Spatial and developmental heterogeneity of calcium signaling in olfactory ensheathing cells

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Abstract

Olfactory ensheathing cells (OECs) are specialized glial cells in the mammalian olfactory system supporting growth of axons from the olfactory epithelium into the olfactory bulb. OECs in the olfactory bulb can be subdivided into OECs of the outer nerve layer and the inner nerve layer according to the expression of marker proteins and their location in the nerve layer. In the present study, we have used confocal calcium imaging of OECs in acute mouse brain slices and olfactory bulbs *in toto* to investigate physiological differences between OEC subpopulations. OECs in the outer nerve layer, but not the inner nerve layer, responded to glutamate, ATP, serotonin, dopamine, carbachol and phenylephrine with increases in the cytosolic calcium concentration. The calcium responses consisted of a transient and a tonic component, the latter being mediated by store-operated calcium entry. Calcium measurements in OECs during the first three postnatal weeks revealed a down-regulation of $mGluR_1$ and $P2Y_1$ receptor-mediated calcium signaling within the first two weeks, suggesting that the expression of these receptors is developmentally controlled. In addition, electrical stimulation of sensory axons evoked calcium signaling via $mGluR_1$ and $P2Y_1$ only in outer nerve layer OECs. Down-regulation of the receptor-mediated calcium responses in postnatal animals is reflected by a decrease in amplitude of stimulation-evoked calcium transients in OECs from postnatal day 3 to 21. In summary, the results presented reveal striking differences in the development of receptor responses during development and in axon-OEC communication between the two subpopulations of OECs in the olfactory bulb.

Introduction

Assembling neuronal networks by axonal wiring and formation of synapses during development requires coordinated interaction between cells and tissues. In the olfactory system, a lifelong integration of new neurons into established networks persists due to continuous neuronal turnover, making it a valuable model to study basic principles of cell-cell interaction during development and regeneration. A specialized glial cell type, the olfactory ensheathing cell (OEC), is of particular interest with respect to the regenerative capability of the olfactory system, since it has been shown to enhance axon growth and is involved in the guidance of growing axons from the olfactory nerve, where OECs ensheath fascicles of axons of olfactory receptor neurons (ORNs), into the olfactory bulb (Doucette, 1990). OECs perform their growth-promoting properties not only in the olfactory system, but also after transplantation into injured dorsal root ganglia and spinal cord (Ramon-Cueto & Nieto-Sampedro 1994, Li et al., 1997), making them a potential tool in the therapy of spinal cord lesions (Raisman & Li, 2007; Barnett and Riddell, 2007). Still enigmatic is the molecular and physiological background of how OECs perform such regenerative tasks.

Intracellular calcium signaling in OECs is supposed to play a key role in intercellular signaling pathways between OECs and growing axons *in vitro* (Hayat et al., 2003). We have recently shown that in the developing olfactory bulb, calcium signaling in OECs can be evoked by stimulation of olfactory receptor axons *in situ*, which results in vesicular release of glutamate and ATP from the stimulated axons (Rieger et al., 2007; Thyssen et al., 2010). However, other neurotransmitters such as acetylcholine, dopamine and serotonin have been shown to be released from local and centrifugal neurons in the olfactory bulb (Fletcher and Chen, 2010). Therefore, we asked whether OECs are able to respond to neurotransmitters other than glutamate and ATP. Since the presence of at least two different subpopulations of OECs have been demonstrated in the rodent olfactory nerve layer by means of marker protein expression, namely OECs of the outer part and OECs of the inner part of the nerve layer (Au

et al., 2002), we have focussed on studying differences in calcium signaling between these two cell populations. We also followed changes in glutamate- and ATP-dependent calcium signaling during the first three postnatal weeks, a time range in which the olfactory system matures with respect to its morphology and functioning. The results of our study indicate that only OECs of the outer nerve layer, but not of the inner nerve layer, respond to neurotransmitters such as glutamate, ATP, dopamine, acetylcholine and serotonin. Glutamate- and ATP-induced calcium signaling was most prominent in the first postnatal week and decreased thereafter, suggesting a particular role of these responses for the early development of the olfactory epithelium to olfactory bulb pathway.

Materials and Methods

Preparation of olfactory bulb slices and olfactory bulbs in toto

NMRI mice of both genders at postnatal day (P) 0-20 were obtained from the animal facilities of the universities of Kaiserslautern and Hamburg. Animals were decapitated in accordance with the EU animal welfare guidelines. The skull was opened along the mid-sagittal line and bone hemispheres removed laterally. The olfactory bulbs were removed and glued to the stage of a vibroslicer (Leica VT1000). Sagittal slices of 250 µm thickness were cut in chilled preparation solution (in mM: 130 NaCl, 2.5 KCl, 26 $NaHCO_3$, 25 glucose, 1.1 NaH_2PO_4, 2.5 $MgCl_2$, 0.5 $CaCl_2$,) and kept afterwards for 1 h at 30 °C in preparation solution gassed with carbogen (95 % O_2, 5 % CO_2). Bulk loading with the calcium-sensitive dye Fluo-4 was performed by the AM-ester derivative (2 µM for 1 h at room temperature). Slices were placed in a recording chamber, fixed with a platinum grid and perfused continuously with gassed artificial cerebrospinal fluid (ACSF, 130 NaCl, 5 KCl, 25 $NaHCO_3$, 25 glucose, 10 NaH_2PO_4, 2 $MgCl_2$, 1 $CaCl_2$, 0.05 Na-L-Lactate). In calcium-free solution, $CaCl_2$ was replaced by $MgCl_2$, and residual calcium was buffered by addition of 1 mM EGTA.

In-toto preparations of olfactory bulbs were prepared as described before (Stavermann et al., 2012). Olfactory bulbs were removed from the opened cranium, glued onto a small coverslip and placed in a perfusion chamber. Fluo-4 AM (200 µM) was filled into a micropipette pulled with a patch-pipette puller (Sutter Instruments, Novato, CA, USA), the pipette was inserted into the nerve layer and Fluo-4 AM was injected into the tissue with pressure (0.7 bar) for 10 s. Fluo-4 was allowed to diffuse into the cells for 30 min.

Cultured OECs

Purified cultures of OECs were generated from 7-day old S100β-DsRed mice (Windus et al, 2007). In these mice OECs express DsRed fluorescent protein and are easily visualized in culture using fluorescent microscopy. To obtain OECs, the olfactory bulbs were removed from the cranial cavity and the nerve fibre layer from the entire bulb was dissected out. The NFL tissue was incubated in plastic 24-well plates coated with Matrigel basement membrane matrix (10 mg/ml; BD Biosciences, San Jose, CA) and maintained in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, G5 supplement (Gibco), gentamicin (Gibco, 50 mg/ml) and L-glutamine (200 µM) at 37°C with 5% CO_2 for 3-5 days. Contaminating macrophages were removed by incubation with TrypLE Express (Gibco) for 2 min. OECs were incubated with TrypLE Express for a further 4-5 min and then replated in the same medium. These cultures routinely give purity of ~90% OECs which have previously been shown to be immunoreactive for S100ß and p75NTR which are markers of OECs (Windus et al., 2007, Windus et al., 2010). For Ca^{2+}-imaging, cells on cover slips were stained with 1.5 µM Fluo4-AM (30 min) in an incubator at 37°C in a 5 % humidified atmosphere, transferred into a perfusion chamber and continuously superfused with CO_2-independent medium (1x, Gibco, Invitrogen Corp, Melbourne, Australia). Time series were acquired using a Zeiss Axioobserver Z1 microscope with a CCD camera (AxioCam MRm, Zeiss, Goettingen, Germany) at a frequency of 0.2 Hz. Images were compiled using Zeiss Axiovision Rel 4.6.3.

