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## RESEARCH ARTICLE

## SUCKLING-INDUCED BURST DISCHARGES OF SUPRAOPTIC OXYTOCIN NEURONS IN RATS: PROSTAGLANDIN MEDIATION OF OXYTOCIN ACTIONS

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### Abstract

Pulsatile release of hormones and neurotransmitters is widespread in mammals and typical of oxytocin secretion during lactation. Suckling-evoked pulsatile release of oxytocin follows a transient activation of hypothalamic oxytocin neurons in a burst-like firing pattern and leads to a bolus release of oxytocin and milk ejections. Prostaglandin (PG), a non-hormonal messenger cytokine, has been implicated in the activation of oxytocin neurons. To clarify the role of PGs in the activation of oxytocin neurons, we made the following observations. In patch-clamp recordings of oxytocin neurons in the supraoptic nucleus of brain slices from lactating rats, bath application of PGE<sub>2</sub> evoked a significantly higher incidence of bursts than oxytocin did. Indomethacin, an inhibitor of PG synthesis, totally blocked oxytocin- but not PGE<sub>2</sub>-evoked bursts. Moreover, suckling-induced milk ejections and increased molecular association between extracellular signal-regulated protein kinase (ERK) 1/2 and actin were blocked by intracerebroventricular application of indomethacin. Intracerebroventricular administration of PD98059, a blocker of ERK 1/2 phosphorylation/activation, also blocked suckling-induced cyclooxygenase 2 (Cox-2, a PG synthetase) in oxytocin neurons and suckling-increased molecular association between ERK 1/2 and Cox-2. These results indicate that PGs are essential signaling links in the suckling-evoked bursting of oxytocin neurons; however, to evoke full bursts, PGs need other signaling events downstream to oxytocin receptors. This finding highlights the mediator effect of PGs on oxytocin-evoked pulsatile oxytocin release, and provides a model of signaling process of hormonal actions and therapeutic target of hormone-related diseases.

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### Introduction:-

Patterned neural activity is widespread in mammalian neuroendocrine systems and appears to underlie pulsatile release of hormones and neurotransmitters in response to environmental challenges. For instance, the hypothalamus can use intermittently released nitric oxide to coordinate both gonadotropin-releasing hormone (GnRH) neuron activity and GnRH release during the estrous cycle (Bellefontaine et al., 2011). Among many hormones and neurotransmitters with pulsatile features, oxytocin release during suckling is the most representative one. The secretion of oxytocin results from a burst-like discharges of hypothalamic oxytocin neurons in the supraoptic (SON) and paraventricular (PVN) nuclei (Wang et al., 1997; Wang et al., 2013a), which is similar to the burst firing in GnRH neurons but more compact (Schauer et al., 2015). Thus, oxytocin neuron activity during lactation has been

used as a model for studying mechanisms underlying the pulsatile release of hormones for decades (Burbach et al., 2001).

One key to understanding mechanisms underlying the patterned activity is to identify a common mediator of numerous burst-evoking agents, either an intracellular signal or a cytokine. In studying the burst firing pattern of oxytocin neurons, several critical signals have been identified, including G protein  $\beta\gamma$  subunits (Wang & Hatton, 2007a), phosphorylated extracellular signal-regulated protein kinase (pERK) 1/2 and the actin cytoskeleton (Wang & Hatton, 2007b) that are mobilized or increased following the activation of oxytocin receptors (OXTRs). However, their effects are limited to individual cells. In contrast, cytokines, like nitric oxide (Okere et al., 1996) and prostaglandin (PG, Shibuya et al., 2000) can be released from oxytocin neurons and act on their neighbors to change the firing activity of supraoptic neurons. In OXTR signaling processes, PG is a down-stream signal of pERK 1/2 and up-stream modulator of actin polymerization (Hatton & Wang, 2008). Using indomethacin to block cyclooxygenase 2 (Cox-2, a PG synthetase) can remarkably reduce spontaneous and oxytocin-evoked excitation of oxytocin neurons; both oxytocin and phenylephrine, the two burst-inducing agents (Hatton & Wang, 2008), can induce Cox-2 expression (Wang & Hatton, 2006). These findings are consistent with the facilitatory effect of PGs in oxytocin secretion (Gillespie et al., 1972; Knigge et al., 2003). However, it remains to verify whether PGs are a critical mediator in burst-evoking signaling cascade in oxytocin neurons.

To identify roles of PGs in burst firing of oxytocin neurons, we observed the effect of PGE<sub>2</sub>, the most potent oxytocin-releasing PG derivative (Knigge et al., 2003), on evoked bursts of oxytocin neurons in supraoptic brain slices from lactating rats. We then compared PG effects to those of oxytocin with and without a pretreatment with indomethacin, an inhibitor of Cox-2. We also assessed the dependency of oxytocin secretion and milk ejections on suckling-evoked expression of Cox-2 and pERK 1/2 as well as molecular associations among ERK 1/2, Cox-2, and actin. Our results support the hypothesis that PGs mediate suckling-elicited milk ejections by evoking burst discharges following activation of OXTRs in supraoptic oxytocin neurons.

### **Materials and Methods:-**

Our experimental procedures were in compliance with the guidelines on the use and care of laboratory animals set by NIH and approved by Institutional Animal Care and Use Committees of the University of California, Riverside and Harbin Medical University, respectively.

#### **Slice preparation and patch-clamp recordings:-**

Sprague-Dawley rats (300-350g), lactating for 8-12 days, were used for this study. Rats were decapitated with a guillotine to minimize distress. Brains were quickly removed from the skull and then placed in ice-cold oxygenated, artificial cerebrospinal fluid (aCSF) for ~1 min. Hypothalami were dissected from the brain and cut coronally at 300  $\mu$ m on a vibratome. After pre-incubation at room temperature (RT, 22  $\pm$  1  $^{\circ}$ C) for at least 1 h, patch-clamp recordings of oxytocin neurons were performed at 35  $^{\circ}$ C. The solutions and recording methods were the same as previously described (Wang & Hatton, 2006; 2007b). Electrical signals were filtered and sampled at 5 kHz. Series resistance compensation was between 60-80%.

