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OT inhibits ASICs in primary sensory neurons

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## RESEARCH PAPER

# Oxytocin inhibits the activity of acid-sensing ion channels through the vasopressin, V<sub>1A</sub> receptor in primary sensory neurons

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### Keywords

oxytocin; acid-sensing ion channel; proton-gated current; vasopressin, V<sub>1A</sub> receptor; dorsal root ganglion neuron; electrophysiology; pain

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## BACKGROUND AND PURPOSE

A growing number of studies have demonstrated that oxytocin (OT) plays an analgesic role in modulation of nociception and pain. Most work to date has focused on the central mechanisms of OT analgesia, but little is known about whether peripheral mechanisms are also involved. Acid-sensing ion channels (ASICs) are distributed in peripheral sensory neurons and participate in nociception. Here, we investigated the effects of OT on the activity of ASICs in dorsal root ganglion (DRG) neurons.

## EXPERIMENTAL APPROACH

Electrophysiological experiments were performed on neurons from rat DRG. Nociceptive behaviour was induced by acetic acid in rats and mice lacking vasopressin, V<sub>1A</sub> receptors.

## KEY RESULTS

OT inhibited the functional activity of native ASICs. Firstly, OT dose-dependently decreased the amplitude of ASIC currents in DRG neurons. Secondly, OT inhibition of ASIC currents was mimicked by arginine vasopressin (AVP) and completely blocked by the V<sub>1A</sub> receptor antagonist SR49059, but not by the OT receptor antagonist L-368899. Thirdly, OT altered acidosis-evoked membrane excitability of DRG neurons and significantly decreased the amplitude of the depolarization and number of action potentials induced by acid stimuli. Finally, peripherally administered OT or AVP inhibited nociceptive responses to intraplantar injection of acetic acid in rats. Both OT and AVP also induced an analgesic effect on acidosis-evoked pain in wild-type mice, but not in V<sub>1A</sub> receptor knockout mice.

## CONCLUSIONS AND IMPLICATIONS

These results reveal a novel peripheral mechanism for the analgesic effect of OT involving the modulation of native ASICs in primary sensory neurons mediated by V<sub>1A</sub> receptors.

## Abbreviations

ASIC, acid-sensing ion channels; AVP, arginine vasopressin; DRG, dorsal root ganglion;  $I_{pH}$ , proton-gated current; OT, oxytocin; TRPV1, transient receptor potential vanilloid channel type 1; TTX, tetrodotoxin; V<sub>1A</sub> receptor, vasopressin type 1A receptor; V<sub>1A</sub><sup>-/-</sup> mice, V<sub>1A</sub> receptor knockout mice; WT, wild-type

## Introduction

Acid-sensing ion channels (ASICs) are members of proton-gated cation channels and are expressed in both central and peripheral nervous systems (Waldmann *et al.*, 1997b). In peripheral sensory neurons, ASICs have been found on cell bodies and sensory terminals, where they have been suggested to be important for nociception (Alvarez de la Rosa *et al.*, 2002; Benson *et al.*, 2002; Wemmie *et al.*, 2013). As pH sensors, ASICs are activated by a decrease in extracellular pH and depolarize the terminals of nociceptive primary sensory neurons to trigger pain sensation. Direct perfusion of acidic solutions into the skin causes pain in humans (Steen *et al.*, 1995; Ugawa *et al.*, 2002; Jones *et al.*, 2004). Protons, canonical ligands for ASICs, are released and cause tissue acidosis under multiple pathological conditions such as inflammation, tissue injury, ischaemic stroke and cancer (Deval *et al.*, 2010). It is well known that the local extracellular pH levels drop to 5.4 in acute inflammation and 6.3 or lower in severe ischaemia (Smith *et al.*, 1986; Steen *et al.*, 1992; Kweon and Suh, 2013). The accumulating protons are sufficient to activate nociceptors by activating proton-sensitive ion channels such as ASICs and transient receptor potential vanilloid receptors type 1 (TRPV1: for nomenclature see Alexander *et al.*, 2013b) (Steen *et al.*, 1992; Frey Law *et al.*, 2008). Studies have suggested that ASICs, rather than TRPV1, mainly mediate pain sensation induced by acid injection, because the pain sensation is significantly attenuated by the non-selective ASIC inhibitor amiloride (Ugawa *et al.*, 2002; Wemmie *et al.*, 2006; Deval *et al.*, 2008). It has been shown that inflammation and tissue injury increase the expression levels of ASIC mRNA in dorsal root ganglion (DRG) neurons, which contribute to hyperalgesia (Voilley *et al.*, 2001; Hori *et al.*, 2010; Chen *et al.*, 2011). Together, increasing evidence has shown that ASICs play an important role in various pain conditions such as inflammatory pain and post-operative pain (Deval *et al.*, 2011; Wemmie *et al.*, 2013).

Oxytocin (OT) is a nine amino acid neuropeptide that is synthesized in the paraventricular and supraoptic nuclei of the hypothalamus (Sofroniew, 1983; Viero *et al.*, 2010). OT is released into the CNS as a modulator of neuronal transmission. Additionally, it is also transported down axons of the posterior pituitary and is secreted into bloodstream as a neurohormone. OT is best known for its roles in female parturition and lactation (Gimpl and Fahrenholz, 2001). Some evidence has suggested that OT plays an important role in pain modulation and nociceptive transmission (Rash *et al.*, 2013). OT has been found to have analgesic effects in a variety of pain tests when administered into the brain and the spinal cord (Yu *et al.*, 2003; Gao and Yu, 2004; Miranda-Cardenas *et al.*, 2006; Condes-Lara *et al.*, 2007). Up to now, most studies have focused on the spinal cord as the site of OT analgesia. In the spinal cord, OT may inhibit nociceptive neuronal responses indirectly by activating inhibitory GABA interneurons, or directly by inhibiting second-order neurons (Robinson *et al.*, 2002; Rojas-Piloni *et al.*, 2007; Breton *et al.*, 2009; Condes-Lara *et al.*, 2009). Recently, Hobo *et al.* (2012) reported that OT targets central terminals of primary sensory afferents and may reduce neurotransmitter release. It has been shown that spinal release or intrathecal injection of OT can relieve both acute and chronic pain in human and rodent

species (Rash *et al.*, 2013). OT also exerts an analgesic effect after systemic administration (Lundeberg *et al.*, 1994; Schorscher-Petcu *et al.*, 2010). However, it is not known whether the hormonal effect of OT on nociceptive processing occurs in peripheral terminals of primary sensory afferents.

We report here that OT inhibited the activity of ASICs in DRG neurons and relieved acidosis-evoked pain when administered peripherally. Moreover, the effects of OT on ASICs were mimicked by arginine vasopressin (AVP) and mediated by the vasopressin type1A  $V_{1A}$  receptor (for nomenclature see Alexander *et al.*, 2013a), but not by the OT receptor. These conclusions were further confirmed by transgenic knockout mice lacking  $V_{1A}R$ . Peripherally administered OT and AVP failed to influence nociceptive behaviours induced by acidosis in  $V_{1A}R$  knockout ( $V_{1A}^{-/-}$ ) mice.

