Noradrenergic modulation of calcium currents and synaptic transmission in the olfactory bulb of *Xenopus laevis* tadpoles

Dirk Czesnik, Leonid Nezlin, Jörg Rabba, Birgitt Müller and Detlev Schild  
Physiologisches Institut, Universität Göttingen, Humboldtallee 23 D 37073 Göttingen, Germany

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**Abstract**

Norepinephrine (NE) has various modulatory roles in both the peripheral and the central nervous systems. Here we investigate the function of the locus coeruleus efferent fibres in the olfactory bulb of *Xenopus laevis* tadpoles. In order to distinguish unambiguously between mitral cells and granule cells of the main olfactory bulb and the accessory olfactory bulb, we used a slice preparation. The two neuron types were distinguished on the basis of their location in the slice, their typical branching pattern and by electrophysiological criteria. At NE concentrations lower than 5 μM there was only one effect of NE upon voltage-gated conductances; NE blocked a high-voltage-activated Ca2+-current in mitral cells of both the main and the accessory olfactory bulbs. No such effect was observed in granule cells. The effect of NE upon mitral cell Ca2+-currents was mimicked by the α2-receptor agonists clonidine and α-methyl-NE. As a second effect, NE or clonidine blocked spontaneous synaptic activity in granule cells of both the main and the accessory olfactory bulbs. NE or clonidine also blocked the spontaneous synaptic activity in mitral cells of either olfactory bulb. The amplitude of glutamate-induced currents in granule cells was modulated neither by clonidine nor by α-methyl-NE. Taken together, the main effect of the noradrenergic, presynaptic, α2-receptor-mediated block of Ca2+-currents in mitral cells appeared to be a wide-spread disinhibition of mitral cells in the accessory olfactory bulb as well as in the main olfactory bulb.

**Introduction**


Regarding the cellular and molecular mechanisms of the noradrenergic effects in the OB, the following pieces of evidence have been accumulated so far. (i) Norepinephrine (NE; 1 mM) evokes an inward current at −60 mV in periglomerular cells of the rabbit OB (Bufler *et al.*, 1992); (ii) NE induces an increase in [Ca2+]i in cultured interneurons of the mouse OB (Tani *et al.*, 1992); (iii) application of NE leads to a block of a high-voltage-activated (HVA) Ca2+-current in cultured mitral cells (MCs) and granule cells (GCs) of rat (Trombley, 1992); (iv) NE decreases the rate of cultured rat granule cell inhibitory postsynaptic currents (IPSCs) (Trombley & Shepherd, 1992); (v) depolarization-induced increase of [Ca2+]i, in cultured MCs in *Xenopus* is reduced during NE or clonidine application, whereby this reduction occurs predominantly in dendritic compartments (Bischofberger & Schild, 1995); and (vi) the activity of MC responses to weak stimuli is enhanced by NE acting via α1-receptors (Ciombor *et al.*, 1999).

It is as yet unknown whether the NE-mediated block of calcium channels as observed in cultured MCs (Trombley, 1992; Bischofberger & Schild, 1995) is also present *in vivo* or in the tissue slice. It would have important functional consequences if NE also blocked synaptic calcium channels in interneurons. It is further unknown whether the NE-induced calcium channel block takes place in the accessory olfactory bulb (AOB), in the main olfactory bulb (MOB) or in both. The α2-receptor mediated modulation described earlier (iii, iv and v) were all measured in cell culture, in which the synaptic connectivity may differ markedly from the connectivity *in vivo* or in the tissue slice, and in which the different neuron types of the AOB and MOB cannot be distinguished unambiguously.

To circumvent these problems, we used a slice preparation of the OB. We made whole-cell recordings of Ca2+-currents and spontaneous synaptic currents from MCs and granule cells (GCs) in both the MOB and the AOB upon application of NE. Our results indicate that NE leads to a markedly reduced Ca2+-current in MCs but not in GCs. This effect occurred in both the MOB and AOB with a subsequent decrease of synaptic currents in GCs and MCs. While our findings do not reveal a mechanism of olfactory learning and olfactory memory formation, they suggest how NE might alter the bulb’s signal processing and how this might facilitate olfactory learning and imprinting to odourants.