#### **Intracerebroventricular administration of drugs:-**

Detailed methods for intracerebroventricular drug administration have been described previously (Wang & Hatton, 2007b). In brief, the mother rats were separated from a litter of 10 pups until 30 min after drug or vehicle applications. The mother was anesthetized with urethane (1.25g/kg body weight, i.p.) and fixed in a prone position on a stereotaxic frame to expose the skull. A stainless steel guide cannula was placed in the third ventricle according to the atlas of Paxinos and Watson (Paxinos & Watson, 1986). The probes were secured in place with dental cement and a tight-fitting stylet was kept inside of the guide cannula to prevent occlusion and losses of CSF. For intracerebroventricular drug applications, a modified 27 gauge stainless steel injector was advanced into and extended 2 mm past the end of the guide cannula in the third ventricle. Correct locations were confirmed by outflow of CSF during cannulations. Drugs were administered at ~0.2  $\mu$ l/min for 10 min using a 10  $\mu$ l Hamilton syringe, and the injector was left in place for 20 min after completion of injections. PD98059 [2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one] and U0126 [1,4-diamino-2,3-dicyano-1,4-bis (o-aminophenylmercapto) butadiene], inhibitors of mitogen-activated protein kinase kinase (MEK or ERK1/2 kinase), were dissolved in dimethyl sulfoxide and then diluted into working solution with aCSF. Considering the milk-ejection reflex is very susceptible to many variables including injection stress, we also injected the vehicle solution into the ventricle before testing drug effects, which did not interrupt the milk ejections or the ensuing intramammary pressure changes. The number of milk ejections

and litter body weight gains were initially recorded for evaluation of the milk-ejection reflex and milk availability. To validate the result of visualized milk ejections, mammary gland cannulation was done to measure intramammary pressure during suckling and oxytocin (1 mU, 0.1 ml) was delivered through a right atrium cannula to verify the sensitivity of mammary glands to oxytocin at the beginning and end of 1 h suckling in two rats.

After aforementioned treatments, rats were killed by overdose of Nembutal (100 mg/kg body weight) and the SON dissected from the brain was used for immunostaining, Western blots, and co-immunoprecipitation (IP), respectively, at the designed times or events as indicated in the **Results**.

#### **Immunohistochemistry (IHC) and confocal microscopy:-**

The methods used were the same as previously described (Wang & Hatton, 2007b). Following 5-10 min of suckling activity with or without intracerebroventricular injection of drugs, rat brains were removed and fixed in 4% paraformaldehyde (PFA) at 4 °C for 72 h. Here we selected submersion fixation, but not vascular perfusion, to fix the tissue because, 1) the brain tissues used for IHC from the same animals were also used for protein analysis, which is not appropriate in PFA-containing intravascular perfusion samples, and 2) we had found that intravascular perfusion with a hyperosmotic 4% PFA solution (>800 mEq/Kg water) could transiently activate SON neurons including increased pERK 1/2 and Cox-2 expressions, which would compromise our interpretation of suckling effects. To avoid potential metabolic disturbance of a longer penetration time in the submersion fixation, we dipped the brain tissue in cold aCSF first to stop metabolic activity following a dissection of the brain within 30 seconds before transferring into the cold PFA solution. The hypothalami were then dissected and sectioned into 50 µm-thick slices with a cryostat and prepared for immunostaining. The slices were treated with 0.3% gelatin for 1 h and 0.3% Triton X-100 in 0.1 M PBS for 30 min. Rabbit polyclonal antibody against Cox-2 (SC7951, 1: 200 dilution, 4 h) and goat polyclonal antibody against oxytocin-neurophysin (OT-NP, SC-7810, 1: 400 dilution) were added to the incubation. All primary antibodies were products of Santa Cruz Biotechnology, Inc. Secondary antibodies from Invitrogen were applied for 1.5 h to label species-matched primary antibodies, including donkey anti-goat antibody (Alexa Fluor® 647 labeled, 1:1000) and donkey anti-rabbit antibody (Alexa Fluor® 488 labeled, 1:1000). Finally, Hoechst (bisbenzimidazole, 0.25 µg/ml) was applied for 30 min to label the nuclei. To eliminate nonspecific staining, controls of no-primary or no-secondary antibody reactions were also performed.

#### **Western blots:-**

Methods for Western blots were modified from a previous report (Wang & Hatton, 2006). Briefly, 60 µg of proteins in tissue lysates was loaded and separated on a 10% SDS-PAGE gel and then transferred onto a polyvinylidene difluoride membrane. Protein membranes were pre-treated with 5% milk solids for 1 h at room temperature to block non-specific binding sites and then incubated with primary antibodies against Cox-2 (1:300 dilution) or actin (SC1616, 1: 500 dilution). Protein bands were visualized using horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Piscataway, NJ) and ECL chemiluminescence reagents (Bio-Rad, Hercules, CA). To identify Cox-2 bands, the protein blots were compared to Cox-2 positive control (Cox-2-expressing cell lysates, SC-2212) when multiple bands presented. Actin immunoreactivity was used as loading control.

#### **Co- IP:-**

The method of Co- IP is the same as previously described (Wang et al., 2013c) with minor modifications. Noteworthy is that the lysis buffer for co-IP was the same as that for Western blots except the replacement of Triton X-100 and EGTA with 1% (v/v) NP-40 and 1 mM EDTA, respectively. The immunoprecipitating antibody against total (t)ERK 1/2 or ERK 1/2 (SC-135900) was added to the SON lysate containing 1.0-1.5 mg of protein to form immune complexes. The final denatured proteins were detected in Western blotting. Positive and negative controls consisted of total lysates and non-specific IgG (mock IP), respectively.

