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# ATP EXCITES MOUSE VOMERONASAL SENSORY NEURONS THROUGH ACTIVATION OF P2X RECEPTORS

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**Abstract—Purinergic signaling through activation of P2X and P2Y receptors is critically important in the chemical senses. In the mouse main olfactory epithelium (MOE), adenosine 5′-triphosphate (ATP) elicits an increase in intracellular calcium ($[Ca^{2+}]_i$) and reduces the responsiveness of olfactory sensory neurons to odorants through activation of P2X and P2Y receptors. We investigated the role of purinergic signaling in vomeronasal sensory neuron (VSN)s from the mouse vomeronasal organ (VNO), an olfactory organ distinct from the MOE that responds to many conspecific chemical cues. Using a combination of calcium imaging and patch-clamp electrophysiology with isolated VSNs, we demonstrated that ATP elicits an increase in $[Ca^{2+}]_i$ and an inward current with similar $EC_{50}$s. Neither adenosine nor the P2Y receptor ligands adenosine 5′-diphosphate, uridine 5′-triphosphate, and uridine-5′-disphosphate could mimic either effect of ATP. Moreover, the increase in $[Ca^{2+}]_i$ required the presence of extracellular calcium and the inward current elicited by ATP was partially blocked by the P2X receptor antagonists pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate and 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate. Consistent with the activation of P2X receptors, we detected gene expression of the P2X1 and 3 receptors in the VNO by Reverse transcription polymerase chain reaction (RT-PCR). When co-delivered with dilute urine, a natural stimulus, ATP significantly increased the inward current above that elicited by dilute urine or ATP alone. Mechanical stimulation of the VNO induced the release of ATP, detected by luciferin–luciferase luminometry, and this release of ATP was completely abolished in the presence of the connexin/pannexin hemichannel blocker, carbenoxolone. We conclude that the release of ATP could occur during the activity of the vasomotor pump that facilitates the movement of chemicals into the VNO for detection by VSNs. This mechanism could lead to a global increase in excitability and the chemosensory response in VSNs through activation of P2X receptors.**


**Key words: pheromones, vomeronasal organ, adenosine 5′-triphosphate, purinergic receptors, excitability, perforated patch clamp.**

## INTRODUCTION

The mammalian vomeronasal organ (VNO), the primary sensory organ of the accessory olfactory system, is critical for normal aggressive and reproductive behaviors. Ablation of the VNO results in a severe disruption of sexual behaviors in male and female rodents, such that mounting and lordosis are no longer preferentially directed toward the opposite sex (Stowers et al., 2002; Samuelsen and Meredith, 2009). The rodent VNO is a paired tubular structure encased in a bony capsule at the caudal tip of the nose that is anatomically segregated from the rest of the nasal cavity. The sensory epithelium of the VNO is richly populated by vomeronasal sensory neuron (VSN)s, supporting cells, and basal cells. VSNs are bipolar sensory neurons with a single dendrite that extends into the lumen of the VNO and a single axon that projects to the accessory olfactory bulb (Liman and Corey, 1996). It is within the microvilli of the dendritic knob that the chemosensory signal transduction machinery is located (Menco et al., 2001). Apical VSNs express V1R receptors and $G_{\alpha i}$ while basal VSNs express V2R receptors and $G_{\alpha o}$ (Berghard and Buck, 1996). In mice, many conspecific chemical cues are released into urine and can be detected by VSNs (Holy et al., 2000). These chemicals can activate V1R/V2R receptors, which lead to the production of diacylglycerol (DAG) and inositol-1,4,5-triphosphate ($IP_3$) by phospholipase C (PLC) (Krieger et al., 1999). DAG opens transient receptor potential channel type 2 (TRPC2) and produces an influx of sodium and calcium into the microvilli, the latter of which can activate calcium-activated chloride channel (CACC)s and amplify the primary response (Yang and Delay, 2010; Kim et al., 2011).