## Methods

### Isolation of the DRG neurons

The experimental protocol was approved by the animal research ethics committee of Hubei University of Science and Technology. All procedures conformed to international guidelines on the ethical use of animals, and every effort was made to minimize the number of animals used and their suffering. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Ten- to 12-week-old Sprague-Dawley female rats were anaesthetized with ethyl ether and then decapitated. The DRGs were taken out and transferred immediately into DMEM (Sigma-Aldrich, St. Louis, MO, USA) at pH 7.4. After the removal of the surrounding connective tissues, the DRGs were minced with fine spring scissors and the ganglion fragments were placed in a flask containing 5 mL of DMEM in which trypsin (type II-S, Sigma-Aldrich)  $0.5 \text{ mg}\cdot\text{mL}^{-1}$ , collagenase (type I-A, Sigma-Aldrich)  $1.0 \text{ mg}\cdot\text{mL}^{-1}$  and DNase (type IV, Sigma-Aldrich)  $0.1 \text{ mg}\cdot\text{mL}^{-1}$  had been dissolved, and incubated at  $35^{\circ}\text{C}$  in a shaking water bath for 25–30 min. Soybean trypsin inhibitor (type II-S, Sigma-Aldrich)  $1.25 \text{ mg}\cdot\text{mL}^{-1}$  was then added to stop trypsin digestion. Dissociated neurons were placed into a 35 mm Petri dish and kept for at least another 60 min before electrophysiological recordings. The neurons selected for the electrophysiological experiments were 15–35  $\mu\text{m}$  in diameter.

### Electrophysiological recordings

Whole-cell patch-clamp and voltage-clamp recordings were carried out at room temperature ( $22\text{--}25^{\circ}\text{C}$ ) using a MultiClamp-700B amplifier and Digidata-1440A A/D converter (Axon Instruments, Foster City, CA, USA). Recording pipettes were pulled using a Sutter P-97 puller (Sutter Instruments, Novato, CA, USA). The micropipettes were filled with internal solution containing KCl 140 mM,  $\text{MgCl}_2$  2.5 mM, HEPES 10 mM, EGTA 11 mM and ATP 5 mM; its pH was adjusted to 7.2 with KOH, and osmolarity was adjusted to  $310 \text{ mOsm}\cdot\text{L}^{-1}$  with sucrose. Cells were bathed in an external solution containing NaCl 150 mM, KCl 5 mM,  $\text{CaCl}_2$  2.5 mM,  $\text{MgCl}_2$  2 mM, HEPES 10 mM and d-glucose 10 mM; its osmolarity was adjusted to  $330 \text{ mOsm}\cdot\text{L}^{-1}$  with sucrose

and pH to 7.4. The resistance of the recording pipette was in the range of 3–6 M $\Omega$ . A small patch of membrane underneath the tip of the pipette was aspirated to form a gigaseal and then negative pressure was applied to rupture it, thus establishing a whole-cell configuration. The adjustment of capacitance compensation and series resistance compensation was performed before recording the membrane currents. The membrane voltage was maintained at –60 mV in all voltage-clamp experiments unless otherwise specified. Current-clamp recordings were obtained by switching to current-clamp mode after a stable whole-cell configuration was formed in voltage-clamp mode. Only cells with a stable resting membrane potential (more negative than –50 mV) were used in the study. Signals were sampled at 10–50 kHz and filtered at 2–10 kHz, and the data were stored in compatible personal computer (PC) for off/online analysis using the pCLAMP 10 acquisition software (Axon Instruments).

### Drug application

All chemicals were purchased from Sigma-Aldrich. The drugs used were hydrochloric acid, OT, AVP, amiloride, APETx2, SR49059, L-368899, YM-254890, capsazepine and tetrodotoxin (TTX). Stocks of drugs were made up and diluted daily in the external solution at a minimum of 1:1000 to a final working concentration. Next, they were held in a linear array of fused silica tubes (o.d./i.d. = 500/200  $\mu$ m) connected to a series of independent reservoirs. The application pipette tips were positioned 30  $\mu$ m away from the recorded neurons. The application of each drug was driven by gravity and controlled by the corresponding valve, and rapid solution exchange could be achieved within about 100 ms by shifting the tubes horizontally with a PC-controlled micromanipulator. Cells were constantly bathed in normal external solution flowing from one tube connected to a larger reservoir between drug applications. To functionally characterize ASIC activity, we used capsazepine (10  $\mu$ M) to block TRPV1 channels (Poirot *et al.*, 2006).

### Animals and nociceptive behaviour induced by acetic acid

All behavioural measurements were performed using Sprague-Dawley rats (female, 10–12 weeks old), littermate wild type (WT) and  $V_{1A}^{-/-}$  mice (female, 6–8 weeks old, and in C57BL/6 background). The generation of  $V_{1A}^{-/-}$  mice by homologous recombination has been described previously (Hu *et al.*, 2003). All animals were kept on a 12 h light/dark cycle at a temperature of 22–24°C and humidity of 50–60%; and with *ad libitum* access to food and water. Animals were placed in a 30  $\times$  30  $\times$  30 cm<sup>3</sup> Plexiglas chamber (ProBeCare, Wuhan, China) and allowed to habituate for at least 30 min before nociceptive behaviour experiments. After pretreatment with 10  $\mu$ L capsazepine (100  $\mu$ M), a double-blind experiment was carried out. In rats, 20  $\mu$ L of acetic acid solution (0.6%) together with 20  $\mu$ L of external solution, amiloride, OT, AVP and SR 49059 were coded, and the other experimenters administered them s.c. into the dorsal face of the hind paw using a 30-gauge needle connected to a 100  $\mu$ L Hamilton syringe (Hamilton Co., Reno, NV, USA). In mice, the volume per injection was 10  $\mu$ L. Nociceptive behaviour (i.e. number of flinches) was counted over a 5 min period starting immediately after the injection (Deval *et al.*, 2008; Omori *et al.*, 2008).

### Data analysis

Data were statistically compared using Student's *t*-test or ANOVA, followed by Bonferroni's *post hoc* test. Statistical analysis of concentration–response data was performed using non-linear curve-fitting program ALLFIT. Data are expressed as mean  $\pm$  SEM.