**Materials and methods**

**Slice preparation**

Tadpoles of *Xenopus laevis* (stages 49–54) (Nieuwkoop & Faber, 1956) were anaesthetized and immobilized in a mixture of ice and water. A block of tissue containing the mucosa, the olfactory nerves and the anterior two-thirds of the brain was cut out and kept in frog
ringer solution \((B_1, \text{Table 1})\). The tissue block was glued onto the stage of a vibrislicer (VT 1000; Leica, Bensheim, Germany) and cut horizontally into 150-µm-thick slices. The slices were placed under a harp-like grid in a recording chamber and viewed in an Axioskop 2 microscope equipped with differential interfluorescent contrast (DIC; Zeiss, Göttingen, Germany). MCs and GCs were chosen according to the layering of the OB (Nezlin & Schild, 2000).

**Recording**

Patch electrodes with a tip diameter of 1–2 μm and a resistance of approximately 7 MΩ were fabricated from borosilicate glass (1.8 mm outer diameter; Hilgenreiner, Malsfeld, Germany) using a two-stage electrode puller (Narishige, Tokyo, Japan). The pipettes were fire-polished. Pulse protocols, data acquisition and evaluation programs were written in ‘C’. Pulses were delivered from a microcontroller (Schild et al., 1996) to a D to A converter and then to the patch-clamp amplifier (EPC7; List, Darmstadt, Germany). Currents and voltages were recorded on videotape using a PCM unit (Instrutech, Elmont, NY, USA). The data were digitized off-line using an eight-pole Bessel filter, an A to D converter and a PC. Further data analysis was performed on a PC under LINUX (SuSE GmbH, Nürnberg, Germany).

**Solutions and stimulus application**

The compositions of the bath solutions \((B_1-B_5)\) and the intracellular solutions \((I_1-I_5)\) are listed in Table 1. All solutions were at pH 7.8 (Howell et al., 1970). The osmolarities of the bath and pipette solutions were adjusted to 230 mOsmol and 190 mOsmol, respectively. Tetrodotoxin (TTX), NE, clonidine and α-methyl-NE were dissolved in the bath solutions, which were applied by gravity feed to the recording chamber. The flow was 250 μL/min. All chemicals except TTX (Molecular Probes, Leiden, The Netherlands) were purchased from Sigma (Munich, Germany). The experiments were carried out at room temperature (about 22 °C).

To visualize cell processes, 0.5% biocytin (Molecular Probes) was added to the pipette solution. After the experiment, cells were filled using depolarizing current (2-s-long pulses at 0.25 Hz for 5–10 min). The preparations were left in fresh saline for 20–30 min and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at 4 °C, rinsed in PB, left overnight in PB with 0.5% triton X100 (TX), and then immersed in a solution of avidin Alexa Fluor 488 conjugate (5 μg/mL, Molecular Probes) in PB-TX for 4–6 h at room temperature. The specimens were rinsed several times in PB, embedded in PB-glycerol (1:1) and examined using a laser scanning confocal microscope (LSM 510, Zeiss, Göttingen, Germany).

**Synaptic activity**

As a measure of synaptic activity we calculated the integral Q of the excitatory postsynaptic current, i.e. the sum over the products given by the postsynaptic current amplitudes times sample time. The average noise level was taken as the baseline and subtracted from each current amplitude value. The integral Q over postsynaptic current was calculated every 10 s as a gliding average over 50 s, e.g., the first value at \(t = 25\) s represented the integrated postsynaptic current average over the interval \([0\ s,\ 50\ s]\). Calculating the postsynaptic charge Q is convenient, first, because it takes into account the rate, amplitude and decay phase of synaptic currents and, second, because it gives a smooth representation of the data without distorting the kinetics of the drug effects. By choosing shorter averaging periods we have ascertained that Q reflected the synaptic activity reliably as long as the averaging time was smaller than the drug-induced changes of synaptic activity.