Drugs

All drugs were dissolved directly before use in ACSF. Glutamate, serotonin, carbachol, dopamine, phenylephrine, adenosine, ATP, 2MeSATP, γ-S-ATP, UTP, UDP, and IDP were obtained from Sigma-Aldrich (Germany). CHPG, DHPG, CPCCOEt, cyclopiazonic acid, and YM298198 were obtained from Ascent Scientific (Bristol, UK). JNJ16259685, MRS2179, and PPADS were obtained from Tocris (Bristol, UK). ARC69931MX was a gift from the Medicines Company (Waltham, MA, USA). All drugs were applied to the preparation via the perfusion system.

Calcium Imaging

Fluo-4-loaded cells were imaged using confocal microscopy (Zeiss LSM 510 for brain slices, Nikon eC1 for in-toto preparations). Time series of images (512x512 pixel) were acquired at a rate of one frame every 3 seconds. Regions of interest covering single glial cell somata were defined, and changes in calcium were analysed throughout the experiment as changes in fluorescence (ΔF) with respect to the resting fluorescence, which was set to 100%. Quantification of the calcium transients was achieved by calculating the amplitude of ΔF, except for quantification of store-operated calcium entry, which is reflected by a sustained fluorescence increase, and was therefore measured as the area under the response curve normalized to the peak amplitude. All values are given as mean values ± standard error of the mean with *n* giving the number of cells investigated. Statistical differences between means were evaluated using Student's t-test at an error probability of $p < 0.05$.

Immunohistochemistry

Sagittal olfactory bulb slices of 250 µm thickness were fixed for 1h in 4% formalin solution, afterwards washed 3 times carefully in phosphate buffered solution (PBS) for 5 min and then

incubated in citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) for 20 min at 90°C. Preincubation in blocking solution (3 % BSA, 10% NGS, 0.1 5 Triton X-100) for 2 h was followed by incubation with the primary antibody (mouse anti-mGluR$_1$, BD Bioscience, 1:500; rabbit anti-S100, Dako, 1:1000; rabbit anti-P75NTR, Chemicon, 1:200) in PBS containing 3% BSA overnight at 4 °C. The next day, slices were washed 3 times in PBS for 5 min and then incubated for 2 h at room temperature with the secondary antibody (goat anti mouse-IgG and goat anti rabbit-IgG coupled with Alexa 488 and Alexa 543, respectively) in PBS containing 10 µM propidium iodide. After a final washing step in PBS (3 times 5 min), slices were mounted on object slides with self-hardening embedding medium (glycerol 40%, polyvinyl alcohol 16%, phenol 0,7%, Tris 0.05 mM). Slices of the olfactory bulb were scanned with a Zeiss LSM 510 at a resolution of 2048x2048 pixels using excitation wavelengths of 488 nm (Alexa 488 and DiA, argon laser) and 534 nm (propidium iodide, helium-neon laser) sequentially. Emission wavelengths were separated by a band pass filter (510-560 nm) and a long pass filter (>560 nm). Axons were traced with DiA as described before (Rieger et al., 2007). In brief, olfactory bulbs were dissected with olfactory epithelia attached, and DiA-coated insect pins were inserted into the epithelium. Specimens were stored for up to 2 months in 2% PFA to allow for dye diffusion along the receptor axons. After that, the bulbs were cut into 250 µm thick slices and analyzed by confocal microscopy. Image stacks of DiA- or antibody-labelled slices were recorded with an axial interval (Z-step) of 0.5 µm, and projections were rendered over a Z-range of 3-5 µm. Control staining without primary antibodies resulted in specimens without significant immunoreactivity, indicating the specificity of the secondary antibodies (not shown).

Results

Histological discrimination of two subpopulations of olfactory ensheathing cells

The nerve layer is the most superficial layer of the olfactory bulb and is clearly distinguishable from the glomerular layer by the lack of glomeruli (Fig. 1A-C). OECs in the nerve layer express the calcium-binding protein S100B, and therefore, S100B can be used as specific marker in young animals in which astrocytes are immature and express too low amounts of S100B to be clearly visible by antibody staining (Raponi et al., 2007; Fig. 1A). In the innermost part of the nerve layer, OECs are only weakly S100B-immunopositive, but can be identified as OECs because they strongly express DsRed under the control of the S100B promoter (Fig. 1A, Windus et al., 2010). Another marker of OECs, the P75 neurotrophin receptor, is highly expressed in OECs of the outer nerve layer (Au et al., 2002; Fig. 1B), but only weakly in OECs of the inner nerve layer, suggesting different subpopulations of OECs. Bundles of olfactory receptor axons enter the outer nerve layer where they sort out before crossing into the inner nerve layer where they intermingle with similar odorant receptor axons and enter glomeruli. This suggests that OECs of the outer nerve layer and OECs in the inner nerve layer interact differently with axons (Fig. 1C).

To test whether these morphological and histological differences between OEC subpopulations are accompanied by physiological differences, we loaded the calcium indicator Fluo-4 into OECs of brain slices for cytosolic calcium signal recording. The nerve layer consists mainly of olfactory receptor axons with OECs as the major source of cell bodies and is identified by the lack of glomeruli. Strikingly, OECs were preferentially loaded with Fluo-4 and thereby highlighted in the confocal image (Fig. 1D), while receptor axons do not take up Fluo-4 (see also Rieger et al., 2007). Fluo-4 did not label the OECs homogeneously, but stained OECs in the outer nerve layer significantly brighter than in the inner nerve layer (Fig. 1D).

Physiological differences between OEC subpopulations

We have recently shown that olfactory receptor axons release glutamate and ATP, which stimulates calcium signaling in OECs in the nerve layer via mGluR and P2Y receptors (Rieger et al., 2007; Thyssen et al., 2010). In the present study, we compared neurotransmitter-evoked calcium signaling in OECs of the outer and inner nerve layer. Bath application of the $mGluR_{1/5}$-selective agonist DHPG (100 µM) resulted in calcium transients in OECs of the outer nerve layer, whereas OECs of the inner nerve layer did not respond to DHPG with calcium signaling (Fig. 2A), although in principle, OECs in the inner nerve layer were able to respond to $InsP_3$ receptor activation with calcium transients (Supplementary Fig. 1; Stavermann et al., 2012). To further analyze the pharmacological identity of the mGluR in outer OECs, we applied the $mGluR_5$-selective agonist CHPG (100 µM), which did not evoke calcium signaling in either outer or inner OECs, suggesting the functional expression of $mGluR_1$ in outer OECs. This was confirmed by the efficient inhibition of DHPG-evoked calcium signaling by the $mGluR_1$-selective antagonists JNJ16259685, CPCCOEt (Fig. 2B, C), YM298198, as well as by MCPG, a broad spectrum mGluR antagonist (Fig. 2 F). DHPG-evoked calcium transients were reduced by JNJ16259685 to 24.5 ± 3.7%, by YM298198 to 20.0 ± 2.6%, by CPCCOEt to 5.9 ± 1.1%, as well as by MCPG to 30.9 ± 4.8 % as compared to the control (100%).