#### **Data analysis:-**

Analyzing data of patch-clamp recordings, imaging, and protein bands used methods similar to those described previously (Wang et al., 2013b). In the SON, vasopressin neurons co-exist with oxytocin neurons. To distinguish vasopressin neurons from oxytocin neurons, neuronal electrical features and responses to oxytocin were analyzed in addition to routinely loading pipette solution with 0.05% Lucifer yellow (K<sup>+</sup> salt) to mark recorded neurons for subsequent IHC identification. Oxytocin neurons were initially identified by their continuous firing pattern and the absence of phasic discharges characteristic of vasopressin neurons. Neuronal identity was then verified by post hoc immunostaining. Burst firing was identified using the criteria previously published (Wang & Hatton, 2007a). Acceptable bursts had to have the following characteristics: a sudden increase in firing rate (at least five times the

average basal firing rate within one minute before a spike cluster) and in spike duration, accompanied by transient, non-plateau depolarization of membrane potential and reduction in spike amplitude during the peak rate of firing (instantaneous frequency 10 times the basal firing rate), all followed by an exponential decay in firing rate after the peak rate, and ending with a period of silence. To discriminate the short-lasting phasic firing of VP neurons from the bursts of oxytocin cells, we set further criteria to distinguish the two. The short phasic bursts of VP neurons had short durations at half-amplitude, short rising  $\tau$  ( $1.5 \pm 0.1$  ms), lower peak firing rates, fast rising  $\tau$  of the spike after hyperpolarization ( $1.9 \pm 0.16$  ms), and abrupt termination of firing ( $0.1 \pm 0.05$  s) from a relatively high firing rate. In addition, there were no significant changes in the spike amplitude and duration around and within the short phasic bursts. In presenting the burst phenomenon, we noted the number of cells or bursts but not the number of rats or slices, since the former were more representative of the ability of oxytocin neurons to produce burst discharges *in vivo* (Wang et al., 1996; Moos et al., 2004) and *in vitro* (Wang & Hatton, 2004; 2005). Measured liquid junction potentials of -8 to -11 mV (potential of pipette solution with respect to the bath) were not corrected in Results.

In quantifying the relative intensities of various elements in the IHC, the luminosity of the cells in a specific channel of each section was assayed with Leica LCS Lite or NIS Elements C2 software. The background level during scanning was set to those of “no-primary antibody” controls. In image analysis, a single optical section was taken. Magnocellular neurons were distinguished from astrocytes by their nuclear morphology as previously defined (Paterson & Leblond, 1977). For the most analyzed magnocellular cells, these optical sections were cut through the middle of the cell and its nucleus. In the same series of experiments, depth of focus, photo multiplier tube intensity (350–600 V), offset (1–4%), pinhole size (1 airy unit corresponding to an optical slice thickness of 300 nm), magnification (63X objective lens), and zoom were kept at the same value. Groups for comparison were scanned in the same experiment. For intensity of immunostaining, the term “strong” means that the image intensity falls in the top one-third of the detectable range (e.g., 1–256); “weak” was used for those falling in the bottom one-third of the range. When visible cytosolic Cox-2 intensity was more than 20% above background levels, we considered the occurrence of “increase.” Control experiments to exclude nonspecific staining included treatment of the slices without no primary control and no secondary antibody.

For quantification of Cox-2 induction of different slices in immunostaining, the background intensity was adjusted to be the same and the intensities were compared between slices with same position in the SON from different groups. In Western blots, the intensity of Cox-2 expression was calibrated with the actin loading control. In co-IP, pulled-down proteins were calibrated with immunoprecipitated proteins or corresponding IgG heavy chains.

Statistical analyses were performed with SigmaPlot 11 software. Non-paired t-test (two tails) was used for two-group comparisons and Repeated Measurement ANOVA followed by the Holm–Sidak test for multiple group comparisons. Significant levels were set at  $P < 0.05$  for t-test and  $P < 0.05$ , 0.025 and 0.017 in multiple comparisons. All measurements were expressed as the mean  $\pm$  SEM and/or their percentage of controls.

## Results:-

In this study, 40 lactating rats were used for patch-clamp recordings and 35 lactating rats were used for *in vivo* observations as well as IHC and Western blot analyses.

### Effects of PGE<sub>2</sub> versus oxytocin on the burst firing of oxytocin neurons:-

In previous work, we found that oxytocin can evoke burst firing of oxytocin neurons in brain slices, which resembles the burst evoked by suckling (Wang & Hatton, 2007a; b). Oxytocin can stimulate PG release following the activation of OXTR and the induction of Cox-2 (Penrod et al., 2013). To evaluate the role of PGs in suckling-evoked burst firing, we first observed the effects of PGE<sub>2</sub> on the electrical activity of oxytocin neurons in current-clamp recordings, and then compared with that of oxytocin. Upon bath application of PGE<sub>2</sub> (0.1  $\mu$ M, 10 min), firing rate of oxytocin neurons increased significantly as previously reported (Wang & Hatton, 2006). Importantly, a significant high proportion of oxytocin neurons (10 of 14) showed burst discharges ( $n = 12$ ) with a latency of  $8.7 \pm 1.0$  min ( $n = 10$ ). These bursts (Fig. 1A) had features similar to those evoked by oxytocin (0.01–0.1 nM, 10 min, Fig. 1B) as previously reported (Wang & Hatton, 2007a; b).