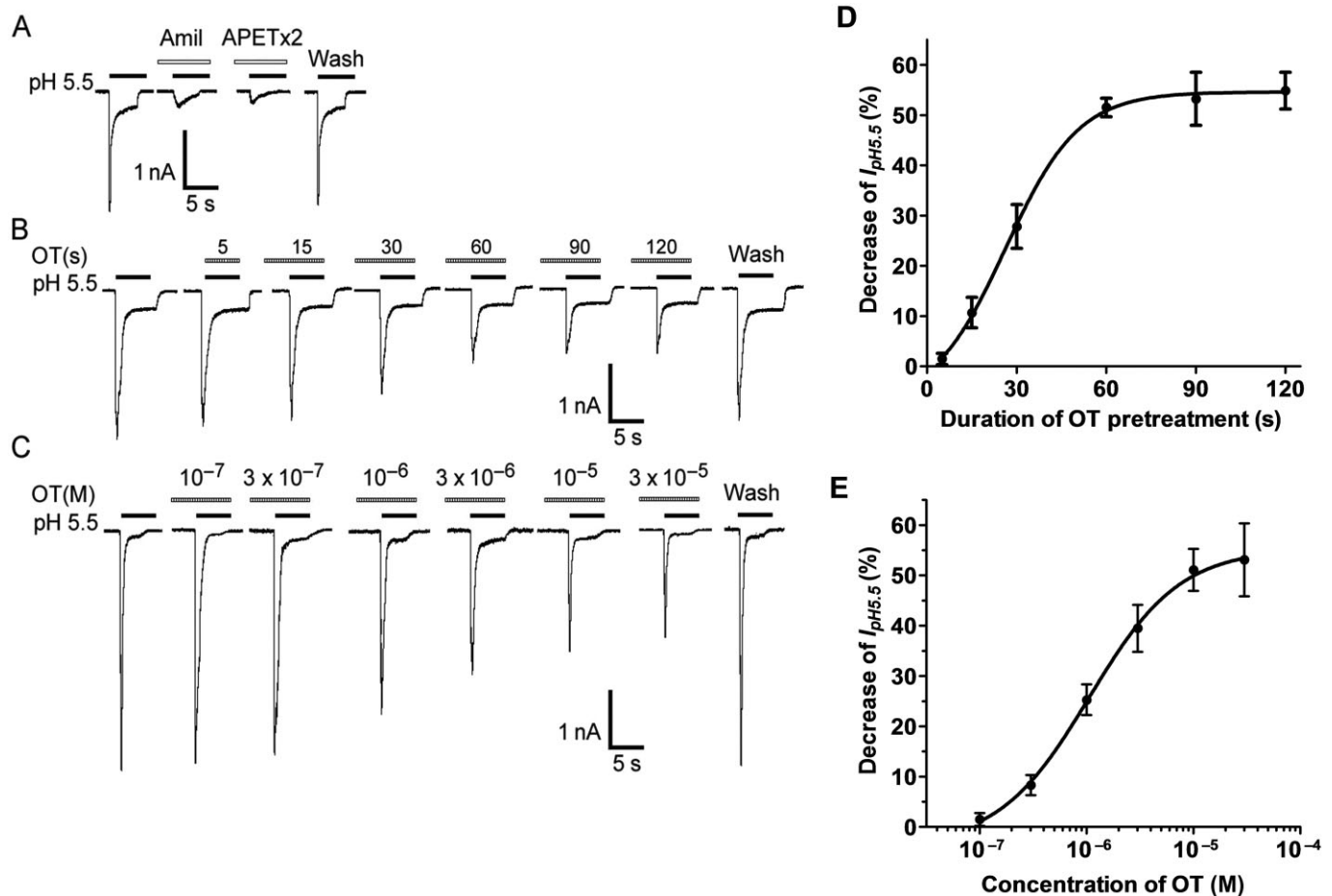
## Results

### OT decreased ASIC currents in rat DRG neurons

All current measurements in this study were performed in small and medium diameter (15–35  $\mu$ m) acutely isolated DRG neurons of adult female rats. To functionally characterize ASIC currents, we measured proton-gated currents ( $I_{pH}$ ) in the presence of capsazepine (10  $\mu$ M) to block proton-induced TRPV1 activation in the whole-cell patch-clamp configuration (Gavva *et al.*, 2005; Poirot *et al.*, 2006). As our previous report showed (Qiu *et al.*, 2012), a rapid reduction of extracellular pH from 7.4 to 5.5 for 5 s evoked an inward current ( $I_{pH\ 5.5}$ ) in most native DRG neurons (79.41%, 135/170). ASICs are the only known channels that mediate the  $I_{pH}$  in the presence of the TRPV1 inhibitor capsazepine, as all observed  $I_{pH}$  could be completely blocked by 100  $\mu$ M of amiloride, a broad-spectrum ASIC channel blocker (Figure 1A). We thus considered them to be ASIC currents because they existed in the presence of capsazepine to block proton-induced TRPV1 activation. Most (72.59%, 98/135) of these acid-evoked currents may be ASIC3-like currents (Figure 1A), which can be characterized by a large transient current followed by fast inactivation and then a small sustained current with no or very slow inactivation (Wang *et al.*, 2013). APETx2, a sea anemone peptide, blocks ASIC3 homomeric and heteromeric channels both in transfected cells and rat primary sensory neuron cultures (Diochot *et al.*, 2004). We found that the ASIC3-like currents were also completely blocked by 3  $\mu$ M of APETx2, further supporting them as ASIC3-like current (Figure 1A). We mainly observed the ASIC3-like currents in this study.

Application of OT (10<sup>–5</sup> M) prior to application of acid solution decreased  $I_{pH\ 5.5}$  in the majority of acid-sensitive neurons (63.27%, 62/98). The level of OT inhibition of  $I_{pH\ 5.5}$  was correlated with the duration of OT pretreatment. As shown in Figure 1B and D, the level of inhibition of  $I_{pH\ 5.5}$  gradually increased as the duration of OT pretreatment increased from 15 to 60 s. The inhibitory effect of OT reached its maximum (51.52  $\pm$  1.82%,  $n$  = 8) at 60 s of pretreatment, after which longer durations had no further effect. However, no inhibitory effects were observed when OT and acidosis of pH 5.5 were co-applied for only 5 s. These results indicate that the inhibition of ASIC currents was dependent on the duration of OT pretreatment.

We next investigated whether the inhibition of ASIC currents was dependent on the concentration of OT. Figure 1C shows that the amplitudes of  $I_{pH\ 5.5}$  decreased as concentration of OT increased from 10<sup>–7</sup> M to 3  $\times$  10<sup>–5</sup> M. Figure 1E shows the dose–response curve for OT in the inhibition of  $I_{pH}$ . OT had a maximum effect (53.10  $\pm$  7.26%,  $n$  = 8) at a concentration of 3  $\times$  10<sup>–5</sup> M. The EC<sub>50</sub> value of the dose–response curve for OT was 1.06  $\pm$  0.19  $\mu$ M.