We also generated a pharmacological profile of purinoceptors in OECs. Application of ATP and ADP, but not of UTP, UDP and IDP elicited calcium signaling in outer OECs (Fig. 2D). Inner OECs did not respond to either of the purinoceptor ligands. ATP- and ADP-evoked calcium signaling in outer OECs was greatly reduced by the $P2Y_1$-selective antagonist MRS2179 to 11.1 ± 2.5% of the control (Fig. 2E), and by the P2 receptor antagonist PPADS to 5.2 ± 4.4% of the control, while the $P2Y_{12}$-specific antagonist ARC69931MX had no effect (Fig. 2F). To test whether neurotransmitter-induced calcium transients in OECs could be mediated by indirect effects by neurotransmitter-dependent neuronal activity, we also applied

receptor ligands to cultured OECs. Cultures of OECs purified from the nerve fibre layer had a purity of over 90% and were immunopositive for S100ß and p75NTR which are markers of OECs from the outer nerve fibre layer (Windus et al., 2007, Windus et al., 2010). We did not detect OECs that were DsRed-positive, but negative for p75 immunostaining, which would indicate that they are OECs from the inner nerve fibre layer (not shown). Hence, the cultures of OECs had a phenotype of outer OECs. In addition, application of ATP and ADP evoked calcium transients in all cultured OECs investigated, in line with a phenotype of OECs of the outer nerve layer (Fig. 2G). Since other neurotransmitters besides glutamate and ATP exist in the olfactory bulb (Shipley et al., 2004), we tested the effect of some canonical neurotransmitters on cultured OECs and OECs in brain slices (P1-P8). Cultured OECs as well as outer OECs, but not inner OECs, responded to glutamate, serotonin, dopamine and phenylephrine (Fig. 2H; Table 1). A complete list of the substances tested on native and cultured OECs, including the mean values of the calcium responses, is given in Table 1. The results suggest that OECs in the outer nerve layer express $mGluR_1$ (but not $mGluR_5$), $P2Y_1$ receptors, 5-HT receptors, dopamine receptors, noradrenalin receptors and muscarinic acetylcholine receptors. Application of adenosine and GABA, however, did not evoke calcium signaling in OECs, in contrast to astrocytes in the olfactory bulb (Doengi et al., 2008; 2009). None of the receptor ligands was able to trigger calcium signaling in OECs of the inner nerve layer, indicating substantial differences in transmitter-evoked calcium signaling between inner and outer OECs.

Store-operated calcium entry contributes to calcium signaling in OECs

We first aimed to test whether calcium release from internal calcium stores mediated by $InsP_3$ receptors is involved in the ATP-evoked calcium signaling in outer OECs. We inhibited $InsP_3$ receptors with 100 µM 2-APB (n=36; Fig. 3A) and depleted internal calcium stores with cyclopiazonic acid (n=125; Fig. 3B). Both, $InsP_3$ receptor inhibition and store depletion,

resulted in a significant reduction of the amplitude of ATP-evoked calcium transients (both $p<0.005$), and both effects were not reversible after 20-30 min. To test whether calcium influx from the extracellular space also contributes to calcium transients evoked by ATP, we compared ATP-induced calcium transients measured in the presence and in the absence of external calcium. In calcium-containing saline, application of 100 μM ATP for 2 min resulted in a fast transient calcium rise that eventually passed into a plateau, i.e. the response had phasic and tonic characteristics (Fig. 3A). In calcium-free saline, the tonic response disappeared, and the calcium concentration declined to the baseline before ATP was washed out, suggesting a contribution of calcium influx to the late, tonic phase of the calcium response (Fig. 3C, thick line). In astrocytes, store-operated calcium entry (SOCE) has been shown to contribute to the calcium signaling repertoire and could be blocked by SOCE channel blockers 2-APB and BTP2 (Singaravelu et al., 2006). To test the involvement of SOCE in calcium signaling in OECs, we applied ATP in the presence of 20 μM 2-APB, a concentration sufficient to block SOCE channels, but not $InsP_3$ receptors, and BTP2 (20 μM). Both, 2-APB and BTP2, suppressed the tonic phase of the calcium response (Fig. 3C,E). To quantify the data, we calculated the area under the calcium curve, normalized to the peak amplitude, and found that the effects of calcium withdrawal, 2-APB and BTP2 on ATP-evoked calcium signaling were highly significant ($p<0.005$) (Fig. 3F). The normalized area was reduced to 47.4 ± 4.5% (n=46) by calcium withdrawal, to 52.5 ± 4.9% (n=54) by 2-APB, and to 63.6 ± 6.0% (n=73) by BTP-2, with respect to the control (100%). The results indicate that ATP initially triggers calcium release from internal stores via $InsP_3$ receptors, which then leads to activation of SOCE channels and thus calcium influx from the extracellular space.

Development of $mGluR_1$ and $P2Y_1$ receptor-mediated calcium signaling

Massive receptor axon ingrowth into the olfactory bulb persists after birth and is accompanied by continuous refinement of glomeruli (Bailey et al., 1999). After the first postnatal week, the gross morphology of the nerve layer and glomerular layer is established, but the size of the glomeruli and the olfactory bulb continue to increase until adulthood (Bailey et al., 1999). We were interested whether these developmental changes in morphology are accompanied by changes in calcium signaling in OECs. The amplitude of DHPG-evoked calcium transients in OECs in the outer nerve layer averaged 42.7 ± 3.8% ΔF in newborn animals (P0-2) and steadily decreased to 33.2 ± 2.8% ΔF at an age of P3-5, to 18.9 ± 2.3% ΔF at P6-8, and finally to 11.3 ± 1.4% ΔF in animals older than P13 (Fig. 4A). The decline in the response amplitude from one age to the next was significant in all cases ($p<0.05$) (Fig. 4B). The decrease in calcium signaling could be due to a decrease in receptor density, or to a modification of the intracellular signaling cascade leading to DHPG-triggered calcium release. We tested the expression of $mGluR_1$ using an anti-$mGluR_1$ antibody in brain slices of different ages. mGluR1-immunoreactivity was co-localized with P75-immunoreactivity, indicating that mGluR1 was expressed by OECs in the outer nerve layer (Fig. 4C). For the first postnatal days (P0-2), prominent $mGluR_1$-like immunoreactivity was detected in the outer nerve layer, in addition to an intense labeling in the glomerular layer which has been documented before (Martin et al., 1992; Fig. 4C). In the inner nerve layer, however, no immunolabeling above background level could be detected. The intensity of the immunoreactivity in the outer nerve layer was still clearly detectable at P3-5, and then decreased until P6-8. $MGluR_1$–like immunoreactivity was under the detection limit at ages older then P13, while $mGluR_1$-immunoreactivity was still visible in the glomerular layer, which served as a control for positive antibody staining (Fig. 4D). These results suggest that a decrease in mGluR expression is responsible for the decrease in amplitude of DHPG-evoked calcium signaling in outer OECs.

Measurements using ATP to stimulate $P2Y_1$ receptors argue against an impairment of the intracellular calcium signaling cascade downstream the receptor, since the amplitude of ATP-evoked calcium signaling did not change significantly during the first postnatal week and amounted to 57.1 ± 4.3% ΔF at P0-2, 61.0 ± 4.1% ΔF at P3-5, and 59.8 ± 3.6% ΔF at P6-8 (Fig. 4E). The amplitude then decreased significantly to 28.7 ± 1.8% ΔF until P14-20 ($p<0.005$; Fig. 4F). Developmental profiles of other neurotransmitters were not established.

