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# OXYTOCIN-INDUCED MEMBRANE HYPERPOLARIZATION IN PAIN-SENSITIVE DORSAL ROOT GANGLIA NEURONS MEDIATED BY $CA^{2+}$/NNOS/NO/$K_{ATP}$ PATHWAY

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**Abstract—Oxytocin (OT) plays an important role in pain modulation and antinociception in the central nervous system. However, little is known about its peripheral effects. This study was conducted to investigate the effect of OT on the electrical properties of neurons in the dorsal root ganglia (DRG) and the underlying mechanisms. DRG neurons from adult rats were acutely dissociated and cultured. Intracellular $Ca^{2+}$ was determined by fluorescent microscopy using an indicator dye. The electrical properties of DRG neurons were tested by patch-clamp recording. The oxytocin receptor (OTR) and neuronal nitric oxide synthase (nNOS) on DRG neurons were assessed with immunofluorescence assays. OTR co-localized with nNOS in most of Isolectin B4 (IB4)-binding cultured DRG neurons in rats. OT decreased the excitability, increased the outward current, and evoked the membrane hyperpolarization in cultured DRG neurons. Sodium nitroprusside (SNP), the donor of nitric oxide (NO), exerted similar effects as OT on the membrane potential of cultured DRG neurons. OT increased the production of NO in DRGs and cultured DRG neurons. Pre-treatment of the OTR antagonist atosiban or the selective nNOS inhibitor N-Propyl-L-arginine (NPLA) significantly attenuated the hyperpolarization effect evoked by OT. OT produced a concentration-dependent increase in intracellular $Ca^{2+}$ in DRG neurons that responds to capsaicin, which can be attenuated by atosiban, but not by NPLA. OT-evoked membrane hyperpolarization and increase of outward current were distinctly attenuated by glibenclamide, a blocker of ATP-sensitive $K^+$ ($K_{ATP}$) channel. OT might be an endogenous antinociceptive agent and the peripheral antinociceptive effects of OT are mediated by activation of the $Ca^{2+}$/nNOS/NO/$K_{ATP}$ pathway in DRG neurons.** 

**Key words: oxytocin, $Ca^{2+}$, nitric oxide, $K_{ATP}$ channel, hyperpolarization, pain.**

## INTRODUCTION

Oxytocin (OT), a nine amino acid neuropeptide, is synthesized in the paraventricular and supraoptic nuclei of the hypothalamus and is released into the central nervous system (CNS) and the bloodstream (Sofroniew, 1983; Rash et al., 2014). In addition to its best known roles in parturition and lactation (Sala et al., 1974; Arrowsmith and Wray, 2014), OT is involved in the regulation of a wide variety of physiological and pathological functions such as social memory and attachment, sexual and maternal behavior, and human bonding and trust (Lee et al., 2009). Some studies have also demonstrated that OT could modulate nociception and pain, and most work has focused on the CNS, especially on the spinal cord as the site of OT analgesia (Robinson et al., 2002; Condes-Lara et al., 2003; Yu et al., 2003; Yang et al., 2007, 2011b; Breton et al., 2008; Russo et al., 2012). Besides that, OT plays an important role in peripheral analgesia (Yang et al., 2002; Reeta et al., 2006; Black et al., 2009; Qiu et al., 2014). However, the mechanisms underlying this peripheral effect of OT have not yet been fully elucidated.

Oxytocin receptor (OTR), a member of the G-protein-coupled receptor family, is distributed in the brain and peripheral tissues (Gimpl and Fahrenholz, 2001; Kimura et al., 2003; Lv et al., 2010; Viero et al., 2010; Boccia et al., 2013). It is known that the OTR is expressed in the dorsal root ganglia (DRG), principally in non-peptidergic C-fiber cell bodies (Moreno-Lopez et al., 2013). OT inhibition on the increase in intracellular calcium from membrane depolarization and on ATP-activated currents is mediated by OT receptors in membranes of DRG neurons (Yang et al., 2002; Hobo et al., 2012), besides that, our lab has shown that OT could hyperpolarize myenteric intrinsic primary afferent neurons (IPANs) through binding OTR (Che et al., 2012). Therefore, we speculate that OT

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*Abbreviations:* AP, action potential; CNS, central nervous system; DAF-FM DA, 3-Amino,4-aminomethyl-2′,7′-difluorescein; DMSO, dimethylsulfoxide; DRG, dorsal root ganglia; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IB4, Isolectin B4; IgG, immunoglobulin G; IPANs, intrinsic primary afferent neurons; $K_{ATP}$, ATP-sensitive $K^+$; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; NPLA, N-Propyl-L-arginine; OT, oxytocin; OTR, oxytocin receptor; PBS, phosphate-buffered saline.

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might induce membrane hyperpolarization of DRG neurons via OTR.

OT caused an increase of intracellular $Ca^{2+}$ of DRG neurons in normal KCl concentration buffer (Hobo et al., 2012; Ayar et al., 2014). Intracellular $Ca^{2+}$ involves in the inhibition of OT on ATP-activated current in DRG neurons, which might modulate the transfer of nociceptive or non-nociceptive sensory information (Yang et al., 2002). However, it is not yet known how the increased intracellular $Ca^{2+}$ of DRG neurons following OT administration produces analgesia.

