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# Blocking P2X receptors can inhibit the injury-induced proliferation of olfactory epithelium progenitor cells in adult mouse

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ABSTRACT

*Objective:* The olfactory epithelium (OE) is unusual for its remarkable regenerative capacity and sustained neurogenesis of olfactory receptor neurons (ORNs) throughout adult life. Regeneration of ORNs is accomplished by basal cells in the OE, including stem cells and progenitor cells. Although there is considerable knowledge about the roles of OE basal cells in ORN turnover, the molecular mechanism that regulates the proliferation and differentiation of adult OE basal cells is not fully understood. As intercellular signaling molecules, purines have been reported to meditate proliferation, differentiation and migration of many kinds of neural stem cells. However, it is still unclear whether ATP, which could be released by injured ORNs, plays a role in regulating neurogenesis in ORN turnover.

*Methods:* RT-PCR and immunohistochemistry were used to detect the expression of ionotropic purinergic receptors-P2X receptors in adult mouse OE. By using the olfactory bulbectomy model and *in vivo* administration of P2X receptors antagonists, the function of P2X receptors in regulating the proliferation of OE progenitor cell was evaluated.

*Results:* We found that basal cells in the adult mouse OE express functional P2X receptors, and blocking the activities of P2X receptors can significantly inhibit the injury-induced proliferation of OE basal cells.

*Conclusion:* Our research provides evidence in support of the hypothesis that purinergic signaling can serve as a paracrine signal in regulating the neurogenesis of OE in adult mouse.



## 1. Introduction

The olfactory epithelium (OE) is unusual for its remarkable regenerative capacity and sustained neurogenesis of olfactory receptor neurons (ORNs) throughout adult life [1]. Regenerated ORNs are differentiated from the basal cells in OE. OE basal cells can be morphologically divided to horizontal basal cells (HBCs) and global basal cells (GBCs) [2]. HBCs are very quiescent but with multipotency. HBSc are the stem cells of OE and can be activated when OE is damaged severely [3]. In contrast, in normal turnover process of ORNs, GBCs are active and are the main source of newly generated ORNs [4]. Based on these characters, GBCs are called as the olfactory epithelium progenitors or olfactory progenitor cells [2]. In the turnover of ORNs, neurogenesis is prompted by injury of ORNs [5]. Many factors participate in the regulation of this process. It has been reported that leukemia inhibitory factor (LIF), bFGF, EGF, and TGF-α all serve as proliferative factors in stimulating the injury-induced proliferation of adult OE progenitors [6,7]. Purinergic signaling has been reported to play important roles in meditating proliferation, differentiation and migration of neural stem cells (reviewed in [8]). But it is not clear whether purinergic signaling can function in ORN turnover.

Two recent publications suggest that purinergic signaling is involved in regulating the proliferation of OE progenitor cells. HassenklÖver et al. reported that nucleotides could induce $Ca^{2+}$ signaling and regulate cell turnover of OE progenitor cells in larval *Xenopus laevis* [9]. Another publication indicates that nasal instillation of ATP can increase the proliferation of OE progenitor cells in normal adult mouse [10]. But it still remains unclear whether purinergic signaling is involved in regulating injury-induced proliferation of OE basal cells in adult mouse.

Purinergic receptors were separated to P1 and P2 receptors (P1R and P2R) based on their different ligands adenosine and ATP/ADP, respectively. P2R were subdivided into P2X and P2Y subtypes on the basis of pharmacology. P2X receptors are ionotropic purinergic receptors and permeable to $Na^+$, $K^+$ and $Ca^{2+}$ when

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**Table 1**
Details of primers used for RT-PCR.