Similar to oxytocin-evoked bursts, PGE<sub>2</sub>-evoked bursts quickly reached peak firing rate based on a transient depolarization, followed by an exponentially decreased firing rate and gradual repolarization of the membrane potential before its ending in a post-burst inhibition. Compared to the number of bursting neurons in oxytocin-treated cells (4/15, 26.7%, 1 burst/cell), the ratio of bursting neurons in PGE<sub>2</sub>-treated cells (71.4%, 1.2 burst/cell) was significantly higher (chi-square test,  $P = 0.043$ ). There was no significant difference in other parameters

including the latency of the first burst onset, amplitude, duration, intra-burst frequency, peak firing time, peak depolarization, peak firing rate, after hyperpolarization, and duration of post-burst inhibition (Fig. 2). This result is in agreement with the facilitatory effect of PGs on oxytocin secretion (Knigge et al., 2003).

#### **Burst-evoking effects of PGE<sub>2</sub> versus oxytocin following pretreatment with indomethacin:-**

Oxytocin is long known as a permissive factor of the milk ejection reflex. However, it remains unknown whether PG is an essential link in OXTR signaling cascade that leads to burst firing. To establish a causal relationship between burst firing and endogenous PGs during oxytocin treatment, we further analyzed the effects of oxytocin on burst firing in brain slices after blocking PG synthesis. After 10-minute application of indomethacin (1  $\mu$ M), PGE<sub>2</sub> (0.1  $\mu$ M, 10 min) still triggered four bursts in 2 of 7 oxytocin neurons (Fig. 2A). This incidence of burst cells (2/7cells) is insignificantly different from that in the absence of indomethacin (10/14 cells, chi-square test,  $P = 0.061$ ). Moreover, in the presence of indomethacin, none of the 17 neurons that received oxytocin treatment showed burst discharge (Fig. 2B), the burst incidence is not only significantly lower than that evoked by oxytocin only (4/15 cells, chi-square test,  $P = 0.023$ ) but also lower than that evoked by PGE<sub>2</sub> in the presence of indomethacin (chi-square test,  $P = 0.021$ ). Lastly, the features of bursts evoked by PGE<sub>2</sub> in the presence of indomethacin did not significantly differ from that by PGE<sub>2</sub> only except the delay of peak firing time (Fig. 2C). This finding is consistent with the hypothesis that PGs are essential mediators of oxytocin-evoked burst discharges.

#### **Effects of blocking ERK 1/2 phosphorylation on Cox-2 induction:-**

To link the inhibitory effect of indomethacin to OXTR signaling, we further examined Cox-2 expression in the SON following disturbance of burst-evoking OXTR signaling cascade in confocal microscopy of immunostaining and in Western blots. It is known that pERK 1/2 is essential for oxytocin-induced Cox-2 expression and PG synthesis (Terzidou et al., 2011) and that ERK 1/2 phosphorylation can be blocked by PD98059 (Wang & Hatton, 2007b). Thus, intracerebroventricular administration of PD98059 was used to validate that suppression of Cox-2 induction in OXTR signaling is responsible for indomethacin blockade of milk ejections. The result showed that Cox-2 expression was low in the SON and almost absent in the cytosol of oxytocin neurons in non-suckling rats ( $n = 4$ ). Cox-2 induction was significantly increased following 5-10 min of suckling ( $n = 4$ ), clearly in the cytosolic portion of oxytocin neurons. However, 30 min after intracerebroventricular administration of PD98059 (5  $\mu$ g, 2 $\mu$ l,  $n = 4$ ), suckling-induced Cox-2 expression was blocked. This result is consistent with the finding that pERK 1/2 mediates oxytocin-induced Cox-2 expression (Terzidou et al., 2011). Fig. 3 shows a typical set of immunostaining of Cox-2 in the dorsal SON from lactating rats with non-suckling, suckling and PD98059 before suckling.

Many stimuli activate oxytocin cells without inducing bursts. To verify that oxytocin-induced Cox-2 expression has specific role in burst firing, we analyzed the expression of Cox-2 positive cells in different types of SON neurons in eight hemi-slices from four rats in each group. Counting the number of cytosolic Cox-2-positive cells revealed that suckling increased the number of cytosolic Cox-2 from 16.7% to 88.3% (non-suckling versus suckling) in oxytocin neurons ( $n = 60$  in each group), 44.4% to 60.3% in non-oxytocin neurons (putative vasopressin neurons, each  $n = 63$ ), and 16% to 56% in non-magnocellular cells (putative astrocytes, each  $n = 25$ ). This result is similar to the effect of oxytocin on Cox-2 expression in SON cells in brain slices (Wang & Hatton, 2006) but shows a more significant inducing effect ( $P < 0.01$ , by Chi-square test) on oxytocin neurons (43/60) than on VP neurons (10/63). There was no significant difference ( $P > 0.05$  by Chi-square test) in the newly-induced number of cytosolic Cox-2 between oxytocin neurons (71.7%) and astrocytes (40%). After application of PD98059, cytosolic Cox-2 was reduced to 25% ( $n=60$ ) in oxytocin neurons.

After assessing Cox-2 immunostaining in confocal microscopy, we further quantified Cox-2 protein levels through Western blotting (Fig. 3B) in the same 12 rats that were used for IHC above. The result showed that suckling significantly changed Cox-2 levels ( $n = 4$ ,  $F = 8.084$ ,  $P = 0.02$  by ANOVA with Holm-Side post hoc analysis). Compared to non-suckling controls, suckling significantly increased Cox-2 levels ( $149.1 \pm 7.5\%$ ,  $t = 3.825$ ,  $P = 0.009$ ), which was blocked by the pretreatment with intracerebroventricular PD98059 ( $117.5 \pm 13.8\%$  control,  $t = 0.84$ ,  $P = 0.433$ ) 30 min before a 5-10 min suckling stimulation. This result is consistent with the finding of confocal microscopy of Cox-2 immunostaining.