**Results**

**Identification of mitral and granule cells**

We have performed voltage clamp recordings in MCs and GCs in both the MOB and AOB of X. laevis tadpoles in order to study the effects of the noradrenergic innervation of the OB. MCs were distinguished from GCs by their localization, dendritic branching patterns (Fig. 1) and different inward currents. The Xenopus OB consists of the AOB (shaded in the inset of Fig. 1) and the MOB, both of which show a distinct glomerular layer (GL) and a mitral cell layer (MCL). In this, as well as in other, amphibian species, the MCL coincides with the external plexiform layer (EPL) and a mitral cell layer (MCL). To illustrate locations and branching patterns of MCs and GCs, we overlaid the images of four biocytin–avidin-stained neurons onto Fig. 1. In MCs, the whole-cell current showed characteristically delayed, repetitive, transient and TTX-sensitive inward currents (Fig. 2A) superimposed on outward currents that increased with voltage. On the other hand, GCs consistently showed a Hodgkin–Huxley-like Na+ current appearing without delay (Fig. 2B).

**Norepinephrine blocks an high-voltage-activated Ca2+-current in mitral cells**

The effect of NE was best observed after blocking the K+-currents by adding Cs+ to the pipette solution (solution I2) and TTX to the bath. The resulting current was a typical high-voltage-activated (HVA) Ca2+-current (Fig. 2C and D), which could be blocked by Cd2+

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**Table 1. Ion composition of each solution used**

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<th>CoCl₂</th>
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(200 μM) added to the bath solution (Fig. 2D). This current was reduced to 61.3 ± 5.9% (mean ± standard error of mean) by the application of NE (500 nM, Fig. 3A). Recovery from the block was usually incomplete (81.0 ± 3.9%), mainly because of the 'washout' of the Ca2+-current. The high affinity of the adrenergic receptors together with slow diffusion of NE in the slice might also have contributed to the incomplete recovery.

The blockage of an HVA Ca2+-current in MCs, physiologically triggered by NE, appeared to be mediated by α2-receptors, as the effect was mimicked by α2-agonists; with α-methyl-NE the current was reduced to 69.8 ± 4.1% and recovered to 85.8 ± 4.3% (10 μM, Fig. 3B) and with clonidine it reduced to 27.0 ± 8.2% and recovered to 63.3 ± 10.3% (20 nM, Fig. 3C and D). Though clonidine is known as an α2-agonist (Ruffolo et al., 1994; Jasper et al., 1998), it could also act via imidazoline receptors (Ruffolo et al., 1994; Eglen et al., 1998). However, in our system the α2-specific agonist α-methyl-NE, as well as noradrenaline itself – both nonligands at I1-receptors – exert the same effect as clonidine. It may therefore be assumed that the effects were mediated via α2-receptors. We did not measure the dose-response curves of the α2-mediated block of the Ca2+-current, as the precise drug concentrations at the site of the receptors are difficult to determine in tissue slices.

The described Ca2+-current suppression was observed in 27/46 MCs in the MOB and 10/25 MCs in the AOB. NE, α-methyl-NE and clonidine were applied in 31, 12 and 28 neurons, respectively. Notably, in GCs, which show an HVA current similar to that in MCs (not shown), we have never observed any noradrenergic effect on the HVA Ca2+-current (seven GCs in the AOB and eight GCs in the MOB).