| Protein | Primers (5′ → 3′) | Product length (bp) | Tm (°C) | GenBank accession number |
|---|---|---|---|---|
| $P2X_1$ | AAAGCCCAAGGTATTCGCACAGG<br>AGGTTCTTCTCCCCGTACAGTCCAT | 437 | 59 | NM_008771 |
| $P2X_2$ | GGGGCAGTGTAGTCAGCATCA<br>GTCGAGCCTCCGGAAAGAATA | 557 | 57 | NM_153400 |
| $P2X_3$ | ACCGGCCGCTGCGTGAACTAC<br>CCGAGCTGATGATGGTGGGAATGAT | 577 | 60 | NM_145526 |

binding to ATP [11]. Here, our study demonstrates that $P2X_{1-3}$ receptors are expressed in the basal compartment of adult mouse OE, where the olfactory neural progenitors reside. Blocking purinergic receptors can significantly inhibit the injury-induced proliferation of OE progenitors *in vivo*. In contrast, inhibition of purinergic receptors did not influence apoptosis or removal of injured ORNs. This study demonstrates that purinergic signaling can regulate injury-induced neurogenesis of ORNs in adult mice.

## 2. Materials and methods

### 2.1. RT-PCR analysis

All protocols of animal experiments followed were approved by the Committee of Animal Use for Research and Education of the Second Military Medical University. Adult C57BL/6 male mice (8 weeks old) were euthanized with a lethal dose of pentobarbital sodium. Total RNA was isolated from the olfactory mucosa using TRIzol (Gibco BRL) and the RNA concentration was measured photometrically. After RNA extraction, samples were digested with RNase-free DNase I and cDNA was synthesized using RevertAid™ First Strand cDNA Synthesis Kit (Fernentas). Details of $P2X_{1-3}$ receptor PCR primers are described in Table 1. Primers for GAPDH and BiP were designed as reported [12]. In order to exclude contamination with genomic DNA, a primer pair for the immunoglobulin heavy chain binding protein (BiP) was used as reported previously, and the binding sites of these primers were separated by an intron in the BiP gene [12].

### 2.2. Olfactory bulbectomies (OBX) and tissue preparation

Unilateral or bilateral OBX on adult C57BL/6 male mice (8 weeks old, ≥25 g) were performed as previously described [13]. Mice were killed and perfused with 4% paraformaldehyde (PFA) in 0.1 M ice-cold phosphate buffer after 4 days OBX. Nasal cavity tissues were collected and placed in 4% PFA for 2 h in 4 °C, and then sequentially bathed in 10% and 30% sucrose for cryoprotection. Tissues were embedded in OCT compound (Tissue-Tek, USA), sectioned at 10 μm and then frozen for storage.

### 2.3. Immunohistochemistry

Immunohistochemistry for $P2X_{1-3}$ receptors, type III neuron-specific tubulin (TuJ1), and cleaved caspase-3 was performed using polyclonal rabbit anti-$P2X_{1-3}$ (Roche Bioscience), monoclonal mouse anti-TuJ1 (1:2000, Promega) and monoclonal rabbit anti-cleaved caspase-3 (1:200, Cell Signaling). The specificity of the P2X antibody has been verified previously [14]. Frozen tissue sections were rinsed with 0.1 M PBS for 5 min, permeabilized with 0.3% Triton X-100 and blocked with normal goat serum in 0.1 M PBS. Then tissue sections were incubated with primary antibody at 4 °C overnight. After washing with PBS, FITC or TRITC conjugated secondary antibodies (Jackson ImmunoResearch) were added for 1 h at room temperature. Slides were washed, mounted and examined using a Nikon fluorescent microscope.

### 2.4. In vivo administration of P2X antagonists

Suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) are functional selective antagonists for P2 receptors [15]. To investigate the functions of P2X receptors in regulating OE progenitor proliferation, we used a modified method from a previous study [16], such that bilateral OBX adult C57BL/6 male mice were injected twice daily with 50 μmol/kg suramin + PPADS or an equivalent volume of Ringer's solution.

### 2.5. Bromodeoxyuridine incorporation and detection

Bromodeoxyuridine (BrdU) incorporation assay was carried out as reported previously [17]. Mice were injected with 30 mg/kg BrdU at 3 and 1 h before perfusion. BrdU detection was processed according to a standard immunohistochemistry protocol with the exception that a 30 min pretreatment in 3 M HCl before the incubation with monoclonal mouse anti-BrdU (1:200, NeoMarkers). OE within the third–fourth endoturbinates and nasal septum (Fig. 3A) were used to assess the OE thickness and the number of basal BrdU positive cells as reported previously [17]. Each group contained four mice, and four non-serial sections from each mouse were used for immunohistochemistry. Statistical analysis was performed using an unpaired Student's *t*-test. All data are presented as mean ± s.d.