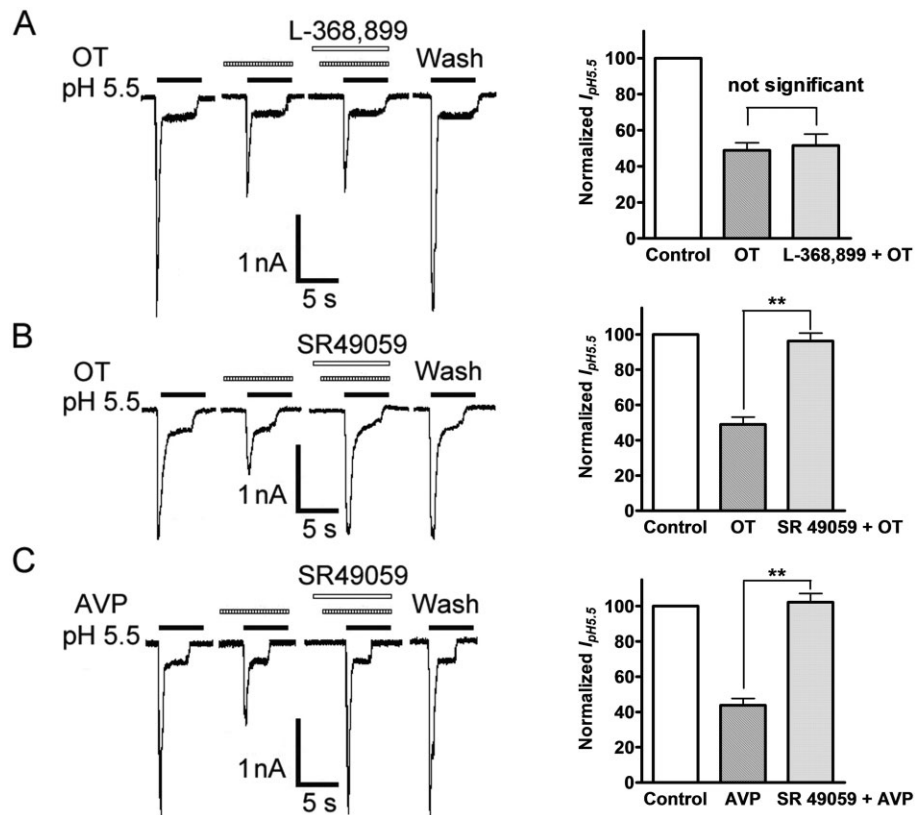
**Figure 1**

Inhibition of ASIC currents by OT in rat DRG neurons. (A) Representative current traces were evoked by extracellular application of a pH 5.5 solution for 5 s in the presence of capsazepine ( $10 \mu\text{M}$ ) to block proton-induced TRPV1 activation. The proton-induced current could be completely blocked by  $100 \mu\text{M}$  amiloride (Amil), a broad-spectrum ASIC channel blocker. It was also blocked by  $3 \mu\text{M}$  APETx2, an ASIC3 blocker. (B and D) Representative current traces and summary data show the effect of OT ( $10^{-5} \text{ M}$ ) pretreatment duration on  $I_{pH}$  in a DRG cell. The graph shows that the inhibitory effect of OT ( $10^{-5} \text{ M}$ ) increased as OT pretreatment duration increased from 15 to 120 s ( $n = 8$ ). (C and E) Representative current traces and summary data show a concentration-dependent inhibition of the peak amplitude of  $I_{pH}$  by OT. The sequential current traces illustrate the inhibition of  $I_{pH}$  by different concentrations of OT ( $10^{-7} \text{ M} - 3 \times 10^{-5} \text{ M}$ ). All data were obtained from a single DRG neuron. The graph shows OT decreased  $I_{pH}$  in a concentration-dependent manner. Each point represents the mean  $\pm$  SEM of seven to nine neurons. DRG neurons with membrane potential clamped at  $-60 \text{ mV}$ .

### The $V_{1A}$ receptor is involved in OT inhibition of ASIC currents

The inhibition of ASIC currents by OT may involve intracellular signal transduction because this inhibition is a time-consuming process and there was no inhibitory effect observed when OT and acidosis were co-applied. The slow onset of OT inhibition may point to a role of GPCRs in modulating  $I_{pH5.5}$ . First, we observed the effect of YM-254890, a specific  $G_{q/11}$  inhibitor, on the inhibition of OT (Uemura *et al.*, 2006). In  $5 \mu\text{M}$  YM-254890-pretreated neurons, OT only produced an inhibition of  $8.36 \pm 0.94\%$  on ASIC currents, which is significantly less than  $52.12 \pm 4.17\%$  of control conditions ( $P < 0.01$ , unpaired *t*-test,  $n = 9$ ). The results indicated that  $G_{q/11}$  protein couple receptors are involved in blocking OT inhibition. We further verified

whether the inhibition of  $I_{pH5.5}$  by OT was mediated by OT receptors. Contrary to our expectations, addition of L-368899 ( $3 \times 10^{-5} \text{ M}$ ), a non-peptide OT receptor antagonist (Vrachnis *et al.*, 2011), failed to block the OT inhibition of  $I_{pH5.5}$  (Figure 2A). However, the OT inhibition of  $I_{pH5.5}$  was completely blocked by co-treatment of OT with SR49059 ( $3 \times 10^{-5} \text{ M}$ ), a selective  $V_{1A}$  receptor antagonist (Serradeil-Le Gal *et al.*, 1993). In addition to OT, we further observed whether other  $V_{1A}$  receptor agonists also decreased ASIC currents. AVP, another  $V_{1A}$  receptor agonist, was added before the acid solution and also exerted a similar inhibitory effect on  $I_{pH5.5}$  (Figure 2C). The amplitude of  $I_{pH5.5}$  decreased to  $43.78 \pm 3.77\%$  of control condition after  $10^{-5} \text{ M}$  AVP pretreatment for 60 s ( $n = 8$ ,  $P < 0.01$ , pairing *t*-test). Likewise, the  $V_{1A}$  receptor antagonist SR49059 ( $3 \times 10^{-5} \text{ M}$ ) completely blocked the AVP inhibition of  $I_{pH5.5}$  (Figure 2C). Together,



**Figure 2**

Involvement of the  $V_{1A}$  receptor in OT inhibition of ASIC currents. The current traces in A and B show that the inhibition of  $I_{pH\ 5.5}$  by OT ( $10^{-5}$  M) pre-applied alone was abolished by the co-application of OT and SR49059 ( $3 \times 10^{-5}$  M), a selective  $V_{1A}$  receptor antagonist, but not by addition of L-368899 ( $3 \times 10^{-5}$  M), a non-peptide OT receptor antagonist. The current traces in (C) show that AVP has a similar inhibitory effect on  $I_{pH\ 5.5}$ , and the inhibition was also blocked by SR49059. Bar graphs in the right panel show currents normalized to control (100%). Data in all bar graphs are shown relative to control. Error bars show  $\pm$ SEM. Statistical tests were performed on raw data using paired  $t$ -test, and significance is shown as follows:  $*P < 0.05$ ,  $**P < 0.01$ .  $n = 8$  in each column.

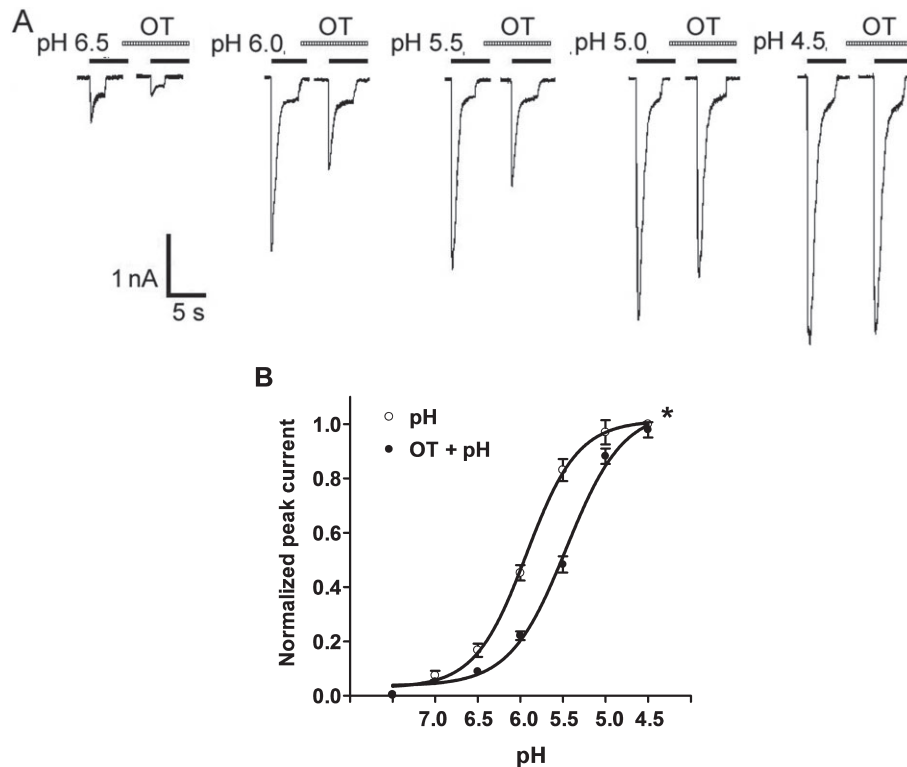
these results indicate that the OT inhibition of ASIC currents is mediated by  $V_{1A}$  receptors, but not by OT receptors.