#### **Molecular interactions among Cox-2, ERK 1/2 and actin:-**

As previously reviewed, there are extensive interactions between the burst-associated signals of oxytocin neurons in the milk-ejection reflex (Hatton & Wang, 2008), including the upstream pERK 1/2 and downstream actin cytoskeleton (Wang & Hatton, 2007b). To verify that Cox-2 signaling is causally associated with these burst-

evoking signals, we examined molecular interactions among Cox-2, ERK 1/2 and actin in suckling process (Fig 4) in 16 lactating rats including the 12 used above.

Relative to four non-suckling rats, suckling significantly increased ( $n = 4$ ,  $P < 0.05$  relative to non-suckling) Cox-2 and actin levels that were pulled down by tERK 1/2 in co-IP. These increased associations were blocked by intracerebroventricular PD98059 ( $n = 4$ ,  $P > 0.05$  relative to non-suckling). This finding is in agreement with that pERK 1/2 is responsible for Cox-2 induction (Zhong et al., 2003) and filamentous actin reorganization (Wang & Hatton, 2007b). Finally, intracerebroventricular indomethacin blocked suckling-induced increases in the co-IP between total ERK 1/2 and actin ( $n = 4$ ,  $P > 0.05$  relative to non-suckling), and decreased the significant level of suckling-evoked increases in the association between ERK 1/2 and Cox-2 ( $n = 4$ , from  $P < 0.01$  to  $P < 0.05$  relative to non-suckling). These findings are consistent with the conclusion that PGs have a positive feedback influence on pERK 1/2 and Cox-2 levels and their actions (Chen et al., 2012).

#### **Effects of blocking Cox-2 induction and ERK 1/2 phosphorylation on milk ejections:-**

The occurrence of milk ejections depends on synchronized burst firing (Wang et al., 1997; Wang et al., 2013a). Thus, if PGs are an essential mediator of burst firing in OXTR signaling, burst-dependent milk ejections should also be blocked by inhibition of PG synthesis in the SON and other nuclei containing oxytocin neurons around the third ventricle. To test this hypothesis, we observed the effects of blocking PG synthesis with intracerebroventricular application of indomethacin (100  $\mu$ M, 2  $\mu$ l) on milk ejections 3 h after the surgery. Thirty minutes after application of indomethacin into the third ventricle, pups were reunited with three lactating rats, and then the milk-ejection reflex and litter body weight gains were observed. Throughout 1 h suckling with 10 pups, none of the three rats showed clear milk ejections as indicated by the lack of simultaneous stretching or sucking responses of pups. Consistently, no significant body weight gain was observed in the three litters (increase by  $0.07 \pm 0.32$ g over 1 h suckling). In contrast, in 6 control rats with intracerebroventricular application of aCSF, litter body weight gains were  $3.1 \pm 0.5$  g with all dams showing milk ejections ( $8.2 \pm 1.5$  times over 1 h suckling). There are significant differences between the two groups in litter body weight gains (non-paired t-test, two tails,  $P = 0.0014$ ) and in the incidence of milk ejections (chi-square test,  $P = 0.0027$ ).

To validity the result observed above, we further measured milk-ejections by monitoring intramammary pressure changes in three rats before and after i.c.v. administration of indomethacin. As shown in Fig. 5, while indomethacin blocked the stretch responses of pups and litter body weight gains, it also blocked the periodic increases in intramammary pressure that occurred regularly before the inhibition of Cox-2 activity and were not interrupted by vehicle injections (Fig. 5). The inhibitory effects of indomethacin on milk ejections of the dams and milk availability to pups are consistent with its inhibition of burst firing in oxytocin neurons.

#### **Discussion:-**

The present study reveals that PGE<sub>2</sub> can evoke burst-like firing in supraoptic oxytocin neurons that depends on PG production. Moreover, suckling-evoked oxytocin secretion and the resultant milk ejections are closely related to Cox-2 induction through increases in pERK 1/2 in the OXTR signaling cascade. Conversely, pERK 1/2 association with Cox-2 and actin reorganization is modulated by PG production. Thus, PGs are essential mediator of suckling-elicited burst firing of oxytocin neurons in lactating rats. These findings suggest that PGs could be a target for the pharmacological modulation of pulsatile activity in the neuroendocrine system.

#### **PG mediation of suckling-evoked activation of oxytocin neurons:-**

It is well documented that many neuroactive substances, including glutamate, GABA, and noradrenalin can modulate oxytocin secretion and/or milk ejections and even trigger burst firing in oxytocin neurons (Crowley & Armstrong, 1992). Although PGs are not considered as neurotransmitters or classical hormones (Rivest, 2010), their autocrine and paracrine functions make them unique messenger molecules to mediate the effect of diverse, biologically active substances. PGs can be induced by cholinergic, adrenergic and histaminergic neurotransmitters as well as by neuropeptides such as vasopressin (Bugajski et al., 2004) and oxytocin (Wang & Hatton, 2006). During lactation, when burst firing is present, neurotransmission that promotes PG production is also increased. For instance, there are significant increases in noradrenergic innervations of oxytocin neurons during lactation (Michaloudi et al., 1997), which accounts for the adrenergic dependence of suckling-evoked oxytocin release (Bealer et al., 2010). PGs can be induced by activating  $\alpha_1$ -adrenergic receptors (Wang & Hatton, 2006) that evoke *in vitro* bursts (Wang & Hatton, 2004; 2005). Thus, PG could serve as a mediator in burst generation.