## 3. Results

### 3.1. $P2X_{1-3}$ are expressed in the basal progenitors of adult mouse OE

Our RT-PCR results revealed that all of the ionotropic purinergic receptors $P2X_{1-3}$ were expressed in the adult mouse olfactory mucosa (Fig. 1A). The PCR product of spliced BiP mRNA was 560 bp and product of non-spliced genome was 876 bp [12]. BiP was amplified from the OE cDNA and only the 560 bp PCR product was

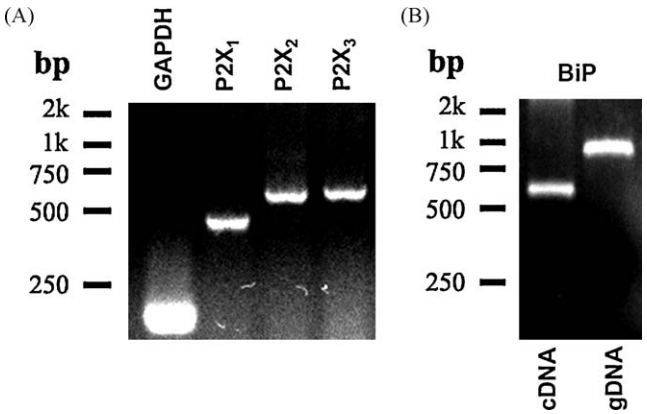


**Fig. 1.** RT-PCR analysis of P2X receptor expression in the adult mouse mucosa. (A) Adult mouse OE expresses mRNA for $P2X_{1-3}$ receptors and (B) GAPDH as a positive control. Using the cDNA of olfactory mucosa, a 560 bp sequence was amplified. With olfactory mucosa genomic DNA (gDNA), a 876 bp PCR product was amplified.