### OT decreased ASIC currents in a pH-dependent manner

We then investigated whether the inhibition of OT was dependent on pH. Figure 3A shows the effect of OT pretreatment for 60 s on currents evoked by different pHs. The decrease in current caused by OT pretreatment was most pronounced at pH 6.0 and 5.5, whereas at pH 4.5 there was no difference between currents recorded in the presence and absence of OT pretreatment. This is illustrated in Figure 3B, which shows the concentration–response curve to protons in the presence and absence of OT. The  $pH_{0.5}$  value of curve changed from  $5.93 \pm 0.04$  to  $5.43 \pm 0.04$  in the presence of OT ( $n = 8$ ,  $P < 0.05$ , Bonferroni's *post hoc* test). However, the maximal current response to protons and the threshold pH values of both curves had no significant difference in the presence and absence of OT. These results indicated that OT decreased ASIC currents in a pH-dependent manner.

### OT decreased proton-induced membrane excitability of rat DRG neurons

Activation of ASICs by protons induces sodium influx, resulting in membrane depolarization and neuronal excitation. Further experiments were performed to record DRG neuron excitability in current-clamp model in the presence of capsaizepine ( $10 \mu\text{M}$ ) to block proton-induced TRPV1 activation. As shown in Figure 4A, a steep pH drop from 7.4 to 5.5 for 5 s could trigger bursts of action potentials under current-clamp conditions in the tested neuron, whereas the whole-cell inward current was also induced by pH 5.5 in the same cell with voltage-clamp recording. Importantly, pretreatment of OT ( $10^{-5}$  M) for 60 s decreased the number of action potentials evoked by acidosis. The mean number of action potentials decreased from  $12.57 \pm 1.30$  of control condition to  $8.39 \pm 1.08$  with pretreatment of OT in the 10 neurons tested ( $P < 0.05$ , paired  $t$ -test) (Figure 4C). After a washout of OT for 10 min, the mean number of action potentials evoked by acidosis was  $11.59 \pm 1.96$ , which was not significantly different from the control condition ( $12.57 \pm 1.30$ , paired  $t$ -test,  $P > 0.1$ ,  $n = 10$ ; Figure 4A and C).



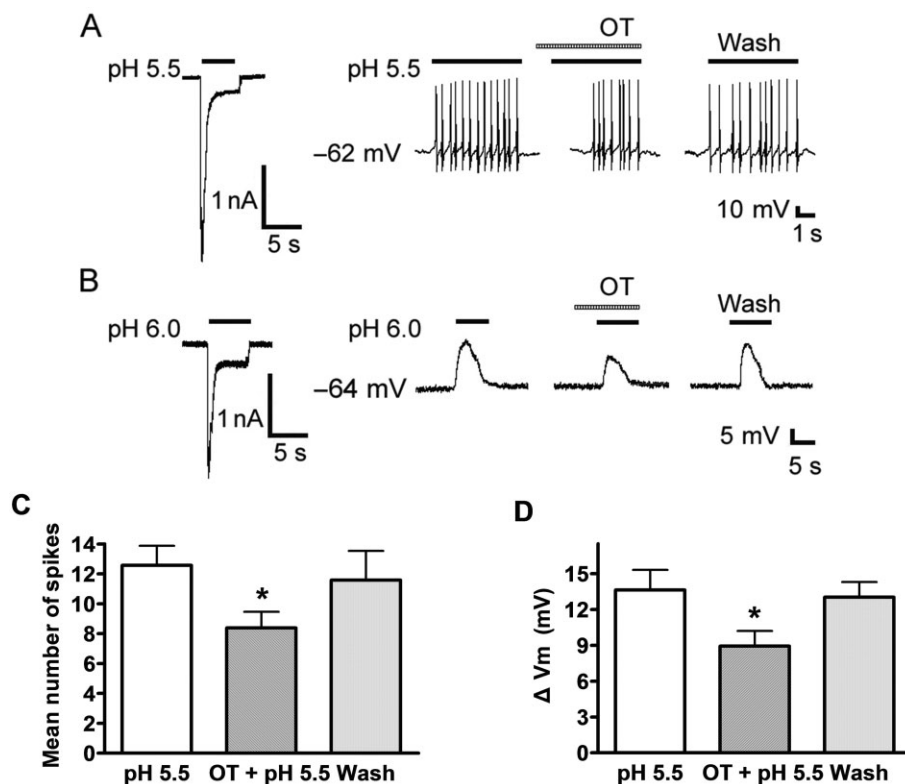
**Figure 3**

Concentration–response relationship for protons with or without the pre-application of OT. (A) Sequential currents evoked by different pHs in the absence or presence of OT. (B) The concentration–response curves for protons with or without  $10^{-5}$  M OT as a pretreatment. Each point represents the mean  $\pm$  SEM of 7–11 neurons. All current values were normalized to the current response induced by pH 4.5 applied alone (marked with asterisk). The curves shown are a best fit of the data to the logistic equation  $I = I_{\max}/[1 + (pH_{0.5}/C)^{n_H}]$ , where C is the concentration of protons, I is the normalized current response value,  $pH_{0.5}$  is the proton concentration that produced half the maximal current response to protons, and  $n_H$  is the Hill coefficient. The curves for protons without and with OT pretreatment were drawn according to the equation described above.

It was shown that TTX is not effective in blocking the  $I_{pH}$ , although it blocks the majority of voltage-gated  $Na^+$  currents (Lilley *et al.*, 2004). We then observed the membrane potential of DRG neurons in the presence of capsazepine (10  $\mu$ M) and TTX (1  $\mu$ M) to block proton-induced TRPV1 activation and  $Na^+$  channel-mediated action potentials respectively. A pH 5.5 acid stimulus induced an inward current in a DRG neuron tested with voltage-clamp recording, whereas it also produced a depolarization of the resting membrane potential in the same cell under current-clamp recording ( $I = 0$  pA) conditions (Figure 4B). In this particular cell, pretreatment of OT ( $10^{-5}$  M) for 60 s decreased the depolarization of the membrane potential evoked by pH 5.5 acid stimuli (Figure 4B). In 10 neurons tested, membrane potential depolarized from  $61.58 \pm 5.33$  to  $47.95 \pm 4.92$  mV after exposure to pH 5.5. In contrast, OT decreased the magnitude of acidosis-induced depolarization from  $13.63 \pm 1.69$  to  $8.94 \pm 1.27$  mV (paired *t*-test,  $P < 0.05$ ,  $n = 10$ ) (Figure 4D). After washout of OT for 10 min, the acidosis-evoked depolarization of membrane potential recovered to control condition ( $13.02 \pm 1.28$  mV, paired *t*-test,  $P > 0.1$ , compared with  $13.63 \pm 1.69$  mV of control condition,  $n = 10$ ; Figure 4B and D). Collectively, these results indicated that OT reversibly decreased proton-induced membrane excitability of rat DRG neurons.