Developmental changes of axon stimulation-induced calcium signaling in OECs

Electrical stimulation of the olfactory nerve layer triggers vesicular release of glutamate and ATP from receptor axons, which results in calcium signaling in OECs (Thyssen et al., 2010). We used *in toto* preparations of the olfactory bulb to clarify if OEC calcium signaling following axonal stimulation is subpopulation-specific. Cells were loaded with Fluo-4, and axons were stimulated with a stimulation pipette inserted into the nerve layer (Fig. 5A). Focussing approximately 50-70 µm into the tissue, until the first glomeruli appeared as dark regions lacking Fluo-4-labelled cell bodies, we were able to record simultaneously from the outer nerve layer, the inner nerve layer, and the glomerular layer (Fig. 5B). Electrical stimulation of axons for 3 s at 20 Hz (30 V) resulted in calcium transients in OECs of the outer nerve layer, whereas OECs in the inner nerve layer did not respond to axonal stimulation (Fig. 5C). Electrical stimulation triggered calcium signaling also in juxtaglomerular astrocytes and neurons (Fig. 5C, ROI 3), indicating that the lack of responsiveness in inner OECs is not a result of failure of action potential propagation into deeper layers of the olfactory bulb.

In mice of the first postnatal week, calcium signaling in outer OECs upon axonal stimulation was reduced by 53.3 ± 4.6% ($n = 49$) in the presence of the $mGluR_1$ antagonist CPCCOEt, and was entirely blocked when the $P2Y_1$ receptor blocker MRS 2179 was applied in addition, confirming the activation of $mGluR_1$ and $P2Y_1$ receptors in outer OECs (Fig. 5D). To test

whether the decrease in $mGluR_1$ and $P2Y_1$ receptor expression in OECs during postnatal development is reflected by a decrease in stimulation-induced calcium signaling, we evoked calcium signaling in outer OECs of different ages (Fig. 5E). At P0-2, stimulation evoked calcium transients with an amplitude of 133.9 ± 7.6% ΔF (n=208). The amplitude significantly increased to 176.1 ± 8.6% ΔF (n=295) at P3-5, but then decreased to 123.3 ± 6.1% ΔF (n=304) at P6-8, and to 79.1 ± 4.7% ΔF (n=156) at P14-20, the differences being statistically significant ($p<0.005$), suggesting that the neurotransmitter-dependent communication between olfactory receptor axons and outer OECs is developmentally regulated (Fig. 5F).

Discussion

As described previously (Au et al., 2002), the labelling pattern of p75NTR, a neurotrophin receptor that is involved in many developmental processes such as neurite outgrowth, differentiation and cell death (Underwood and Coulson, 2008), shows a clear division into two subpopulations of OECs, with OECs only in the outer nerve layer being labelled by the p75NTR antibody. In the present study, we could show that OECs in the outer nerve layer respond to different agonists, such as glutamate, ATP, serotonin, acetylcholine and dopamine with an intracellular calcium increase. In contrast, the OEC population of the inner nerve layer was not immunopositive for p75NTR, and agonist-evoked calcium signals could not be detected. Hence, this is the first study that demonstrates physiological differences between subpopulations of OECs in the olfactory bulb.

Physiological heterogeneity of OECs

OECs are considered to promote the growth of olfactory receptor axons that originate in the olfactory epithelium and enter the central nervous system at the level of the olfactory bulb.

When transplanted into cut nerve ends of dorsal roots or into spinal cord lesions, OECs enable axon growth and functional reconstitution of limb mobility and somatic sensation (Ramon-Cueto & Nieto-Sampedro 1994, Li et al., 1997). In some studies, however, transplantation of OECs failed to enhance axon regeneration (Ridell et al., 2004; Gomez et al., 2003; Ramer et al., 2004; Lu et al., 2006). Some of the discrepancies between the different studies could be explained by the usage of different OEC subpopulations for transplantation. Roskams and co-workers, e.g., used OECs from the lamina propria (Ramer et al, 2004; Lu et al., 2006), while Raisman and co-workers as well as Ramon-Cueto and Nieto-Sampedro used OECs from the olfactory nerve and olfactory bulb nerve layer (Li et al., 1997; 1998; Ramon-Cueto & Nieto-Sampedro, 1994). Recent studies, using magnetic beads and fluorescent activated cell sorting (FACS) to purify P75-positive OECs from different regions of the olfactory pathway, demonstrate differences in gene expression profiles and cell proliferation between OECs of the mucosa (lamina propria) and the olfactory bulb (Guerout et al., 2010; Kueh et al., 2011) and between subpopulations in the olfactory bulb nerve layer (Honoré et al., 2012). Although differences between OEC subpopulations in terms of expression of marker proteins and signaling molecules are well known also from other studies (Au et al., 2002; Windus et al., 2010; Wang et al., 2008; Hisaoka et al., 2004), physiological properties of OEC subpopulations have not been measured and analysed comparatively. Calcium signaling and voltage-dependent membrane currents have been measured in olfactory bulb OECs (Rieger et al., 2007; Rela et al., 2010), but the exact position of the studied cells within the nerve layer has not been addressed in these studies. We found striking differences in the functional expression of neurotransmitter receptors between OECs of the outer and inner nerve layer. While OECs in the outer nerve layer respond to a variety of neurotransmitters, inner OECs were insensitive to these substances, with respect to cytosolic calcium responses. Outer OECs not only responded to transmitters applied via the perfusion system, but also to glutamate and ATP endogenously released from olfactory receptor axons, suggesting that neurotransmitters

indeed contribute to the orchestration of axon-OEC interactions (Rieger et al., 2007; Thyssen et al., 2010). In particular ATP could be an important messenger in communication between regenerating axons and transplanted OECs in traumatic spinal cord, since ATP is not only a neurotransmitter, but also a mediator of tissue damage and inflammation (Burnstock, 2009). In addition, other neuromodulators shown to affect calcium signaling in OECs might influence the regenerative capacity of transplanted OECs in spinal cord lesions.

OECs in olfactory bulb development: A role for calcium signaling?

In many glial cell types, elevations in intracellular calcium following activation of metabotropic receptors trigger various kinds of responses, e.g. the secretion of transmitter molecules or growth factors (Ramamoorthy and Whim, 2008; Marchaland et al., 2008; Montana et al., 2006) as well as changes in gene expression (Finkbeiner, 1993). An involvement of intracellular calcium signaling in promoting neuronal axonal growth has been observed in OEC-neuron co-culture (Hayat et al., 2003). Furthermore, there is evidence that secretion of neuroactive substances like BDNF, NGF and neuregulin is employed in OEC interactions with neuronal processes (Pastrana et al., 2007, Boruch et al., 2001), emphasizing their role in axon regeneration. However, the mechanisms by which OECs are activated to interact with axons are not yet elucidated, and calcium signaling is one likely candidate to translate stimulation of OECs into OEC-to-axon communication.

In the present study, we could show calcium signaling in OECs mediated by glutamate, the principle neurotransmitter released by ORN (Berkovizc et al., 1994). The pharmacological profile suggests the involvement of $mGluR_1$, since the agonist of mGluR subgroup I (comprising $mGluR_1$ and mGluR5), DHPG, evoked calcium signals in OECS, but not the mGluR5 agonist, CHPG. The lack of effect of CHPG is in line with the lack of mGluR5-like immunoreactivity in the olfactory nerve layer (Romano *et al.*, 1995, Sahara *et al.*, 2001).