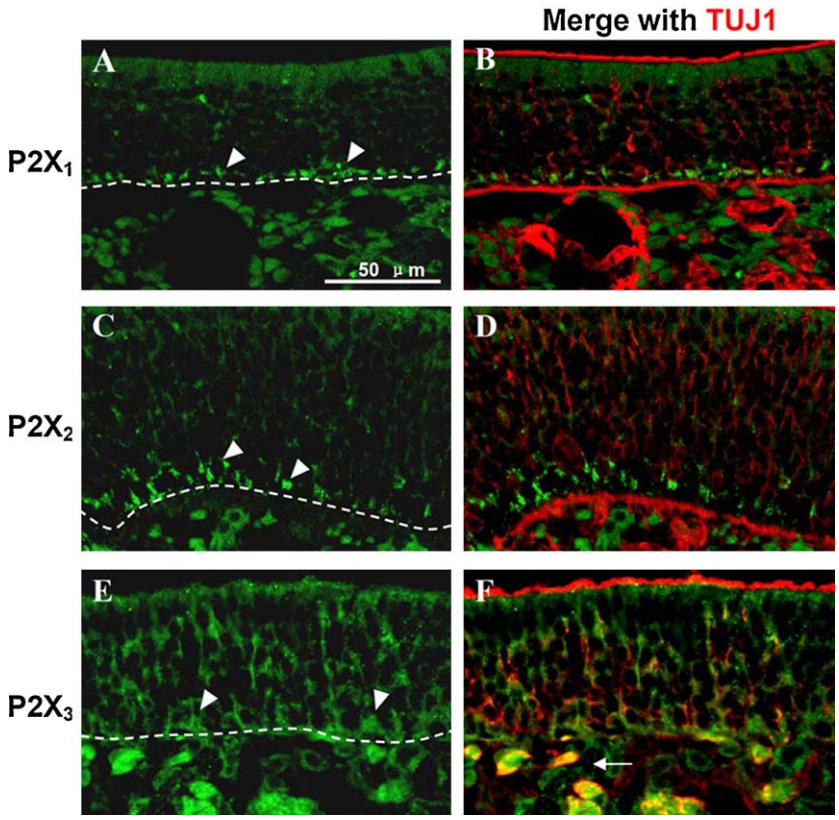


**Fig. 2.** Expression pattern of $P2X_1$, $P2X_2$ and $P2X_3$ receptors in adult mouse OE. (A, C) $P2X_1$ and $P2X_2$ receptors are expressed in the basal cells (arrow heads), soma of sustentacular cells, and mesenchymal cells of the lamina propria. (B, D) No detectable $P2X_{1-2}$ expression was seen in the ORNs when merged with TuJ1. (E) $P2X_3$ receptors are expressed in the basal cells (arrow heads) and sustentacular cells and (F) high expression of $P2X_3$ receptors can be detected in the olfactory nerves (indicated by arrows). Scales bar in (A–F) = 50 μm, dashed lines indicate the basal lamina of olfactory mucosa.

obtained (Fig. 1B), indicating that the mRNA was not contaminated with genome DNA. We also found mRNA expression of $P2X_{4-7}$ in the adult mouse olfactory mucosa (data not shown).

To identify the localization of P2X receptors in OE, tissue sections were co-stained with $P2X_{1-3}$ and TuJ1 antibodies. TuJ1 is expressed in the mature and immature ORNs [17]. Our results showed that $P2X_1$, $P2X_2$ and $P2X_3$ receptors were localized at the basal part of OE. $P2X_1$ and $P2X_2$ were mainly localized to the soma of sustentacular cells and basal cells (Fig. 2A and C). No significant colocalization with TuJ1 was detected (Fig. 2B and D). $P2X_3$ receptors were widely localized in the OE, including the sustentacular cells, ORNs and basal cells (Fig. 2E). Strong colocalization of $P2X_3$ with TuJ1 was detected in the olfactory nerve (Fig. 2F). We also found immunoreactivity to $P2X_{4-7}$

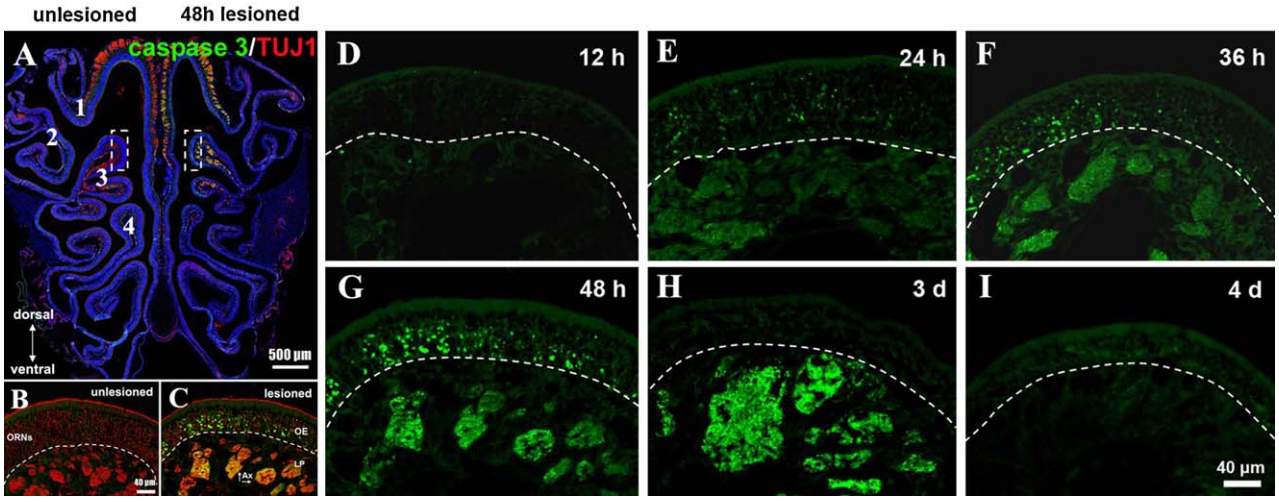


**Fig. 3.** Expression pattern of cleaved caspase-3 in the OE after OBX. (A) Cleaved caspase-3 was detected in the lesioned lateral OE 48 h after OBX and when merged with TuJ1, is localized in the ORNs. (B) No cleaved caspase-3 was detected in the unlesioned lateral. (C) Cleaved caspase-3 is expressed in the soma of ORNs and olfactory nerves (indicated by arrows). OE: olfactory epithelium, LP: lamina propria, AX: axons and (D–I) cleaved caspase-3 started to express in the OE 24 h after OBX (E), and reached expression peak at 48 h (G), only expression in olfactory nerves was detected 3d after OBX (H), no cleaved caspase-3 was detected 4 days after OBX (I). Dashed lines indicate the basal lamina of olfactory mucosa. Scale bar in (A) = 500 μm, in (B–I) = 40 μm.