Purine nucleotides, such as adenosine 5′-triphosphate (ATP), exert their effects on cellular physiology by binding to three classes of receptors (1) ionotropic P2X receptors (P2X1–7), (2) G-protein coupled P2Y receptors (P2Y1, 2, 4, 6, 11, 12, 13, 14), and (3) G-protein coupled adenosine receptors (A1, 2A, 2B, 3) (for review: Burnstock, 2007).

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*Abbreviations:* AC, adenylyl cyclase; ADP, adenosine 5′-disphosphate; ANOVA, analysis of variance; ATP, adenosine 5′-triphosphate; BK, big-conductance calcium-activated potassium channel; cAMP, cyclic adenosine 5′-monophosphate; CACC, calcium-activated chloride channel; ConA, concanavlin A; DAG, diacylglycerol; fura-2, AM, Fura-2, acetoxymethyl; $IP_3$, inositol-1,4,5-trisphosphate; MOE, main olfactory epithelium; OSN, olfactory sensory neuron; PLC, phospholipase C; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate; RT-PCR, Reverse transcription polymerase chain reaction; RLU, relative light unit; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate; TRPC2, transient receptor potential channel type 2; UDP, uridine 5′-disphosphate; UTP, uridine 5′-triphosphate; VDCC, voltage dependent calcium channel; VNO, vomeronasal organ; VSN, vomeronasal sensory neuron.

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*P2X receptors* are trimers, with each monomer consisting of two transmembrane domains and large extracellular loops. Monomer subtypes can form both homomeric (i.e. P2X3) as well as heteromeric (i.e. P2X2/3) P2X receptors. The pore of P2X receptors is permeable to both monovalent and divalent cations, and is opened by the binding of ATP as well as a variety of synthetic, non-hydrolyzable ATP analogs (Ding and Sachs, 1999; Egan et al., 2006). *P2Y receptors* are activated not only by ATP, but by adenosine 5′-diphosphate (ADP), uridine 5′-triphosphate (UTP), uridine 5′-diphosphate (UDP), and UDP-glucose. *In vitro*, P2Y receptors typically (1) activate the cyclic adenosine 5′-monophosphate (cAMP)/adenylyl cyclase (AC) pathway (P2Y11), (2) inhibit the cAMP/AC pathway (P2Y2, 4, 12, 13, 14), or (3) activate the $IP_3$/PLC pathway (P2Y1, 2, 4, 6, 11) (for review: Abbracchio et al., 2006). *Adenosine receptors* are activated by adenosine, which is typically produced by the rapid breakdown of extracellular ATP by transmembrane ATPases (Burnstock, 2007). In the mouse main olfactory epithelium (MOE), P2X and P2Y receptors are expressed in the sensory epithelium. ATP increases intracellular calcium ($[Ca^{2+}]_I$) and reduces the responsiveness of olfactory sensory neuron (OSN)s to odorants through activation of P2X and P2Y receptors (Hegg et al., 2003). Furthermore, ATP induces the differentiation of basal progenitor cells into new OSNs (Hassenklover et al., 2009; Jia et al., 2009; Gao et al., 2010). Exposure to highly concentrated odorants, such as is possible with pollution, appears to result in the release of ATP from the MOE (Hegg and Lucero, 2006). Therefore, purinergic signaling in the MOE is believed to play a neuroprotective and neuroregenerative role by protecting OSNs against further excitotoxic challenge and promoting basal progenitor cells to differentiate into new OSNs. While P2X3 and P2Y2 receptors are expressed in the sensory epithelium of the rat VNO, the role of purinergic signaling in the VNO has not yet been determined (Gayle and Burnstock, 2004).