### *OT and AVP decreased nociceptive responses to intraplantar injection of acetic acid in rats*

Intraplantar injection of acetic acid elicited an intense flinch/shaking response in rats (Deval *et al.*, 2008; Omori *et al.*, 2008). The flinch response mainly occurred during 0–5 min after injection of acetic acid. In the present study, intraplantar injection of acetic acid solution (0.6%, 20  $\mu$ L) in the presence of the TRPV1 inhibitor capsazepine (100  $\mu$ M) caused an intense flinch/shaking response in female rats. The acidosis-evoked pain may be mediated by ASIC3, as it was potentially blocked by treatment with 200  $\mu$ M amiloride and 20  $\mu$ M APETx2 (Figure 5A). Administration of OT significantly decreased flinching behaviour induced by acetic acid in a dose-dependent manner. Quantitative analysis showed that OT decreased the number of flinches from  $11.56 \pm 1.25$  of vehicle treatment to  $10.62 \pm 0.91$ ,  $8.06 \pm 0.62$  and  $6.68 \pm 0.55$  at dose of 0.1, 1 and 10  $\mu$ M, respectively ( $n = 10$ ,  $P < 0.05$  or 0.01, *post hoc* Bonferroni's test) (Figure 5B). In contrast, the analgesic effect of OT on acetic acid-induced pain behaviour was not observed when OT was co-injected together with SR49059, a selective  $V_{1A}$  receptor antagonist. And the number of flinches induced by acetic acid ( $12.73 \pm 1.67$ ) was not significantly different from those induced by vehicle treat-



**Figure 4**

Effect of OT on proton-evoked membrane excitability of rat DRG neurons. (A) Original current and spikes recordings from the same DRG neuron. Left panel: a pH 5.5 acid stimulus induced an inward current with voltage-clamp recording. Right panel: the pH 5.5 acid stimulus produced cell spikes with current-clamp recording in the same neuron in the presence of the TRPV1 inhibitor capsazepine (10  $\mu$ M). The treatment with OT (10<sup>-5</sup> M) for 60 s inhibited the acidosis-induced spiking activity. (B) Original current and membrane potential recordings from the same DRG neuron. Left panel: voltage-clamp recording of current induced by a pH 5.5 acid stimulus. Holding potential was -60 mV. Right panel: current-clamp recording ( $I = 0$  pA) of the depolarization evoked by the pH 5.5 acid stimulus from the same neuron as the left panel. The treatment of OT (10<sup>-5</sup> M) for 60 s decreased the acidosis-induced membrane depolarization. No action potential was triggered by the membrane depolarization in the neuron tested in the presence of capsazepine (10  $\mu$ M) and TTX (1  $\mu$ M) to block proton-induced TRPV1 activation and Na<sup>+</sup> channel-mediated action potentials respectively. (C and D) Bar graphs show the effects of OT on the number of spikes and membrane potential depolarization produced by pH 5.5. The acidosis-evoked depolarization and spikes recovered to control condition after washout of OT for 10 min. \* $P < 0.05$ , paired  $t$ -test, compared with pH alone,  $n = 10$  in each column.

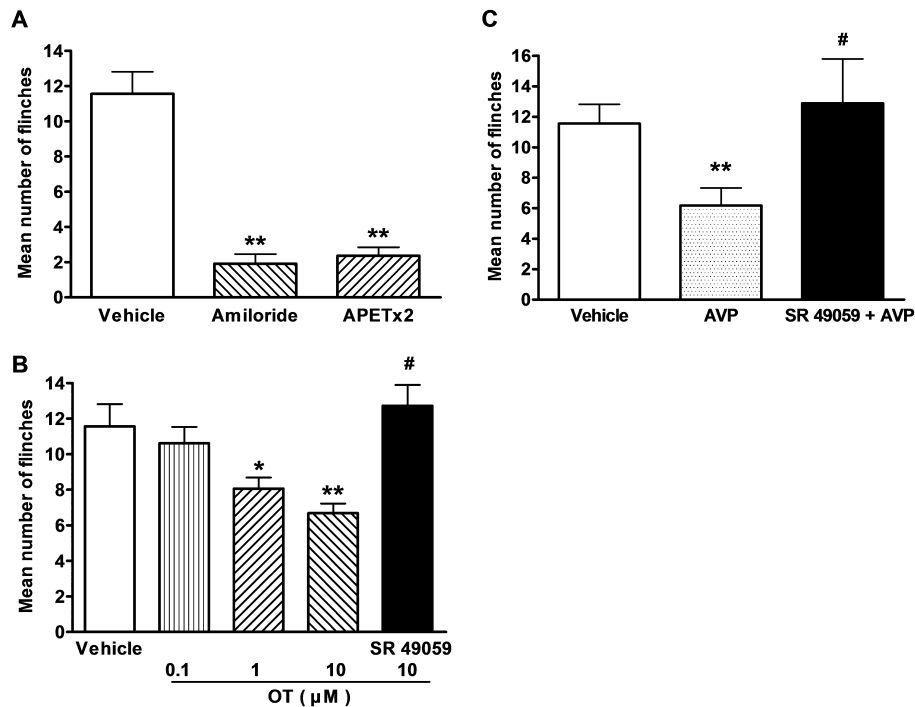
ment ( $P > 0.1$ , *post hoc* Bonferroni's test,  $n = 10$ ). Moreover, administration of AVP also significantly decreased flinching behaviour induced by acetic acid from  $11.56 \pm 1.25$  of vehicle treatment to  $6.18 \pm 1.14$  ( $P < 0.05$ , *post hoc* Bonferroni's test,  $n = 10$ ) (Figure 5C). Likewise, the V<sub>1A</sub> receptor antagonist SR49059 completely blocked the analgesic effect of AVP on acetic acid-induced pain (Figure 5C). These results indicate that the acidosis-evoked pain was relieved by the activation of V<sub>1A</sub> receptors by OT or AVP at the periphery.

#### *OT and AVP failed to decrease nociceptive behaviours induced by acetic acid in mice lacking V<sub>1A</sub> receptors*

To further address the finding that the OT and AVP analgesic effects on acidosis-evoked pain were mediated by the V<sub>1A</sub> receptor, we investigated the roles of OT and AVP in specific V<sub>1A</sub><sup>-/-</sup> mice. Intraplantar injection of acetic acid solution (0.6%, 10  $\mu$ L) in the presence of the TRPV1 inhibitor capsaz-

epine (100  $\mu$ M) also caused an intense flinch/shaking response in female mice. There was no significant difference in flinching behaviour induced by acetic acid between V<sub>1A</sub><sup>-/-</sup> mice and WT littermates. Similar to that observed in rats, administration of 10  $\mu$ M OT or AVP significantly decreased flinching behaviour induced by acetic acid from  $29.56 \pm 2.03$  of vehicle treatment to  $16.89 \pm 1.06$  and  $15.50 \pm 1.68$ , respectively, in V<sub>1A</sub> receptor WT littermates ( $P < 0.1$ , *post hoc* Bonferroni's test,  $n = 10$ ) (Figure 6). However, OT or AVP failed to decrease flinching behaviour induced by acetic acid in V<sub>1A</sub><sup>-/-</sup> mice (Figure 6). After treatment of OT or AVP, the number of flinches induced by acetic acid in V<sub>1A</sub><sup>-/-</sup> mice were  $33.46 \pm 5.37$  and  $26.92 \pm 1.61$ , respectively, which were not significantly different from  $28.76 \pm 1.43$  of vehicle treatment ( $P > 0.1$ , *post hoc* Bonferroni's test,  $n = 10$ ). These results suggest that the OT- and AVP-induced analgesic effects on acidosis-evoked pain were absent in V<sub>1A</sub><sup>-/-</sup> mice, but present in WT littermates.