receptors in the ORNs, and $P2X_5$ receptors only in the axons of ORNs (data not shown). Localization of P2X receptors in the basal progenitor cells of adult mouse OE suggests that purinergic signaling may play a role in regulating adult ORN neurogenesis.

### 3.2. P2X receptors antagonism inhibits the proliferation of the OE basal progenitors after OBX

An olfactory bulbectomy (OBX) model was used to investigate neurogenesis in adult mouse OE. After removal of olfactory bulbs, the ORNs undergo a process of synchronizing apoptosis, and the olfactory neural progenitor cells start to proliferate and differentiate into new ORNs [2]. Firstly, immunohistochemistry of cleaved caspase-3 was performed to investigate the time course of ORN apoptosis after OBX. Immunoreactivity to cleaved caspase-3 was detected in the OE at 24 h following OBX (Fig. 3E), and reached its peak 48 h after OBX (Fig. 3G). At 4 days after OBX, no expression of cleaved caspase-3 was detected in the OE and the OE thickness reached a minimal level (Fig. 3I). Our data indicated that after 4 days OBX, most of the ORNs in the OE had apoptosised. This result was identical to the western blotting analysis of caspase-3 in the OE after OBX as seen in previous studies [18].

Suramin and PPADS are general P2R antagonists and can block the activities of $P2X_2$ and $P2X_3$ receptors [15]. Injured ORNs can release ATP as reported previously [16], and all the injured ORNs had apoptosised after 4 days OBX as mentioned before. So we administered P2R antagonists to the mice for 4 days after OBX, and our data revealed that *in vivo* administration of P2R antagonists for 4 days could inhibit the proliferation of progenitor cells significantly (Fig. 4A and B). After 4 days OBX, the basal BrdU positive cells were 20 cells/mm OE in the control group, which had only been injected with saline buffer (Fig. 4C), and this value is equal to the published one [17]. However, in the group where antagonists were administered, the basal BrdU positive cells were significantly reduced to 12 cells/mm OE (Fig. 4C, $P < 0.05$, $n = 4$). The OE thickness of the test group was thinner than the control group (Fig. 4D), and it suggested that less cells were generated after OBX in the antagonists administration group.