On the other hand, metabotropic purinergic receptors could evoke intracellular calcium release in this study, too. The involvement of purinergic signaling in addition to the main transmitter in a neuron-glia or glia-glia communication is common in the nervous system and sensory organs (Franke et al., 2006; Housley et al., 2009) and already been described for glomerular astrocytes in the olfactory bulb (Doengi et al., 2008). In OECs, $P2Y_1$ receptors appear to mediate ATP-induced calcium signaling, since agonists of other P2Y subtypes such as UTP, UDP and IDP were not effective in triggering calcium signals, and the specific antagonist MRS2179 inhibited ADP-induced calcium signals effectively. These results are in accordance with studies from Simon et al. (1997), where the presence of $P2Y_1$ in the nerve layer could be shown with radioactive ligand binding. In the nerve layer, the source for released ATP and glutamate has been shown to be of axonal vesicular origin (Thyssen et al., 2010), presumably acting as an extracellular signaling molecule of ingrowing axons to the OECs, thereupon triggering attraction or repulsion of axons.

Purinergic signaling is an important component of nervous system development (Zimmermann, 2011). Metabotropic purinergic receptors are strongly regulated during ontogenesis in the central nervous system (Cheung et al., 2003), and their influence on eye development has been described (Masse et al., 2007). A significant role for $P2Y_1$ in the development of radial glial cells and neurons could be shown in the subventricular zone, where proliferation of glial cells and migration of neuronal progenitors depends on ATP-mediated calcium signals (Weissman et al., 2004; Liu et al., 2008). In addition, neuronal activity evokes calcium signaling in glial cells in the insect olfactory nerve and the olfactory lobe (homologous to the vertebrate olfactory bulb) (Hartl et al., 2007; Heil et al., 2007), and this calcium signaling is required for glial cell migration in these structures (Lohr et al., 2005; Koussa et al., 2011). A similar function of $mGluR_1$ and $P2Y_1$ receptor-mediated calcium signaling for migration of OECs from the olfactory placode to the telencephalon, a key step in early development of the olfactory system in mammals (Ekberg et al., 2012), could be

speculated, but needs further investigation. The up-regulation of stimulation-evoked calcium signaling in OECs during the first postnatal days, when a large number of receptor axons is still growing into the olfactory bulb, and the down-regulation of mGluR expression and neurotransmitter-evoked calcium signaling during the second to third postnatal week, after which the gross morphological development of the olfactory bulb is completed, supports the hypothesis that calcium signaling is of particular interest for the development of OECs and the nerve layer.

Acknowledgements

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Tables

Table 1: Efficacy of receptor ligands on cytosolic calcium in OECs (P1-P8) of the outer and inner nerve layer (NL) and in cultured OECs. Values represent mean increases in fluorescence (ΔF), the number in parentheses indicate the number of analyzed cells.

Substance (100 µM)	outer NL (ΔF [%])	inner NL	cultured OECs (ΔF [%])
Glutamate	158.1 ± 1.1 (43)	-	27.5 ± 3.3 (38)
DHPG	70.4 ± 1.1 (72)	-	nd
CHPG	-	-	nd
ATP	61.0 ± 4.1 (119)	-	31.7 ± 5.7 (18)
ADP	70.4 ± 5.3 (54)	-	59.6 ± 5.5 (31)
UTP	-	-	nd
UDP	-	-	nd
IDP	-	-	nd
Adenosine	-	-	nd
GABA	-	-	nd
Dopamine	42.4 ± 4.8 (33)	-	38.1 ± 3.9 (45)
Serotonin	40.8 ± 3.0 (189)	-	78.6 ± 9.5 (75)
Carbachol	80.6 ± 5.6 (278)	-	nd
Phenylephrine	154.7 ± 8.1 (89)	-	93.1 ± 18.6 (28)

-, does not induce calcium signaling; nd, not determined

Figure legends

Fig. 1. Morphology of the nerve layer (NL) in the developing mouse olfactory bulb. (**A)** DsRed expression under control of the S100B promoter (red) and S100 immunoreactivity (green) in the NL. Nuclei were stained with DAPI (blue). Asterisks indicate glomeruli in the glomerular layer (GL). (**B)** P75NTR-immunoreactivity (green) is only found in the outer nerve layer (NL_o), but not in the inner nerve layer (NL_i). Red labelling (propidium iodide) indicate nuclei. (**C)** DiA-labelled receptor axons (green) proceed in straight bundles (arrowhead) in the outer nerve layer, and eventually separate to cross the inner nerve layer (arrows) and enter a glomerulus (asterisk). (**D)** After loading olfactory bulb slices with Fluo-4 AM, OECs in the outer nerve layer were strongly labelled by Fluo-4, whereas OECs in the inner nerve layer were weaker labelled. Scale bars: 50 μm.

Fig. 2. Neurotransmitter-evoked calcium signaling in OEC subpopulations. (**A)** OECs in the outer nerve layer, but not the inner nerve layer, respond to the mGluR agonist DHPG (20 μM) with a calcium transient, while the $mGluR_5$-specific agonist CHPG (100 μM) had no effect on the calcium concentration in either cell type. (**B)** DHPG-induced calcium transients were reduced by the $mGluR_1$-specific antagonists JNJ 16259685, and (**C)** CPCCOEt. (**D)** ATP and ADP, but not UTP, UDP and IDP evoke calcium transients in OECs of the outer nerve layer, while purines did not evoke calcium signaling in OECs in the inner nerve layer. (**E**) Calcium transients evoked by ATP were blocked by the $P2Y_1$ receptor antagonist MRS 2179. (**F**) Summary of antagonistic effects of mGluR and P2Y receptor ligands. The $mGluR_1$-specific antagonists JNJ 16259685 (10 μM), YM 230888 (1 μM) and CPCCOEt (100 μM) as well as the non-specific mGluR antagonist (R,S)-MCPG (1 mM) significantly reduced DHPG-evoked responses in OECs of the outer nerve layer. The non-specific P2 receptor antagonist PPADS (100 μM) and the $P2Y_1$-specific antagonist MRS 2179 (60 μM) reduced ADP/ATP-evoked

calcium transients in OECs in the outer nerve layer, while the $P2Y_{12}$-specific antagonist ARC69931MX (100 μM) had no effect. ***, $p<0.005$. (**G)** In cultured OECs, ATP (100 μM) and ADP (100 μM), as well as serotonin (100 μM) (**H**) elicit calcium transients.

Fig. 3. Calcium stores and store-operated calcium entry in OECs of the outer nerve layer. (**A**) Calcium transients evoked by ATP (100 μM, 1 min) were reduced in amplitude in the presence of 100 μM 2-APB, which blocks $InsP_3$ receptor at that concentration. (**B**) Depletion of calcium stores with 20 μM cyclopiazonic acid (CPA) suppressed ATP-induced calcium transients. (**C**) Application of ATP (100 μM) for 2 minutes evoked a fast calcium peak, followed by a tonic calcium elevation that continued for at least the time of application (thin trace). In the absence of external calcium ($0Ca^{2+}$), the tonic phase was suppressed, and the calcium transient returned to the baseline value during ATP application (thick trace). Traces were normalized to the peak amplitude to accomplish comparability. (**D**) 20 μM 2-APB, which is sufficient to block store-operated calcium channels but not $InsP_3$ receptors, and 20 μM BTP2 (**E**) suppressed the tonic phase of the ATP-induced calcium response. (**F**) The integral of the normalized calcium response, reflecting both the fast calcium peak mediated by intracellular calcium release and the tonic phase reflecting calcium influx, decreased in the absence of external calcium, and in the presence of 2-APB and BTP2. ***, $p<0.005$.