Effects of norepinephrine upon spontaneous synaptic activity in granule cells
If the Ca2+-channels blocked by NE were located at the reciprocal synapses between MCs and GCs, one could expect an effect of NE upon the synaptic activity of GCs as first shown in cultured OB neurons by Trombley & Shepherd (1992). NE applied to the Xenopus OB slice induced a decrease of spontaneous synaptic activity in GCs (Fig. 4). This effect was more pronounced if extracellular Mg2+ was reduced to 0.2 mM confirming the involvement of NMDA receptors in the synaptic transmission from MCs to GCs (Trombley & Shepherd, 1992; Schoppa et al., 1998). Figure 4 shows an example of spontaneous synaptic activity before (A), during (B) and after (C) application of NE. Because example traces like these can easily contain a certain bias and are thus not perfectly representative, we calculated the integral of the postsynaptic current, i.e. the postsynaptic charge, Q, as a measure of synaptic activity (see Materials and methods). Upon application of NE, Q(t) clearly decreased in GCs of the AOB (Fig. 4D, 9/16 cells). Similar results were obtained in 10/14 GCs in the MOB (Fig. 4E).

Effects of norepinephrine and clonidine upon spontaneous synaptic activity in mitral cells
From a system as well as from a coding point of view, the decrease of granule cell synaptic activity would presumably have no effect upon
the information conveyance from receptor neurons to higher brain centres if there were no feedback upon MCs. We therefore tested whether NE or clonidine also affected the synaptic activity of MCs. Figure 5 unambiguously shows that this was the case in both the MOB (Fig. 5A) and AOB (Fig. 5B). Similar results were observed in 6/8 MCs of the MOB and in 6/9 MCs of the AOB.

**Does norepinephrine act post-synaptically on granule cells?**

The NE-mediated blockage of an HVA current in MCs on the one hand and the NE-mediated decrease of synaptic activity in GCs on the other does not necessarily mean that the latter is caused by the former. An alternative explanation for this correlation could be that NE, in addition to blocking an HVA current in MCs, had a direct and suppressive effect on postsynaptic glutamate channels in GCs. We therefore measured the noradrenergic effect upon glutamate-induced current responses of GCs. Figure 6A shows that the inward currents induced by bath application of glutamate (5 mM, solution B4) were virtually identical before and during the application of \( \alpha \)-methyl-NE (20 \( \mu \)M). This experiment was repeated under various conditions. First we applied clonidine (six GCs) and \( \alpha \)-methyl-NE (12 GCs). Second, in 11 GCs we blocked Ca\(^{2+}\) channels by Co\(^{2+}\) (B5) in order to reduce synaptic transmission, in particular from MCs to GCs. In these cases the glutamatergic current amplitudes were smaller than in the control cases, presumably due to a permeation block by Co\(^{2+}\) in NMDA channels (Mayer & Westbrook, 1987). Figure 6B summarizes the glutamate-induced current amplitudes during \( \alpha \)-agonist application as a function of the respective glutamate-induced control response that preceded the agonist application. Linear regression analysis gave a correlation coefficient of 1001. This clearly indicated that the glutamate-mediated current responses of GCs were not affected by \( \alpha \)-methyl-NE or clonidine.
Finally we tested whether clonidine or α-methyl-NE had an effect upon voltage-activated conductances in GCs. Figure 6C shows current responses to voltage ramps from −80 mV to +80 mV before and during clonidine application. We never observed any significant difference between the respective ramp responses.

Discussion

Neuroanatomical (Halasz & Shepherd, 1983; Shipley et al., 1985; McLean et al., 1989; McLean & Shipley, 1992), as well as combined pharmacological and behavioural data (Kaba & Keverne, 1988; Kaba et al., 1989), clearly suggest that the signal processing by the OB is affected by NE. Presumably there is more than one modulatory, NE-dependent pathway (Tani et al., 1992; Trombley & Shepherd, 1992; Ciombor et al., 1999). Data from cultured OB neurons showed that NE blocked a Ca^{2+}-current in MCs and GCs (Trombley, 1992), and suppressed synaptic activity in presumed GCs (Trombley & Shepherd, 1992). Here we followed this line of evidence extending it to another species (X. laevis) and to another preparation using a slice preparation of the OB. The known gross morphology of the Xenopus tadpole OB (Byrd & Burd, 1991; Nezlin & Schild, 2000) allowed us to distinguish between MCs and GCs of both AOB and MOB. We tried to answer the following questions: (i) what kind of NE receptors are involved in Xenopus?; (ii) are the NE-receptors localized at MCs, GCs or both?; (iii) which types of ion channels are affected by the noradrenergic innervation? (iv); does NE act at synapses?; and (v) are the effects of NE confined to the AOB or can they also be observed in the MOB?