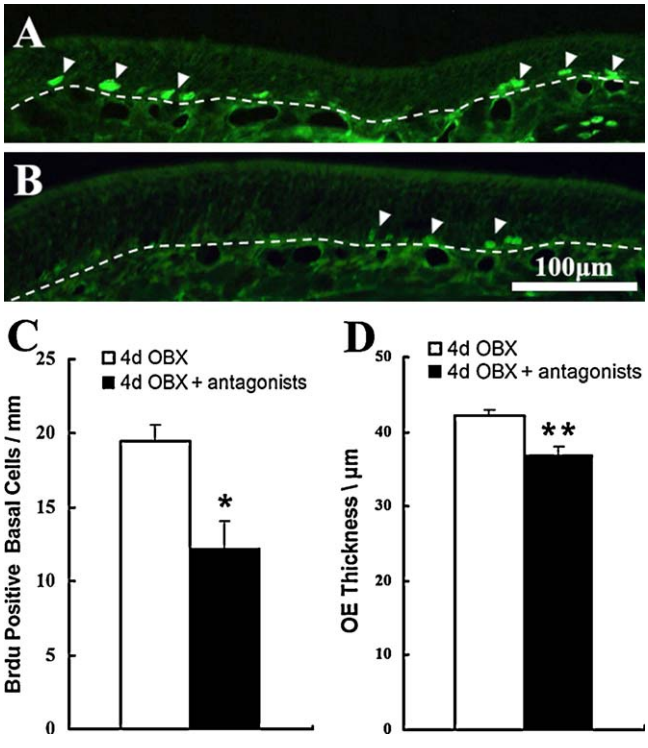


**Fig. 4.** Effect of suramin and PPADS administration on OE progenitor cell proliferation *in vivo*. (A) After 4 days OBX, BrdU positive cells were detected in OE basal cells. (B) *In vivo* administration of suramin and PPADS for 4 days significantly reduced proliferation of OE basal cells. Arrow heads indicate the proliferating cells. Scales bar in (A and B) = 100 μm, dashed lines indicate the basal lamina of olfactory mucosa. (C) Statistical analysis of BrdU positive basal cells of the 4 days OBX group and the antagonists group. The proliferating cell number is reduced to 12 cells/mm OE in the antagonists administration group compared to 20 cells/mm OE in the control group and (D) after 4 days OBX, the OE thickness of the administration group was thinner then the control group. $^*P < 0.05$, $^{**}P < 0.01$, $n = 4$.

## 4. Discussion

This study has demonstrated that adult mouse OE progenitor cells express $P2X_{1-3}$ receptors. No significant immunoreactivity for $P2X_1$ receptors could be detected in the ORNs when co-stained with the TuJ1 antibody. These differences to previous reports may be due to the distinctions between the age and genus of animals used for experiments [19,20]. Immunoreactivity of cleaved caspase-3 was detected in the OE at 24 h after OBX and reached its peak at 48 h after OBX. At 3 days after OBX, cleaved caspase-3 was only expressed in the olfactory nerves. No expression of cleaved caspase-3 was detected in the OE and the OE thickness reached the minimal level at 4 days after OBX. Blocking the activities of $P2X_{2-3}$ receptors by *in vivo* suramin and PPADS administration significantly inhibited the injury-induced proliferation of OE progenitors. As suramin and PPADS cannot block the activity of $P2X_1$, the function of $P2X_1$ in regulating OE progenitor proliferation needs further investigation.

Purines as intercellular factors have been reported to play important roles in meditating proliferation, differentiation and migration of neural stem cells [8]. In the retinal pigment epithelium, ATP is released through efflux by gap junction connexin 43 hemichannels. The released ATP increased the proliferation and stimulated DNA synthesis in neural retinal progenitor cells [21]. ATP application to human neural stem cells induced $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores, which in turn promoted NSC proliferation [22]. The adult subventricular zone (SVZ) expresses defined P2 receptors, whose activation can elevate cytosolic $Ca^{2+}$ concentrations and augment cell proliferation in a synergistic manner when combined with mitogenic growth factors [12]. HassenklÖver et al. recently reported that in larval *X. laevis*, nucleotide-induced $Ca^{2+}$ signaling in OE progenitor cells is involved in the regulation of cell turnover in OE [9]. Whether purines can induce $Ca^{2+}$ signaling in OE progenitor cells of adult mice and what is the downstream effector of purinergic signaling in regulating OE progenitor cells proliferation has yet to be determined.

In physiological condition, ORNs undergo unceasing regeneration in adult vertebrates, and injury of ORNs is a sufficient condition for neurogenesis of adult OE progenitor cells to occur [5]. Previous work has shown that systemic injection of EGF, transforming growth factor-α (TGF-α) or olfactory marker protein (OMP) peptides enhance cell proliferation in OE [23,24]. Jia et al. reported that nasal instillation of ATP can increase the proliferation of OE progenitor cells in normal adult mice [10]. But until now, only LIF has been found as a proliferative factor which is released from injured ORNs and can induce neurogenesis of OE progenitors in the turnover of ORNs [6]. Here we show that ATP, which is released from the injured ORNs [16], can serve as such a factor too.

Taken together, our study supports the notion that purinergic signaling contributes to the injury-induced proliferation of OE progenitor cells in adult mice. In the turnover of ORNs, purines released from damaged ORNs can activate nearby OE progenitors through the activation of P2X receptors. Thus, in physiological

conditions, purinergic signaling may serve as a paracrine signal in regulating injury-induced neurogenesis of OE in adult mammals.

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