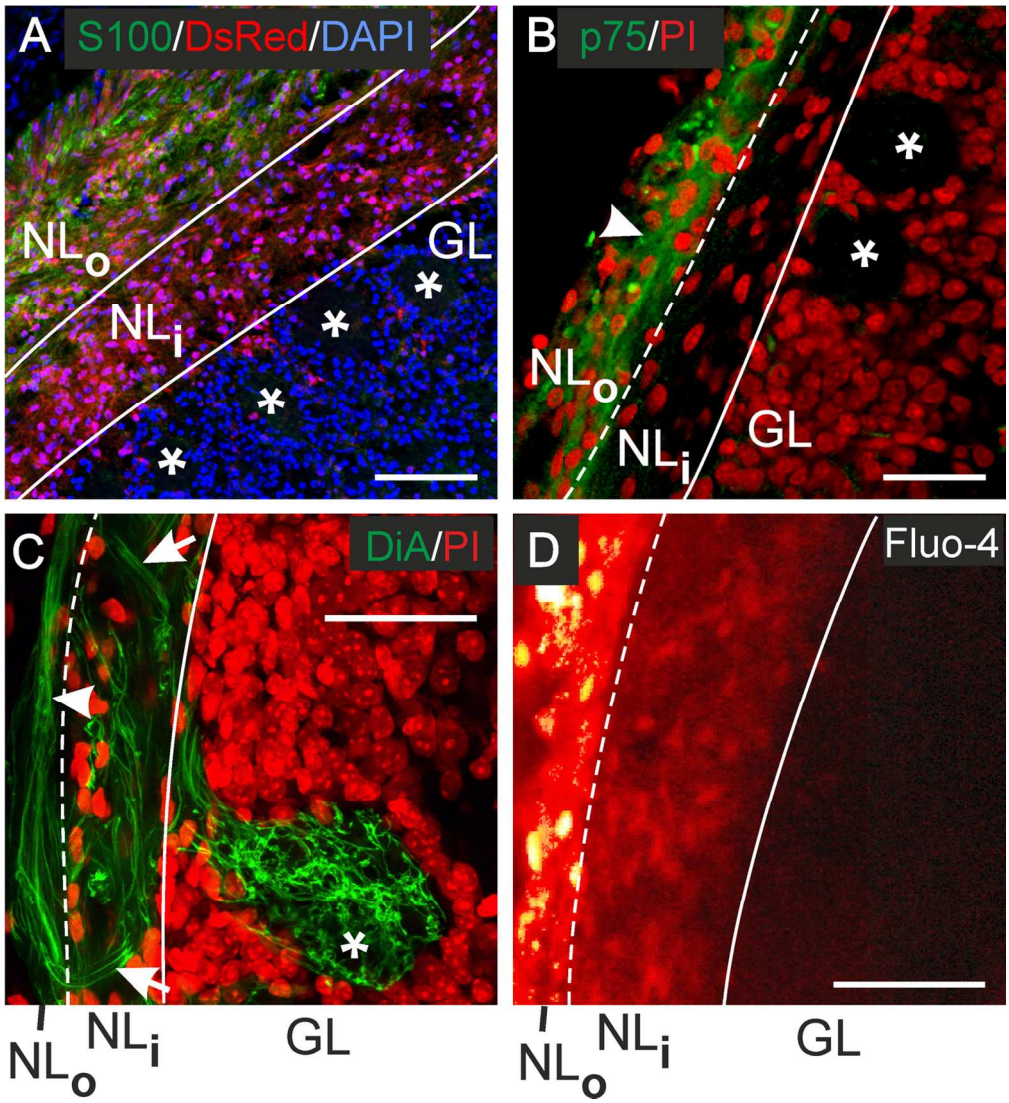
Fig. 4. Development of DHPG- and ATP-evoked calcium signaling in OECs of the outer nerve layer. (**A**) The amplitude of DHPG-evoked calcium transients steadily decreased during the first postnatal weeks. (**B**) Differences in amplitude of DHPG-evoked calcium transients in OECs in four age groups, postnatal days 0-2 (P0-2), P3-5, P6-8 and older than P13, were significantly different as compared to the neighboring age groups. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$. (**C**) Anti-$mGluR_1$ immunostaining (green) co-localizes with anti-P75 immunoreactivity (red) in the nerve layer of an olfactory bulb slice of an animal at P1. OECs

in the outer nerve layer (arrowhead) as well as glomeruli (asterisks) are mGluR1-immunopositive. (**D**) Decreasing number of $mGluR_1$-positive OECs (arrowheads) from P4 to P7 and P16, while the staining in glomeruli remained intense (asterisks). Cell nuclei were stained with propidium iodide (red). (**E**) ATP-evoked calcium transients in OECs of different ages. (**F**) The amplitude of ATP-evoked calcium transients remained constant from P0 to P8, and then decreased between P8 and P14 and older. Scale bars: 20 μm (C), 50 μm (D).

Fig. 5. Development of stimulation-induced calcium transients in OECs in olfactory bulbs *in toto*. (**A**) Experimental setup. (**B**) Original confocal image of the olfactory bulb in which the traces in the regions of interest (1, 2 3) in (**C**) were recorded. Glomeruli are asterisked to indicate the glomerular layer (GL). The arrowhead highlights the stimulation pipette. (**C**) Calcium transients could be evoked by electrical stimulation (20 Hz, 3 s) of receptor axons in OECs of the outer nerve layer (ROI 1) and periglomerular astrocytes (ROI 3), but not in OECs of the inner nerve layer (ROI 2). (**D**) Stimulation-induced calcium transients in OECs of the outer nerve layer were inhibited by combination of CPCCOEt (100 μM) and MRS 2179 (60 μM). (**E**) Stimulation-induced calcium transients at different ages. (**F**) The amplitude of stimulation-evoked calcium transients in OECs of the outer nerve layer increased significantly from birth to P3-P5, and then steadily decreased until P14 and older. ***, $p<0.005$.

Supplementary figure 1. Photolysis of caged $InsP_3$ and application of cyclopiazonic acid evoke calcium signaling in OECs in the inner nerve layer. (**A**) OECs were bulk-loaded by injecting 40 μM of the membrane-permeant ci-$InsP_3$/PM (caged $InsP_3$) together with 200 μM Fluo-4 AM into the tissue. After 30-60 min, during which OECs took up both caged $InsP_3$ and Fluo-4, OECs were illuminated with a 405 nm laser diode for 2 seconds, which resulted in an immediate calcium response of 185.1 ± 22.4 ΔF (60 cells from 6 preparations derived from 3 animals). (**B**) Depletion of internal calcium stores by cyclopiazonic acid (CPA) is reflected by

a transient increase in cytosolic calcium of 64.3 ± 3.6% ΔF (n=59) in OECs of the inner nerve layer. The traces represent individual OECs.