Our data show that following: (i) NE and the two α₂-agonists clonidine and α-methyl-NE acted in both the AOB and MOB; (ii) they blocked an HVA Ca^{2+}-current in MCs but not in GCs The same effect had been reported in cultured OB neurons of Xenopus (Bischofberger & Schild, 1995); (iii) as the effect of NE was...
mimicked by α-methyl-NE (10 μM) and by clonidine (20 nM), the observed effect was presumably mediated by α₂-receptors (Dausse et al., 1981; Ebersolt et al., 1981; Ruffolo et al., 1994; Eglen et al., 1998; Jasper et al., 1998); (iv) NE blocks synaptic activity in GCs and in MCs. The block of synaptic activity in GCs did not appear to be postsynaptic because glutamate-induced currents in GCs were not affected by α-methyl-NE or clonidine.

**Measure of synaptic activity**

Synaptic activity was represented as the integrated postsynaptic current, corresponding to a charge (Schoppa et al., 1998). This measure is particularly convenient when recording spontaneous synaptic activity as it is hard to decide by eye or simple statistics whether some of the recorded synaptic currents are decreased in amplitude or whether the rate of synaptic currents of certain amplitudes are decreased. Basically, histograms of excitatory postsynaptic current (EPSC) amplitudes measured prior to, during

**Fig. 4.** Norepinephrine (NE) decreases spontaneous synaptic activity in granule cells. Spontaneous synaptic activity of a granule cell recorded in the accessory olfactory bulb (AOB) before (A), during (B) and after (C) the application of NE (500 nM, solutions B₁ and I₁). The integrated synaptic current shows a clear reduction in granule cells of both the AOB (D) and main olfactory bulb (E) after application of NE (500 nM), followed by an almost complete recovery after washout. The dotted line approximates the slow rundown of synaptic activity during the experiment.

**Fig. 5.** Norepinephrine (NE) decreases spontaneous synaptic mitral cell activity in the main olfactory bulb (MOB) and accessory olfactory bulb (AOB). (A) Integrated synaptic current of a mitral cell recorded in the MOB. Application of NE (500 nM) markedly decreases the spontaneous synaptic activity. (B) Same effect as in A but here in the AOB. The dotted lines in A and B indicate the overall slight decrease in synaptic activity during the experiment.
and after the drug effect could be used to decide this question. However, a rather low rate of synaptic events, together with slow drug diffusion in the slice and a limited recording time of about 1 h, did not allow the building up of a histogram with sufficiently narrow amplitude bins. The integrated postsynaptic current rather appeared to be the appropriate measure for changes in low spontaneous synaptic activity. Most recordings showed a slow drift of the rate of EPSCs. We did not correct for this drift, so that the slow run-down of synaptic activity can be seen in the respective figures (Figs 4 and 5).

**Lack of $\alpha_2$-mediated effects in granule cells**

The block of an HVA calcium conductance was never observed in GCs, nor did we observe any other effect of clonidine, NE (< 1 $\mu$m) or $\alpha$-methyl-NE upon voltage-activated conductances in GCs. Importantly, glutamate-induced currents in GCs were not affected by the bath application of $\alpha$-methyl-NE or clonidine (Fig. 6A and B). These findings in the *Xenopus* OB are not consistent with the idea that NE influences olfactory learning via direct $\alpha_2$-modulation of GCs, as suggested by Trombley (1992). Trombley’s experiments were, however, carried out in cultured neurons of the rat OB, and high agonist concentrations (30 $\mu$m NE) were applied. There are thus three major differences between his and our experimental conditions, i.e. the species, the preparation and the agonist concentration applied, which may explain the different experimental outcome. Interestingly, the lack of direct, postsynaptic $\alpha_2$-dependent effects in GCs has the consequence that GCs stay sensitive to efferent and recurrent modulation.