To support this conclusion, we verified that 1) the burst was different from spontaneously-produced spike clusters, and 2) that it was evoked in oxytocin neurons rather than vasopressin neurons. To confirm the first point, we set strict criteria to differentiate a burst from a spike cluster. To differentiate oxytocin from vasopressin neurons, particularly those with non-phasic firing and short lasting spike clusters, we identified if they expressed oxytocin and the unique features of burst from identified oxytocin neurons (Wang & Hatton, 2004; 2005). Following these criteria, we have no doubt to present our conclusion, even that the high co-expression rate of oxytocin and vasopressin in the SON in lactating rats may unavoidably make the result include a few of “vasopressinergic” cells in currently reported population. The present study provides further evidence that PGs are not only essential signals in OXTR-mediated burst discharges, but also more direct triggers than pERK 1/2 in oxytocin neurons of the SON, since PGE<sub>2</sub> evoked bursts had a higher incidence than oxytocin-evoked bursts did.

One remaining question is whether PG is a unique burst-inducer. As reported previously, PGE<sub>2</sub> can excite both oxytocin and vasopressin neurons (Wang & Hatton, 2006), however, bursts are only evoked in the former. This is likely attributable to the inherent “milk-ejection burst”-generating capacity of oxytocin neurons rather than an inherent machinery of phasic discharges of vasopressin neurons (Hatton & Wang, 2008). In this study, we did not use a prolonged oxytocin application protocol that had been applied previously, rather, we used a low dose and short-lasting protocol. The dose of oxytocin seems very low. However, it has been proved functional by several *in vitro* observations (Kow et al., 1991; Kuriyama et al., 1993; Wang et al., 2006). It is possible that in the *in vitro* environment, where oxytocin is less likely accumulated to the *in vivo* level, OXTRs are reasonably up-regulated while become more sensitive to lower dose of oxytocin. Certainly, effects of this oxytocin dose are comparable with higher doses or gradually increased doses used previously (Wang & Hatton, 2007b; a) in terms of burst-evoking efficacy. The only difference is likely at its less possible to evoke desensitization of OXTRs in the perfusion system and thus is more appropriate to reveal the inherent burst-evoking capacity of oxytocin.

Noteworthy is that the burst-evoking capacity is inherent to PGs. 1) The different incidences of bursts evoked by oxytocin versus PGE<sub>2</sub> were not due to drug concentration differences. In previous studies, we found that oxytocin applied in either a single concentration (10 pM for 30–45 min) or progressively increasing concentrations (1, 10, and 100 pM, each for 10 min) yielded the same effects on the burst incidence (Wang & Hatton, 2007b; a). Thus, burst-evoking capacity of oxytocin is relatively independent of its levels. 2) PGE<sub>2</sub>, a downstream signal of OXTR activation, will be increased following the induction of Cox-2. 3) Adding to these points, we have previously identified that there is no spontaneous burst in oxytocin neurons in brain slices and oxytocin-evoked burst are mediated by OXTRs. Thus, the higher burst incidence evoked by PGE<sub>2</sub> should reflect a natural result of OXTR activation. It is likely that PGs bypass many upstream signals (including those of inhibitory) to activate burst-generating machinery, thereby playing a mediator role in oxytocin-evoked bursts during suckling. As a result, PGs also trigger a bolus release of oxytocin (our unpublished ELISA assay of serum oxytocin levels during suckling) and thus mediate milk letdown.

#### **Signaling pathways underlying PG-evoked burst firing:-**

As reviewed previously (Hatton & Wang, 2008), suckling-evoked increases in intranuclear oxytocin levels activate OXTRs, which increases pERK 1/2, most likely by activating G protein  $\beta\gamma$  subunit signaling in oxytocin neurons. The present results further clarify the pivotal position of PGs in burst-associated OXTR signaling cascade.

In the present study, we found that there is a molecular association among pERK 1/2, Cox-2 and actin, which increased during suckling and could be interrupted by PD98059 and/or indomethacin. This finding suggests that pERK 1/2 is the upstream signal of Cox-2 induction. Moreover, PG signaling participates in pERK 1/2-associated actin reorganization since indomethacin blocked suckling-induced increases in the co-IP between ERK 1/2 and actin. Thus, pERK 1/2 promotion of filamentous actin networks is under intensive modulation or coordination of Cox-2/PGs. This proposal is consistent with our finding that indomethacin dramatically reduced the number of bursting cells following PGE<sub>2</sub> actions and with the report that PGE<sub>2</sub> activates ERK 1/2 and Cox-2 through activating the EP-2 subtype of PG receptors (Chen et al., 2012). It is not clear yet why blocking the induction of Cox-2 also delayed the peak firing time. It seems that the induced Cox-2 may exert additional effects on the opening of burst-related ion channels other than producing PGs. Further experiments are necessary to substantiate and clarify the details of these interactions.

PGE<sub>2</sub> can elicit bursts via several mechanisms. 1) PGE<sub>2</sub> activates EP4 receptors on postsynaptic neurons while reducing inhibitory postsynaptic currents through EP3 receptors (Shibuya et al., 2000), thereby exciting oxytocin

neurons. 2) At cellular levels, PGs enhance reorganization of the actin cytoskeleton at the cytosolic area close to the plasma membrane (Wang & Hatton, 2006), which is the key to promoting oxytocin-induced burst firing (Wang & Hatton, 2007b). 3) PGs could induce the burst by activating cyclic AMP/protein kinase A (Bugajski et al., 2004; Yang & Chen, 2008), which also mediates PG-evoked actin reorganization (Birukova et al., 2007). This possibility is supported by the burst-evoking effect of cyclic AMP that has association with PGs and oxytocin (Osipovskii & Polesskaia, 1981). 4) PGE<sub>2</sub> can increase the neuroexcitation via cAMP-mediated upregulation of persistent tetrodotoxin-resistant Na<sup>+</sup> currents as shown in female Ah-type trigeminal ganglion neurons in rats (Liu and Duan, 2016), and thus, facilitates burst generation. Here, we propose that suckling-evoked intranuclear release of oxytocin (Bealer et al., 2010) induces PG production and release, and PGs further activate protein kinase A and evoke actin reorganization in coordination with pERK 1/2. As a result, the activity or spatial distribution of burst-associated ion channels is optimized to a pattern that facilitates sudden opening of many cation channels, leading to the burst firing.