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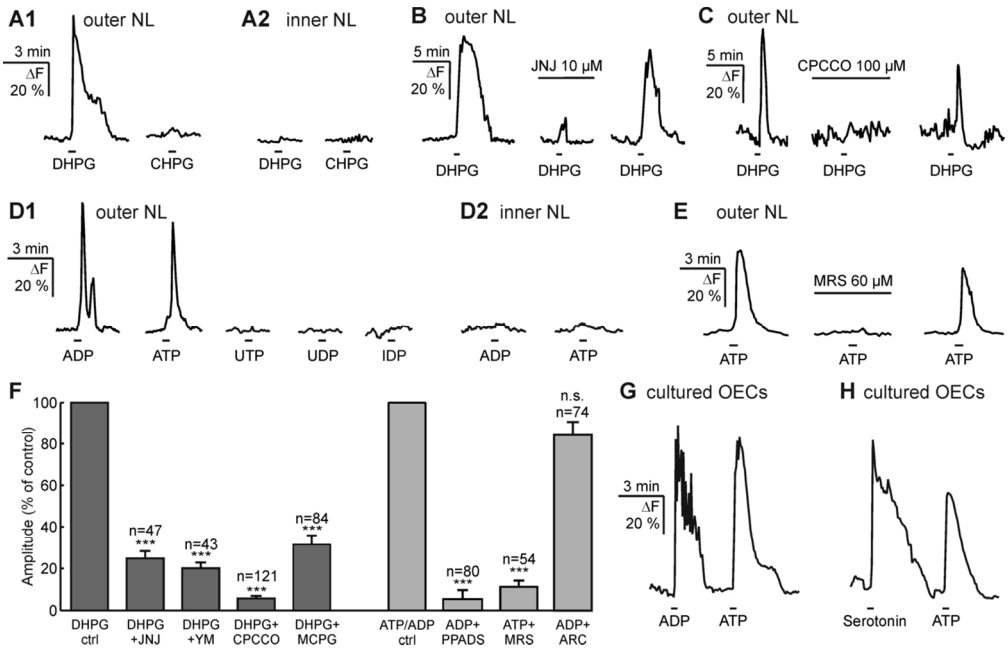

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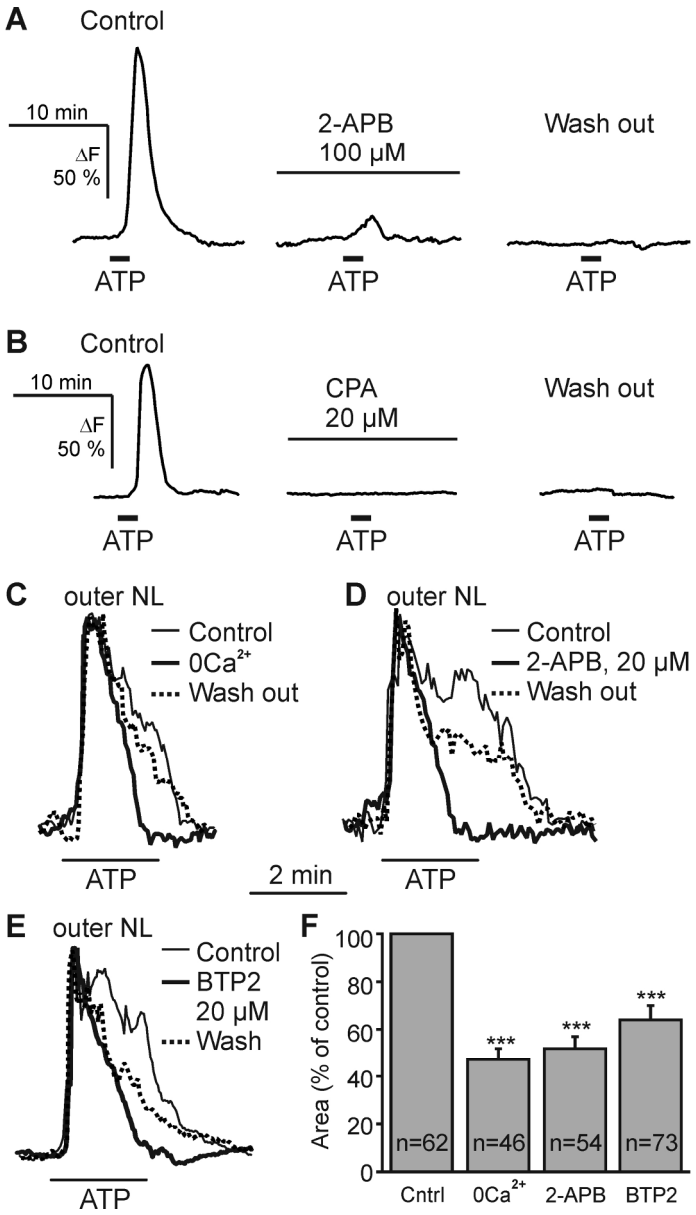

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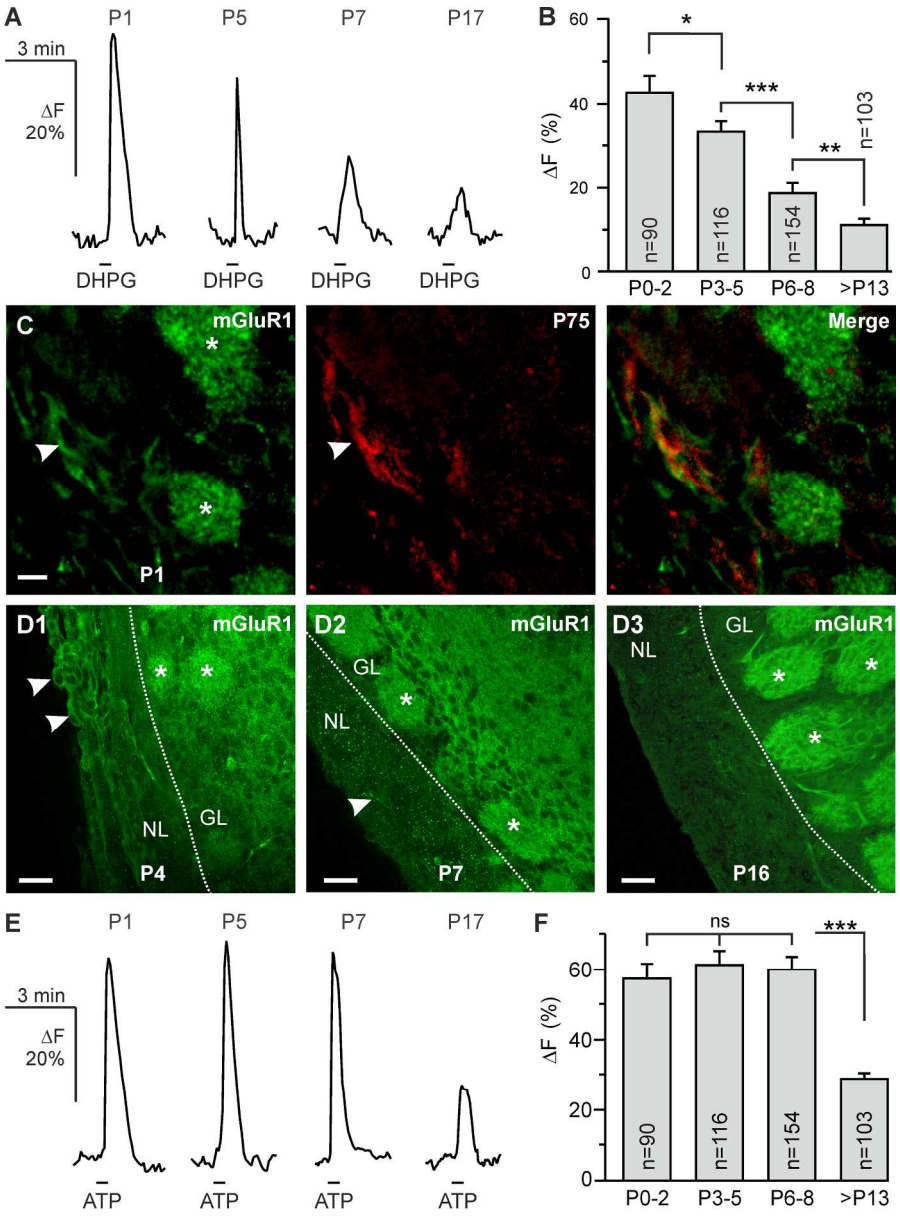