**Figure 5**

Effects of OT and AVP on nociceptive responses to intraplantar injection of acetic acid in rats. Intraplantar injection of acetic acid (0.6%, 20  $\mu$ L) evoked a flinch/shaking response in female rats. The bar graph in (A) shows acidosis-evoked pain was blocked by pretreatment with 200  $\mu$ M amiloride and 20  $\mu$ M APETx2. \*\* $P$  < 0.01, unpaired  $t$ -test, compared with vehicle column.  $n$  = 10 in each column. The bar graph in (B) shows that pretreatment with OT decreased flinching behaviour induced by acetic acid in a dose-dependent manner. The effect of OT was blocked by SR49059, a selective  $V_{1A}$  receptor antagonist. \* $P$  < 0.05, \*\* $P$  < 0.01, one-way ANOVA followed by *post hoc* Bonferroni's test, compared with vehicle column; # $P$  < 0.01, *post hoc* Bonferroni's test, compared with OT (10  $\mu$ M) column.  $n$  = 10 in each column. The bar graph in (C) shows that pretreatment with 10  $\mu$ M AVP also decreased flinching behaviour induced by acetic acid, and SR49059 completely blocked the analgesia of AVP on acetic acid-induced pain. \*\* $P$  < 0.01, one-way ANOVA followed by *post hoc* Bonferroni's test, compared with vehicle column; # $P$  < 0.01, *post hoc* Bonferroni's test, compared with AVP column.  $n$  = 10 in each column. Flinch/shaking of paw was recorded as the number of flinches per observation period (5 min).

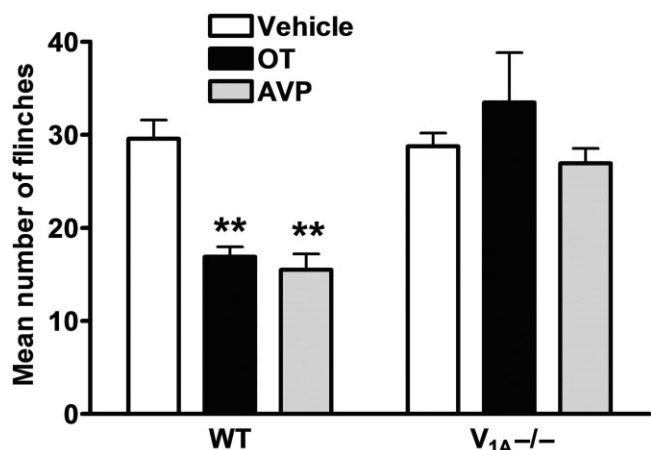
## Discussion

This study demonstrates that OT can exert an inhibitory effect on the functional activity of ASICs. OT decreased the amplitude of ASIC currents and acidosis-evoked membrane excitability in dissociated rat DRG neurons. Peripherally administered OT inhibited nociceptive responses to intraplantar injection of acetic acid *in vivo*. Effects of OT on ASICs could be mimicked by AVP and mediated by  $V_{1A}$  receptors, but not by OT receptors. The conclusion was further supported by the data that OT or AVP-induced analgesic effect on acidosis-evoked pain was completely absent in  $V_{1A}^{-/-}$  mice, but present in WT littermates.

A rapid drop in the extracellular pH from 7.4 to 5.5 for 5 s was found to evoke an inward current in most native DRG neurons. The acidosis-evoked currents may be involved in the activation of ASIC and TRPV1 channels (Bevan and Geppetti, 1994). The contribution of TRPV1 was ruled out as capsazepine was used to block proton-induced TRPV1 activation (Gavva *et al.*, 2005; Poirot *et al.*, 2006). Our previous work showed that the acid-induced currents are not blocked by the TRPV1 blocker AMG 9810 (Qiu *et al.*, 2012). Importantly, these acid-induced currents could be completely blocked by

amiloride, a broad-spectrum ASIC channel blocker, suggested the acid currents were mediated by ASICs. To date, at least four genes encoding seven ASIC subunits have been cloned in mammals (Krishtal, 2003). All ASICs besides ASIC4 are present in DRG neurons (Alvarez de la Rosa *et al.*, 2002; Benson *et al.*, 2002). ASIC3 have emerged as critical pH sensors predominantly expressed in nociceptors (Price *et al.*, 2001; Voilley *et al.*, 2001; Deval *et al.*, 2008). The present acid currents were completely blocked by ASIC3 channel blocker APETx2 and characterized by a large transient current followed by fast inactivation and then a small sustained current (Wang *et al.*, 2013). They may be ASIC3-like currents, although we cannot rule out the possibility of other ASIC subunits. We considered the acid currents as ASIC3 currents in the present study.

Our study showed that OT exerted an inhibitory effect on ASIC currents in a dose-dependent manner in rat DRG neurons. Inhibition of OT was due to reduction of the affinity of ASICs to protons, as shown by a decrease in the  $pH_{0.5}$ . ASICs are extracellular pH sensors and are selectively permeable to cations (Wemmie *et al.*, 2013). Activation of ASICs by a rapid drop in pH induces an inward current, which causes a depolarization of the resting membrane potential and trig-



**Figure 6**

Effects OT and AVP on nociceptive behaviours induced by acetic acid in V<sub>1A</sub><sup>-/-</sup> mice. Intraplantar injection of acetic acid (0.6%, 10  $\mu$ L) evoked a flinch/shaking response in female V<sub>1A</sub><sup>-/-</sup> mice and WT littermates. Administration of 10  $\mu$ M OT or AVP decreased flinching behaviour induced by acetic acid in WT, but not V<sub>1A</sub><sup>-/-</sup> mice. \*\* $P < 0.01$ , one-way ANOVA followed by *post hoc* Bonferroni's test, compared with vehicle column.  $n = 10$  in each column. Flinch/shaking of paw was recorded as the number of flinches per observation period (5 min).

gers bursts of action potentials (Mamet *et al.*, 2002). The current-clamp experiments showed that OT decreased the amplitude of the depolarization and the number of action potentials induced by extracellular acid stimuli. Effect of OT on acidosis-evoked neuronal excitability of DRG neurons appeared to be related to their inhibitory effect on the ASIC current amplitude in voltage-clamp experiments. Consistent with the electrophysiological results, behavioural experiments showed that OT relieved acidosis-evoked pain in a dose-dependent manner. Altogether, the present study indicated that OT inhibited the functional activity of ASICs in primary sensory neurons.