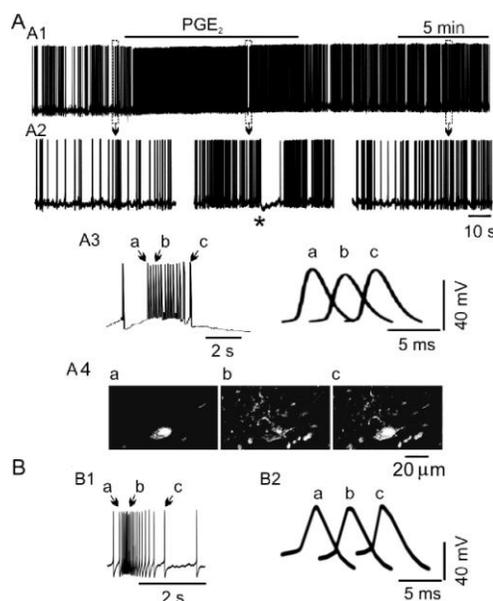
### Implications:-

Pulsatile secretion and the underlying burst firing of many neurons and endocrine cells could share common mechanisms. Understanding the role of PGs in burst generation is critical to establishing the signaling cascade leading to the pulsatile release of oxytocin following the burst discharges during suckling, parturition (Higuchi et al., 1986; Petraglia et al., 2010) and sexual orgasm (Carmichael et al., 1994). Thus, it will help us to understand oxytocin-related pathogenesis and then explore the therapeutic potential of the pulsatile pattern of action, such as prevention of precancerous lesions of the mammary glands by pulsatile release of oxytocin during lactation (Liu et al., 2016). Importantly, PGs are produced throughout the human body and function as locally acting messenger molecules and thus understanding the mediator roles of PGs in primary cytokine provides a useful model for studying the mechanism underlying pulsatile release of other hormones and neurotransmitters, such as GnRH and inflammatory cytokines. For instance, selective inhibition of EP2/EP4 can suppress inflammatory responses and restores endometrial functional receptivity (Arosh et al., 2015). Thus, further studying the regulation of PG production and actions will extensively influence our view of autocrine or paracrine regulation of all life processes through this unique mediator.

### Acknowledgements:-

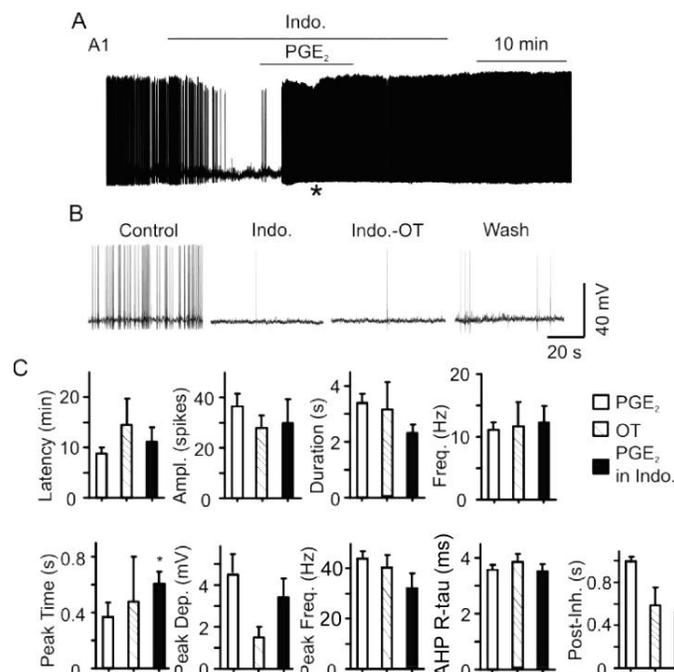
Thanks to late Dr. Glenn I. Hatton for initiating this study at the University of California, Riverside, CA. This research was sponsored by the National Natural Science Foundation of China (Grant No. 31471113 and 31271254), College of Natural and Agricultural Sciences, University of California, Riverside, the Higher Education Talents Funds of Heilongjiang Province (grant No. 002000154) and Natural Science Foundation of Guangdong Province (grant No. S2012010009014).

### Figure legends

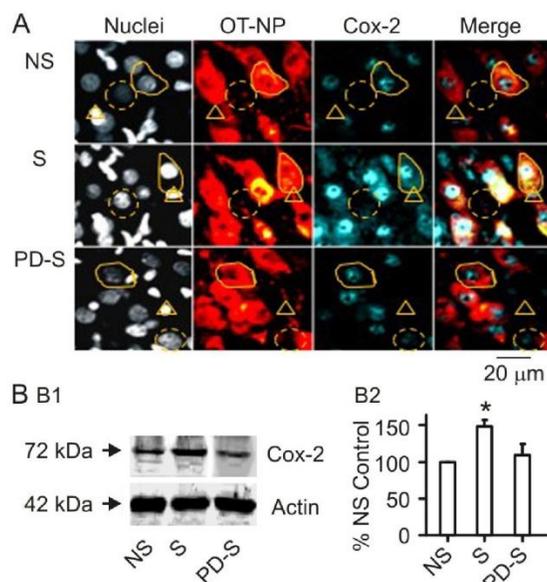


**Figure 1:-** Effects of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) on the firing activity of putative oxytocin (OT) neurons in the supraoptic nucleus (SON) of brain slices from lactating rats. A - B. Current-clamp recordings of the firing activity.