Some evidence has suggested the participation of NO derived from neuronal nitric oxide synthase (nNOS) in the peripheral antinociceptive actions (Leanez et al., 2009; Cunha et al., 2010). The nNOS is constitutively expressed in central and peripheral neurons and some other cell types (Forstermann and Sessa, 2012), which depends upon $Ca^{2+}$/calmodulin for activation (Lee and Stull, 1998; Hayashi et al., 1999). Furthermore, some studies show that the NO/cGMP/$K_{ATP}$ pathway was involved in the peripheral antinociceptive effects of the pertussis toxin (Brito et al., 2006), morphine, dipyrone (Sachs et al., 2004; Cunha et al., 2010), and noradrenaline (Romero et al., 2012). In a recent study, we found that OT modulated the mesenteric afferent response to bradykinin and high-pressure distension via nNOS (Li et al., 2014), so we hypothesized that OT might inhibit the excitability of DRG neurons through the $Ca^{2+}$/nNOS/NO/$K_{ATP}$ pathway. To test this hypothesis, we investigated the effect of OT on the membrane potential, membrane current, intracellular $Ca^{2+}$ and NO content of DRG neurons using multiple methods, including patch-clamp recording, calcium imaging and immunofluorescence. We found that OT hyperpolarized the membrane, increased the outward membrane current and raised NO content in DRG neurons. These preliminary data supported our hypothesis and provided new evidence that OT might modulate nociception through releasing NO in DRG neurons.

## EXPERIMENTAL PROCEDURES

### Animals

Wistar male rats (200–220 g) were provided by the Experimental Animal Center of the Shandong University. All procedures described were approved by the Ethics Committee for Research on Animals, Shandong University School of Medicine.

### DRG neurons culture

Rats were killed by cervical dislocation and the entire spinal columns were removed, transferred to a beaker containing ice-cold sterile Krebs solution, and bisected longitudinally. Bilateral DRG (T7-L5) (Knowles and Aziz, 2009) were dissected and washed twice with L-15 medium (Gibco, Gaithersburg, MD, USA), and then incubated for 50 min in collagenase type I (1 mg/mL, Sigma–Aldrich, St. Louis, MO, USA) and 0.5 mL trypsin (0.25%, Gibco) in 20 mL L-15 medium at 37 °C. DRG were then washed twice and transferred to 2 mL L-15 medium containing 10% FBS. A single-cell suspension was subsequently obtained by repeated trituration using a fire-polished Pasteur pipe. The cells were then cultured at 37 °C in a 5% $CO_2$ incubator (Thermo Forma, Hamilton, NJ, USA).

### Whole-cell patch-clamp recording

Whole-cell patch-clamp recordings were performed using an Axon Instruments Multiclamp 700B amplifier (Molecular Devices, New York, NY, USA) interfaced to Digidata 1440A with the pClamp 10.2 software (Molecular Devices). Glass pipettes filled with an intracellular saline had a resistance of 5–7 MΩ. The external solution was Krebs saline. All recordings were conducted at 30 °C. In this study, we used relatively small DRG neurons (20–27 μm in diameter) (Scroggs and Fox, 1992) that were considered to transmit visceral sensory information.

### Calcium imaging

DRG neurons adhered to the bottom of round glass panes, were incubated with the calcium-sensitive dye fura-2/AM ester (1 μM, 1 mM stock in dimethylsulfoxide (DMSO)) for 60 min in imaging bath solution, and then were washed three to four times for 30 min with Krebs saline. The DRG neurons were exposed to vehicle or different concentrations of OT ($10^{-8}$ – $10^{-5}$ M). Atosiban ($10^{-6}$ M), $CdCl_2$ ($10^{-4}$ M), thapsigargin (5 μM) or N-Propyl-L-arginine (NPLA) ($10^{-6}$ M) was administrated 10 min before OT application. All imaging experiments were performed in the dark, at room temperature (20–25 °C). Fura-2 fluorescence was recorded at 510 nm during alternating excitation at 340 and 380 nm at 1 Hz using a monochromator (Polychrome V, FEI Company, Hillsboro, OR, USA). Regions of interest were defined on a computer connected to a CCD camera, and ratio of emission at 510 nm from excitation at 340 and 380 was analyzed. Only cells with a resting 340/380 fluorescence ratio of 0.55–0.90 were included. Finally, to determine the effect of OT on capsaicin-sensitive DRG neurons, at the end of the experiment, capsaicin (10 μM) was added.

### Immunofluorescence

DRG neurons adhered to the bottom of round glass panes and were rinsed three times with phosphate-buffered saline (PBS), soaked in 4% paraformaldehyde for 30 min at 22–25 °C, and incubated with 10% donkey serum for 1 h. Then the neurons were incubated with a primary antibody mixture including a goat anti-OTR antibody (1:50, ab87312; Abcam) and a rabbit anti-nNOS antibody (1:1000, N7280; Sigma) for 16–18 h at 4 °C. The neurons were washed three times with PBS and incubated with a secondary antibody mixture composed of DyLight 405 AffiniPure Donkey Anti-Goat immunoglobulin G (IgG) (1:50, 705-475-003; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), Alexa Fluor 568-labeled donkey anti-rabbit IgG antibody (1:500, A10042; Invitrogen, Eugene, Oregon, USA), and Isolectin $B_4$ ($IB_4$)-FITC conjugated antibodies (1:100, L2895; Sigma) for 1 h at 22–25 °C. After washing three times with PBS, the neurons were placed