The central and peripheral effects of OT are thought to be mediated by OT's binding to a single isoform of the OT receptor (Gimpl and Fahrenholz, 2001). OT receptors are densely expressed in pain-relevant laminae I and II of the dorsal horn and are a likely target of spinal oxytocinergic projections from the paraventricular nucleus of the hypothalamus (Reiter *et al.*, 1994; Puder and Papka, 2001; Condes-Lara *et al.*, 2007). However, the presence of OT receptors in DRG is controversial. In the mouse, OT receptor mRNA was only barely expressed in this area (Schorsch-Petcu *et al.*, 2010). Instead, it was reported recently that OT receptors are expressed in C-fibre cell bodies, but not in skin nociceptive terminals in rats (Moreno-Lopez *et al.*, 2013). Both OT and AVP, as well as the OT and V<sub>1A</sub> receptor, display a high degree of sequence homology, and both peptides can activate both receptors (Chini and Manning, 2007). Similar to OT, systemic injections of AVP also lead to analgesia (Mogil *et al.*, 2011). AVP analgesia is believed to be mediated by V<sub>1A</sub> receptors, as V<sub>1A</sub> receptor mRNA is reported to be abundantly expressed in mouse DRGs, and V<sub>1A</sub> receptor-positive neurons were predominantly of small and medium diameter

(Schorsch-Petcu *et al.*, 2010). Both OT and AVP are also expressed in the DRG and trigeminal ganglia of the rat and cause an increase in the accumulation of inositol phosphates (IP) through V<sub>1A</sub> receptors (Kai-Kai *et al.*, 1985; Horn and Lightman, 1987; Kai-Kai and Che, 1995). A recent study showed that i.v. injection of a low dose of OT causes an OT receptor-mediated antinociceptive effect whereas a high dose of OT exerts a V<sub>1A</sub> receptor-mediated pronociceptive effect in rats (Juif and Poisbeau, 2013). In contrast, a study in transgenic mice lacking the OT or V<sub>1A</sub> receptor indicates that OT-induced antinociception was mediated by V<sub>1A</sub> receptors, but not by OT receptors, in mice (Schorsch-Petcu *et al.*, 2010). The study showed that i.p. injection of OT still produces mechanical and thermal analgesia in OT receptor-knockout mice as well as in WT littermates, but does not alter nociceptive threshold in V<sub>1A</sub><sup>-/-</sup> mice (Schorsch-Petcu *et al.*, 2010). In the present study, OT inhibition of ASICs appeared to involve V<sub>1A</sub> receptors, but not OT receptors. Firstly, OT inhibition of ASIC currents was blocked by the V<sub>1A</sub> receptor antagonist SR49059, but not by OT receptor antagonist L-368899. Secondly, the effect of OT on ASICs was mimicked by AVP. Thirdly, behavioural experiments showed that analgesia mediated by OT and AVP on acetic acid-induced pain were completely blocked by SR49059. Finally, OT and AVP failed to relieve acidosis-evoked pain in V<sub>1A</sub><sup>-/-</sup> mice, whereas they significantly decreased nociceptive behaviours induced by acetic acid in WT littermates.

The present study showed that the inhibitory effect of OT was mediated by V<sub>1A</sub> receptors. It was reported that activation of the V<sub>1A</sub> receptor by AVP could induce hyperpolarization in most DRG neuronal membranes and increase the membrane conductance of the DRG neurons (Hu *et al.*, 2004). But we observed that the baselines of original membrane current and potential did not change when DRG neurons were treated with OT and AVP in both voltage-clamp and current-clamp recordings, as seen in Figures 1 to 4. ASIC3 is a voltage-insensitive and sodium-selective ion channel, and the effect of OT on ASIC3 currents may not be due to the hyperpolarization. As for the mechanistic link between V<sub>1A</sub> receptors and ASIC3, one plausible explanation may be the involvement of calcineurin signalling, a pathway-modulating calcium-dependent protein phosphorylation. The activation of V<sub>1A</sub> receptors seems to trigger a multifaceted and complex response in which the calcineurin pathway is stimulated (Scicchitano *et al.*, 2005; Toschi *et al.*, 2011). Calcineurin has been reported to be involved in the regulation of ASICs (Chai *et al.*, 2007). Cyclosporin A, a specific inhibitor of calcineurin, induces an increase in ASIC current amplitude in mouse cultured cortical neurons (Chai *et al.*, 2007). This suggests that calcineurin-dependent dephosphorylation could play an important role for ASICs, as these channels have been reported to be in a highly phosphorylated state at basal level (Leonard *et al.*, 2003). Thus, we speculate that the activation of V<sub>1A</sub> receptors by OT or AVP up-regulated calcineurin, which induced calcineurin-dependent dephosphorylation of ASICs and resulted in a decrease in ASIC current amplitude.

Extracellular acidosis is a common feature in pain-generating pathological conditions such as inflammation, tissue injury, ischaemic stroke, infections and cancer (Wemmie *et al.*, 2013). Although both ASICs and TRPV1 could be involved, ASICs are believed to be the primary

mediators of pain caused by extracellular acidification (Wemmie *et al.*, 2006; Deval *et al.*, 2008). It has been shown that acidosis-induced pain is significantly attenuated by the non-selective ASIC inhibitor amiloride, whereas the TRPV1 antagonist capsaizepine failed to influence C-fibre excitability induced by an acidic solution (Habelt *et al.*, 2000; Ugawa *et al.*, 2002). Intraplantar injection of acetic acid elicited an intense flinch/shaking response in rats (Deval *et al.*, 2008; Omori *et al.*, 2008). In the present study, the acidosis-evoked pain was also mediated by ASICs, as it was potently blocked by the ASIC channel blocker amiloride. Among ASICs, ASIC3 is expressed almost exclusively expressed in sensory neurons and predominantly in nocifensive sensory neurons (Waldmann *et al.*, 1997a; Waldmann and Lazdunski, 1998; Krishtal, 2003; Deval *et al.*, 2008). Recently, it was reported that peripheral ASIC3 plays a significant role in post-operative pain (Deval *et al.*, 2011). Pharmacological inhibition of ASIC3 channels with the toxin APETx2 or *in vivo* knockdown of the ASIC3 protein significantly reduced the post-operative spontaneous, thermal and postural pain behaviours (Deval *et al.*, 2011). OT and AVP do not cross the blood–brain barrier (Ermisch *et al.*, 1985). So far, it is not known whether the hormonal role of OT on nociceptive processing occurs in peripheral terminals of primary sensory afferents. One study has shown that OT inhibits ATP-activated currents in rat DRG neurons (Yang *et al.*, 2002). OT is also found to inhibit intracellular calcium increases in capsaicin-sensitive DRG neurons (Hobo *et al.*, 2012). However, it was recently reported that OT activates intracellular calcium signalling in cultured rat primary sensory neurons through a PKC-dependent mechanism (Ayar *et al.*, 2014). In this work, we used the cell body of DRG neurons as a simple and accessible model to examine the characteristics of the membrane of peripheral terminals. Histochemical analysis reveals the presence of ASIC3 in cutaneous nerve endings (Price *et al.*, 2001; Deval *et al.*, 2008). OT inhibition of ASICs hinted that a novel peripheral analgesic mechanism of OT occurs in peripheral terminals of primary sensory neurons. This view was further supported by behavioural experiments that peripheral application of OT relieved acidosis-evoked pain in a dose-dependent manner.

In conclusion, this study shows that OT inhibits the functional activity of ASICs in DRG neurons. The OT inhibition of ASICs was mediated by  $V_{1A}$  receptors, but not by OT receptors. OT relieved acidosis-evoked pain when administered peripherally, whereas OT analgesia was absent in mice lacking  $V_{1A}$  receptors. Our results reveal a novel peripheral mechanism of OT analgesic action by modulating native ASICs in primary sensory neurons. This indicates that OT released while giving birth may have multiple pathways of analgesic effects.

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## Conflict of interest

We have no conflict of interest to declare.

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