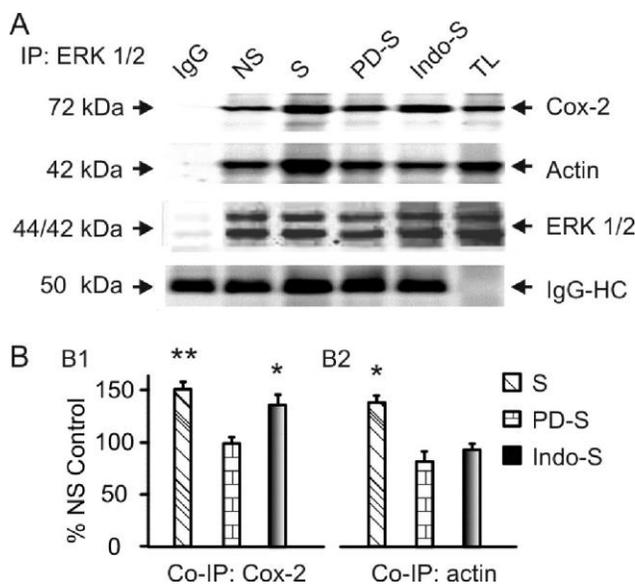
A1. Full recording showing a representative burst response of oxytocin neurons to PGE<sub>2</sub> (0.1 μM). A2. Expanded episodes from the boxed areas of A1 (arrows). A3. Arrow a-c in the left panel indicates the spikes at different portions of the burst: initiation (a), peak firing (b) and end (c). The right panels show three expanded spikes at the different portions. A4. Immunocytochemical identification of oxytocin neurophysin (OT-NP, marker for OT neurons) in Lucifer yellow (LY) labeled putative OT neurons. A4a: LY-labeled cell. A4b: OT-NP-positive cells. A4c: Overlap of the 2 fluorophores (LY and Alexa Fluor 647). B. OT-evoked burst firing (B1) and the expanded spikes at their different portions (B2).



**Figure 2:-** Effects of indomethacin (Indo.) on PGE<sub>2</sub> and OT-evoked burst firing. A-B. Current-clamp recordings of the firing activity. A. Effects of pretreatment with indomethacin (1 μM) on PGE<sub>2</sub> (0.1 μM, 10 min)-evoked changes in firing activity. Note that a burst firing occurred as indicated by the asterisk. B. Effects of pretreatment with indomethacin (1 μM) on OT (Indo-OT, 10 pM, 10 min)-evoked changes in firing activity. C. Bar graphs showing burst characteristics evoked by PGE<sub>2</sub> (open bar, all analyses were based on 12 bursts except the latency that used the first 10 bursts), OT (hatching bar, 4 bursts), and PGE<sub>2</sub> in the presence of indomethacin (solid bar, 4 bursts except the latency using the first 2 bursts). They show the latency for the first burst, burst amplitude (Ampl), duration, average frequency (Freq), time of the peak firing rate from burst onset, membrane depolarization (Dep) at peak firing rate, peak firing rate (Peak Freq.), spike after hyperpolarization (AHP) and duration of post-burst inhibition (Post-Inh), respectively. \*, P < 0.05 compared to PGE<sub>2</sub>-only by two tailed non-paired t-test.



**Figure 3:-** Suckling-induced expression of cyclooxygenase 2 (Cox-2) in OT neurons in the SON of urethane-anesthetized lactating rats. A. Confocal microscopic images taken from the dorsal section of the SON. From left to the right: the Hoechst staining of nuclei, immunostaining of OT-NP, Cox-2 and their merges; from top to the bottom: non-suckling (NS), suckling (S), suckling after intracerebroventricular (i.c.v.) injection of PD98059 (PD-S, 5 µg, 2 µl), respectively. The irregular circles, dashed round circle and triangles represent one OT-NP-positive, one OT-NP-negative and one non-magnocellular cells, respectively. B. Western blots showing the blocking effects of PD98059 on suckling-induced expression of Cox-2. B1, representative bands and B2, bar graph summarizing these effects. \*,  $P < 0.05$  compared to non-suckling control in one way Repeated Measurement ANOVA with Holm-Sidek Post Hoc analysis.



**Figure 4:-** Molecular associations and interactions between extracellular signal-regulated protein kinase (ERK) 1/2, Cox-2, and actin in the SON during suckling. A. Exemplary bands showing co-immunoprecipitation (IP) of ERK 1/2 with Cox-2 and actin following i.c.v. administration of control solution, PD98059 and indomethacin respectively during suckling in lactating rats. The bands from left to the right represent mock IP with non-specific immunoglobulin G (IgG), and with ERK 1/2 antibody in the groups of non-suckling, suckling, suckling following PD98059 (5 µg, 2 µl) or indomethacin (Indo-S, 100 µM, 2 µl), and with total lysate (TL), respectively. B. Bar graphs summarizing the effects of suckling on the molecular associations between ERK 1/2 and Cox-2 (B1) and between ERK 1/2 and actin (B2) after the different treatments. Note, IgG-HC, IgG heavy chain; \*,  $P < 0.05$  and \*\*,  $P < 0.01$

compared to non-suckling control (100%); each group contains samples from four rats. Other annotations refer to Figure 3.



**Figure 5:-** Effects of blocking Cox-2 induction on milk ejections. Effects of i.c.v. application of indomethacin (Indo, 100  $\mu$ M, 2  $\mu$ l) on intramammary pressure (IMP) during suckling. The downward arrows represent starting suckling of 10 pups, i.c.v. injection of vehicle and indo., respectively. The right inset indicates the IMP responses to 1 mU OXT (i.v.) as a biologic assay of the relative amount of endogenous OXT released in response to suckling stimulation. Other annotations refer to Figure 3 and 4.

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