

In addition, in this report we have also found that capsaicin significantly increased the discharge activity of the dl-PAG neurons (Fig. 5). This effect was abolished after blockade of NMDA and non-NMDA receptors with the prior application of AP-5 and CNQX (Fig. 5), indicating that TRPV1 activation elevated the activity of the dl-PAG neurons through augmentation of the excitatory glutamatergic synaptic inputs. It should be noted that AP-5 and CNQX did not significantly alter the firing activity in the dl-PAG neurons in the present experiment. This suggests that the tonic glutamatergic inputs might not be sufficient to alter the neuronal activity of the dl-PAG.

A prior study has shown that capsaicin injected into the PAG produces antinociception and the effect is prevented by pretreatment with a TRPV1 antagonist, capsazepine (McGaraughty et al. 2003; Palazzo et al. 2002). TRPV1 receptor activation in the dl-PAG neurons affects neuronal activity of the rostral ventromedial medulla and contributes to descending modulation of nociception (McGaraughty et al. 2003). The antinociceptive effect induced by capsaicin is
also attenuated after a blockade of glutamate receptors within the PAG (Palazzo et al. 2002). Speculatively, the glutamate release is increased after TRPV1 activation and postsynaptic glutamate receptors are then activated. Activation of glutamate receptors in the PAG has been reported to produce analgesia (Maione et al. 1998; Maione et al. 2000). The results from our current study provide, for the first time, electrophysiological evidence that 1) TRPV1 activation within the dl-PAG neurons increases the spontaneous firing rate of the PAG cells; and 2) the excitatory action of TRPV1 on the neuronal activity is likely to be mediated via the synaptic glutamate release and activation of glutamate receptors.

A large body of evidence has suggested that the dl-PAG has descending neuronal projections to crucial cardiovascular areas in the rostral ventrolateral medulla and plays an important role in regulating cardiovascular activity (Tjen-A-Looi et al. 2006; van Bockstaele et al. 1991; van der Plas et al. 1995; Verberne and Guyenet 1992). Stimulation of the dl-PAG elicits potent increases in arterial blood pressure, heart rate and sympathetic nerve discharge (Bandler et al. 1991). Studies have further shown that glutamate and glutamate-positive terminals appear throughout the PAG (Azkue et al. 1998). Glutamate and its receptors play a role in cardiovascular regulation in the PAG (Hall and Behbehani 1998; Maione et al. 1994). For example, increased glutamate in the dl-PAG elevates blood pressure and the effect is significantly reduced by a pretreatment with AP-5 (Maione et al. 1994). The PAG is also involved in integrating cardiovascular responses to activation of somatic afferent and arterial baroreflex inputs (Li 2004; Li and Mitchell 2000; Li and Mitchell 2003). It has been reported that glutamate is accumulated in synaptic terminals ascending from the spinal cord to the PAG (Azkue et al. 1998). Furthermore, activation of somatic afferent inputs increases glutamate concentration in the dl-PAG (Li and Mitchell 2003). Our current study suggests that TRPV1
activation enhances excitatory glutamatergic synaptic activity in the dl-PAG. Thus it is reasoned that TRPV1 receptors within the PAG may play a role in modulating cardiovascular responses during activation of somatic sensory afferents via glutamate release.

A significant finding from the present study is that i-RTX decreased the mEPSCs of the dl-PAG neurons. The result suggests that TRPV1 receptors exert a tonic influence on glutamate release within the dl-PAG via endogenous factors. A number of endogenous capsaicin-like substances (i.e. proton, anandamide and 12-hydroperoxyeicosatetraenoic acid) have been identified to activate and/or potentiate the activity of TRPV1 receptors (Hwang et al. 2000; Ryu et al. 2003; Zygmunt et al. 1999). For instance, evidence has shown that electrical stimulation of the dorsal regions of the PAG produces analgesia accompanied by a marked increase in the release of anandamide in the PAG (Walker et al. 1999). In the PAG, enhanced level of endogenous anandamide by inhibition of fatty acid amide hydrolase induces both CB1- and TRPV1-mediated analgesia (Maione et al. 2006). However, it is noted that a higher dosage of anandamide is necessary to activate TRPV1 receptors as compared with activation of CB1 receptors (Luo et al. 2002; Morisset and Urban 2001). A precise mechanism by which TRPV1 receptors are activated by endogenous factors needs to be determined.

It has generally been accepted that the glutamate increase by TRPV1 activation results from an increase in intraterminal Ca²⁺ concentration through Ca²⁺ influx into the nerve terminal, as the effect can be abolished after removal of extracellular Ca²⁺ (Li et al. 2004; Marinelli et al. 2002). Thus we speculate that the increased frequency of mEPSCs of the dl-PAG by capsaicin was due to enhanced Ca²⁺ concentration in the nerve terminals in the present study.

Finally, previous studies have shown that there exist TRPV1 receptors on presynaptic nerve terminals in the superficial dorsal horn of the spinal cord and hypothalamus (Guo et al.
Whether TRPV1 receptors are localized on glutamatergic terminals of presynaptic sites has not, to our knowledge, been reported although TRPV1 immunoreactivity and mRNA have been identified in the dl-PAG (McGaraughty et al. 2003; Roberts et al. 2004). Our data from the current experiment demonstrated that activation of TRPV1 receptors on presynaptic site increased glutamate release. This provides electrophysiological evidence that TRPV1 is likely to locate on presynaptic nerve terminals in the dl-PAG. It has been reported that there are neurons with TRPV1 immunostaining in the dl-PAG and capsaicin injected into the dl-PAG induces analgesia (McGaraughty et al. 2003). Thus TRPV1 receptors speculatively exist on postsynaptic membrane. In the present study, our purpose was to determine if activation of TRPV1 receptors would release glutamate from presynaptic nerve terminals. The data from the current experiment support our hypothesis. Furthermore, our results show that the elevated glutamate stimulated glutamate NMDA and non-NMDA receptors on the dl-PAG neurons and increased neuronal activity. Glutamate appears within the dl-PAG as a major excitatory neurotransmitter (Beitz and Williams 1991). We believe that neurons tested in this experiment were likely glutamatergic. Nonetheless, those neurons had NMDA and non-NMDA receptors and were responsive to glutamate. The responsiveness of postsynaptic TRPV1 receptors in the dl-PAG to capsaicin requires additional experiments to be determined.