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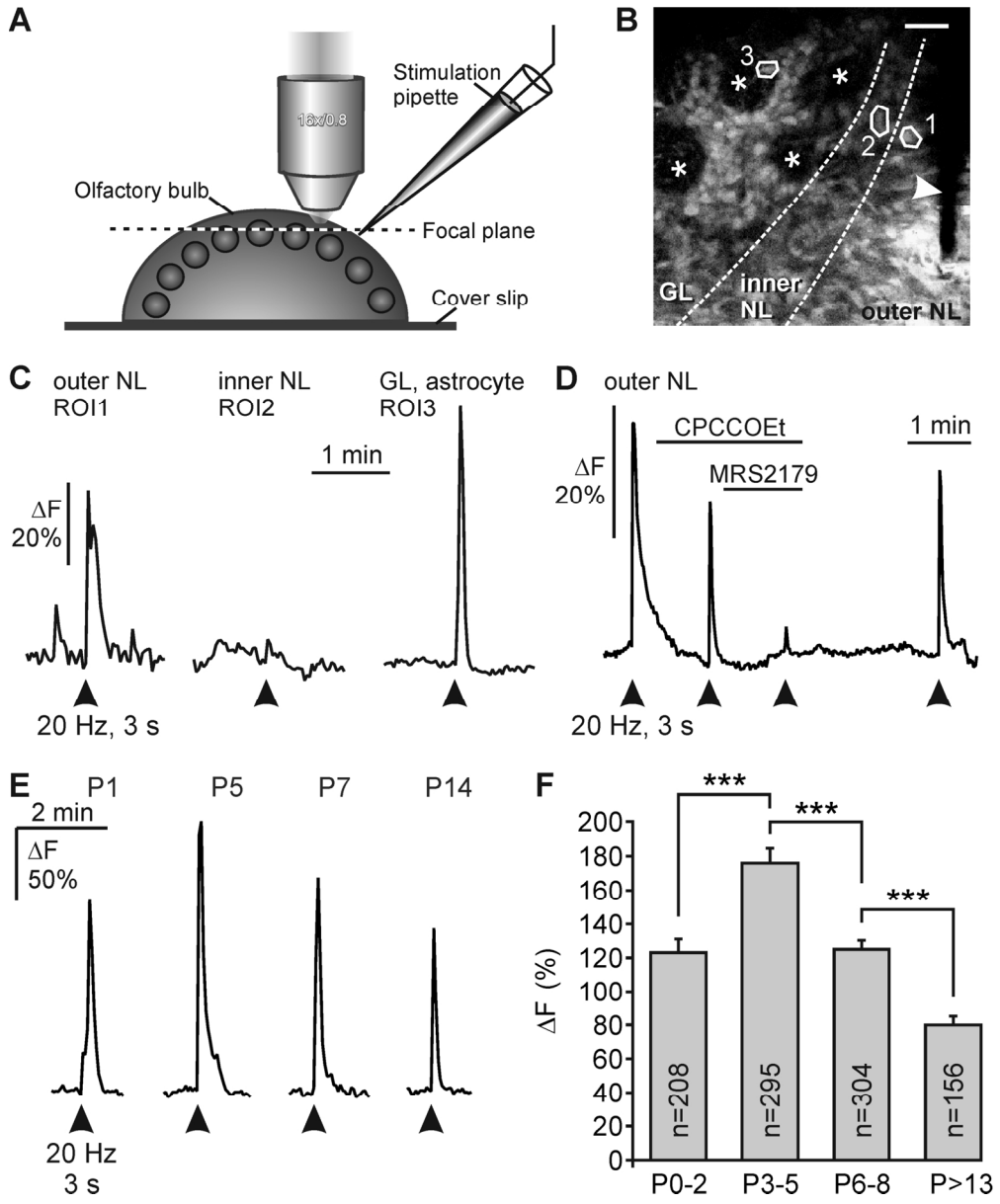

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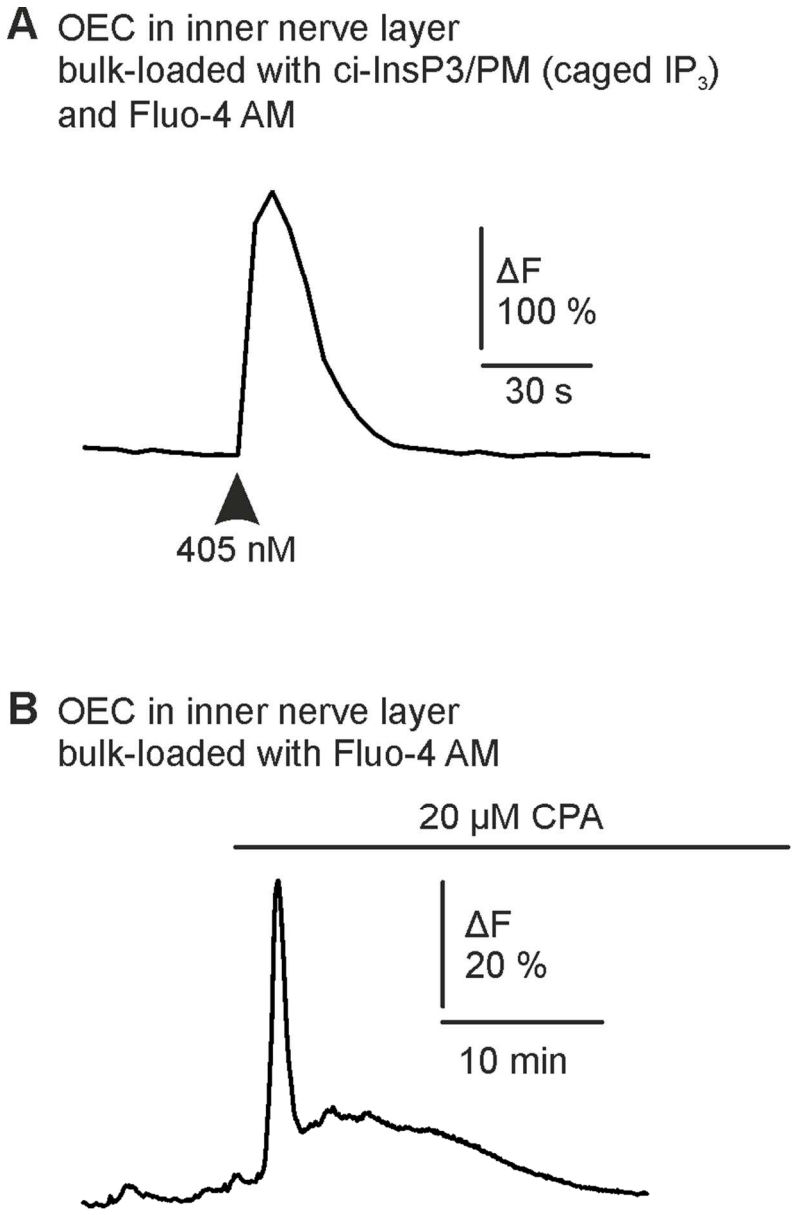

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75x115mm (300 x 300 DPI)