Fluid movement into the VNO lumen of guinea pigs and mice exposed to urine, a natural stimulus, indicates the VNO is able to access the nasal cavity despite its anatomical segregation (Wysocki and Beauchamp, 1980; Ogura et al., 2010). Along the lateral wall of the VNO bony capsule are large blood vessels that are extensively innervated by sympathetic nerves from the superior cervical ganglion (Valeria Canto Soler and Suburo, 1998). Stimulation of the nasopalatine nerves in anesthetized hamsters and mice, along which the sympathetic nerves from the superior cervical ganglion enter the VNO, results in the activation of the vasomotor pump that facilitates the movement of chemicals into the VNO for detection by VSNs (Meredith and O'Connell, 1979; Ben-Shaul et al., 2010). In awake, behaving male hamsters, the pumping of the VNO occurs naturally in the presence of a novel environment or a sexually receptive female hamster (Meredith, 1995). Purine nucleotides are released in a mechanosensitive manner from the retina, urinary bladder, nephron of the kidney, polarized epithelium of the lung, and cochlea (Homolya et al., 2000; Newman, 2001; Zhao et al., 2005; Sipos et al., 2009; Chen et al., 2010). In the nephron of the kidney and cochlea, ATP is released by mechanosensitive connexin hemichannels (Zhao et al., 2005; Sipos et al., 2009). Connexin 36, 31.1, and 30.1 are expressed in the sensory epithelium of the mouse VNO (Zhang and Restrepo, 2003; Zheng-Fischhofer et al., 2007a,b). In this report, we provide evidence that ATP elicited a concentration-dependent increase in $[Ca^{2+}]_I$ and an inward current in isolated mouse VSNs through activation of P2X receptors. Moreover, we show that ATP increased the responsiveness of VSNs to dilute urine. We conclude that ATP could be released from the VNO when mechanically stimulated, and that activation of P2X receptors may provide a means of globally increasing excitability and the chemosensory response in VSNs when the vasomotor pump is active.

## EXPERIMENTAL PROCEDURES

### VNO dissection and VSN isolation

Male and female C57BL/6 and BALB/C mice (Charles River Laboratory, Wilmington, MA) were maintained on a 12-h light/12-h dark cycle with food and water provided *ad libitum* in accordance with the University of Vermont's Institutional Animal Care and Use Committee. Mice were euthanized by $CO_2$ asphyxiation followed by cervical dislocation. The head was removed, cut bilaterally, and the entire VNO was dissected out. The VNO was removed from both halves of the bony capsule and VSNs were isolated by mechanical dissection in nominally divalent cation-free Ringer's solution (in mM: 140 NaCl, 5 KCl, 10 HEPES, 10 glucose; pH 7.4) supplemented with ≈50 μg/mL papain for 10 min. After filtration through a nylon mesh, VSNs were maintained in Ringer's solution (in mM: 138 NaCl, 5 KCl, 10 HEPES, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose; pH 7.4) supplemented with ≈10 μg/mL leupeptin for 15 min to inhibit papain and plated on glass coverslips for calcium imaging and patch-clamp electrophysiology. VSNs remained viable for up to 4 h.

### Chemical/drug preparation and urine collection

All chemicals/drugs were dissolved in Ringer's solution to their final concentrations unless noted otherwise. Urine was diluted to a final concentration of 1:100 at which there is no effect on osmolarity or detectable level of ATP (Turner et al., 2010; Yang and Delay, 2010). ATP magnesium salt, ADP monopotassium salt dihydrate, UTP trisodium salt hydrate, UDP disodium salt hydrate, adenosine, carbenoxolone disodium salt, gramicidin, and dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO). Papain and concanavlin A (ConA) were purchased from Calbiochem (Darmstadt, Germany). Leupeptin was purchased from USB (Cleveland, OH). Fura-2, acetoxymethyl (fura-2, AM) and pluronic F-127 were purchased from Invitrogen (Carlsbad, CA). Pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS) and 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP) were both kind gifts from Dr. John Tompkins of the anatomy and neurobiology department at the University of Vermont (Burlington, VT). Urine was collected from at least 20 male and female mice, and filtered through a 0.2-μm filter before storage in single use aliquots at −80 °C.

### Calcium imaging

VSNs were plated on ConA-coated glass coverslips, incubated in 5 μM fura-2, AM and 0.05% pluronic F-127 dissolved in DMSO in Ringer's solution for 15 min, and washed in Ringer's solution for 10 min before each recording session. Only VSNs with a stable baseline were used. Images were captured every 5 s with