**Does norepinephrine influence lateral inhibition and learning?**

The block of an HVA Ca$^{2+}$-current and the suppression of synaptic currents occurred in 47% and 61% of MCs, respectively. These values might be larger in vivo, because some dendrites and synapses of the MCs were inevitably lost during the sectioning of tissue.

We recorded the spontaneous synaptic activity in MCs using a chloride reversal potential of approximately 0 mV (see Table 1), i.e. the GABAergic synaptic input from GCs to MCs, which is presumably inhibitory under most physiological circumstances (Siklos et al., 1995), was reflected by EPSCs rather than be IPSCs. As this synaptic activity was suppressed during NE application, we conclude that, under most physiological conditions, the majority of MCs are disinhibited by NE. In a first approximation that accounts neither for the exact percentage of disinhibited MCs nor for the strength of disinhibition, this could mean that an odorant, when applied during increased $\alpha_2$-modulation, is perceived with reduced filtering by the complex network of lateral inhibition. The spatial pattern of odorant-activated MCs would thus be affected directly by noradrenergic innervation and one could hypothesize that its correlation with the glomerular activation pattern of receptor afferents becomes higher by this means. The extrapolation of our results to many MCs and GCs suggests that NE may temporarily alter the weights of lateral inhibition, and thereby the overall spatial transmission of the OB. This could be an important condition for learning novel odours.

In this context it is noteworthy that the action of NE was not confined to the AOB. NE rather appears to influence odorant processing in the MOB, too. While the $\alpha_2$-receptor-induced disinhibition in the AOB is consistent with the pharmacological and behavioural experiments mentioned above, analogous effects of NE on the odorant processing in the MOB have as yet not been reported.

**Coupling of $\alpha_2$-receptors to glutamate exocytosis**

Three different ways of inhibition via presynaptic $\alpha_2$-receptors are known (Miller, 1998), i.e. blockage of calcium channels, increase of a $K^+$-conductance and a direct modulation of transmitter exocytosis.
Our data show unambiguously an NE-induced suppression of an HVA Ca2+-current (Fig. 3), and we have no indication for an increase of a K+-conductance. Hence, it is presumed the decrease in Ica diminishes the probability for the exocytotic of synaptic vesicles, eventually leading to a decreased postsynaptic activity in GCs. The mechanism by which α2-adrenoceptors modulate calcium channels needs a more detailed analysis. The molecular and cellular actions of α2-agonists have recently been studied in hippocampus (Boehm, 1999) and postganglionic sympathetic neurons. In these cases, activation of presynaptic α2-receptors blocked an N-type presynaptic calcium channel (Boehm & Huck, 1996) and postsynaptic IPSC amplitudes (Miyazaki et al., 1998; Boehm, 1999). Our observation that α2-receptors couple negatively to glutamatergic synaptic transmission in MCs but not in GCs parallels Boehm’s data in the hippocampus, where NE blocked a Ca2+-current in glutamatergic but not GABAergic neurons (Boehm, 1999). Taken together, these data suggest that the negative coupling between α2-receptor activation and glutamate exocytosis may be a widespread mechanism in the vertebrate central nervous system.

Abbreviations
AOB, accessory olfactory bulb; DIC, ; EPCP, excitatory postsynaptic potential; EPSC, excitatory postsynaptic current; GC, granule cell; GL, glomerular layer; GCL, granule cell layer; HVA, high-voltage activated; IPSC, inhibitory postsynaptic current; MC, mitral cell; MCL, mitral cell layer; MOB, main olfactory bulb; NE, norepinephrine; OB, olfactory bulb; PB, phosphate buffer; PCM, pulse code modulation; TTX, tetrodotoxin; TX, triton X100.

References