In summary, capsaicin significantly increases the frequency of glutamatergic mEPSCs but not GABAergic mIPSCs of the dl-PAG neurons via activation of TRPV1 receptors. The increased glutamatergic synaptic inputs augment the discharge of PAG neurons since this effect by capsaicin is blocked by glutamate NMDA and non-NMDA receptor antagonists. Our data suggest a mechanism by which TRPV1 modulates neuronal activity in the dl-PAG via synaptic glutamate. The current study provides new information that the dl-PAG could be an important
supraspinal site to be involved in TRPV1-related modulation of physiological functions.

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REFERENCES


FIGURE LEGENDS

**Figure 1.** TRPV1 activation increased the frequency of glutamatergic mEPSCs of the dl-PAG neurons. The effect was observed in 12 neurons tested. *A:* Representative tracings from a dl-PAG neuron show that 1 μM of capsaicin increased the frequency of spontaneous mEPSCs as compared with control, and that the mEPSCs recovered during washout. It is noted that the mEPSCs were completely abolished in the presence of 20 μM of CNQX, a non-NMDA receptor antagonist. *B:* Average of 100 consecutive mEPSCs was superimposed during control and capsaicin application. The decay phase of mEPSCs was best fitted by a single exponential function. The decay phase of mEPSCs was identical in control and during capsaicin application. *C&D:* The cumulative probability analysis shows that capsaicin decreased the inter-event interval of mEPSCs but did not alter the distribution pattern of the amplitude of the mEPSCs. *E&F:* Average data show the effect of capsaicin on the frequency and amplitude of mEPSCs of the dl-PAG neurons. *P* < 0.05, vs. control and washout.

**Figure 2.** Effect of TRPV1 blockade on glutamatergic mEPSCs of the dl-PAG (n=8). *A:* Representative tracings from a dl-PAG neuron show that 300 nM of i-RTX (a specific TRPV1 receptor antagonist) decreased the frequency of spontaneous mEPSCs and that the mEPSCs recovered during washout. *B:* The decay phase of mEPSCs was identical in control and during i-RTX application. *C&D:* The cumulative probability analysis shows that i-RTX increased the inter-event interval of mEPSCs but did not alter the distribution pattern of the amplitude of the mEPSCs. *E&F:* Average data show the effect of TRPV1 blockade with i-RTX on the frequency and amplitude of mEPSCs of the dl-PAG neurons. *P* < 0.05, vs. control and washout.
**Figure 3.** Effect of capsaicin on the mEPSCs of the dl-PAG neurons after application of a specific TRPV1 receptor antagonist, i-RTX (n=10). 

A: Representative tracings from a dl-PAG neuron show that 300 nM of i-RTX decreased the frequency of spontaneous mEPSCs and that i-RTX abolished effect of 1 μM of capsaicin. 

B&C: The cumulative probability analysis shows that distribution patterns of neither the inter-event interval nor the amplitude of the mEPSCs was altered by capsaicin after application of i-RTX as compared with i-RTX alone. 

D&E: Average data show the effects of i-RTX and i-RTX plus capsaicin on the frequency and amplitude of mEPSC of the dl-PAG neurons. *P<0.05, vs. control.

**Figure 4.** Effect of TRPV1 activation on GABAergic mIPSCs of the dl-PAG neurons (n=8). 

A: Representative tracings from a dl-PAG neuron show the frequency of spontaneous mIPSCs was not altered by bath application of 1μM capsaicin and that the mIPSCs were completely abolished in the presence of 20 μM of bicuculline, a GABA<sub>A</sub> receptors antagonist. 

B: The decay time constant of mIPSCs was similar during control and during capsaicin application. 

C&D: The cumulative probability analysis shows that capsaicin did not alter the distribution patterns of the inter-event interval and the amplitude of the mIPSC. 

E&F: Average data show capsaicin had no effect on the frequency and amplitude of mIPSC of the dl-PAG neurons.

**Figure 5.** Capsaicin had an excitatory effect on the firing activity of the dl-PAG neurons. 

A: A histogram shows the time course of 1 μM of capsaicin effect in a dl-PAG neuron. 

B: Original tracings from a dl-PAG neuron show the spontaneous discharge activity during control, capsaicin perfusion and washout. 

C: Average data (n=8). *P<0.05, vs. control and recovery. 

D: The time course shows that the increased firing activity of a dl-PAG neuron by capsaicin was
abolished by blocking glutamate NMDA and non-NMDA receptors following perfusion of AP-5 (50 μM) and CNQX (20 μM). E: Original tracings from a dl-PAG neuron show the spontaneous discharge activity during control, CNQX plus AP-5 and capsaicin perfusion with glutamate receptors blockade. F: Average data (n=9) show that effect of TRPV1 activation was blunted after CNQX and AP-5 application.
Figure 1
Figure 2
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