[Title]

Dopaminergic neuromodulation of synaptic transmission between the mitral and granule cells in the teleost olfactory bulb

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Running head: Dopaminergic neuromodulation in teleost olfactory bulb

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Abstract

A growing body of evidence suggests that teleosts are important models for the study of neural processing of olfactory information, and the functional role of dopamine (DA), which is a potent neuromodulator endogenous to the mammalian olfactory bulb, has been one of the strongest focuses in this field. However, the cellular mechanisms of dopaminergic neuromodulation in the olfactory bulbar neural circuits have not been fully understood. We investigated such mechanisms by using the goldfish, which offers several advantages for analyzing the olfactory information processing by electrophysiological methods. First, we found in the olfactory bulb that numerous cell bodies of the dopaminergic neurons are mainly distributed in the mitral cell layer and extend fine processes to the glomerular layer. Next, we made in vitro field potential recordings and showed that synaptic transmissions from the mitral to granule cells were suppressed by DA application. DA also increased the paired-pulse ratio, suggesting that the suppression of synaptic transmission is caused by a decrease in the presynaptic glutamate release from the mitral cells. Furthermore, DA significantly suppressed the oscillatory activity of the olfactory bulb in response to the olfactory stimuli. Although DA suppresses the synaptic inputs from the olfactory nerve to the olfactory bulbar neurons in mammals, such phenomenon was not observed in the goldfish. These findings indicate that the suppression of the mitral to granule cell synaptic transmission in the reciprocal synapses plays an important role in the negative regulation of olfactory responsiveness in the goldfish olfactory bulb.

Keywords

olfaction, dopamine, neuromodulator, goldfish
Introduction

The olfactory bulb is the primary neural circuit for olfactory information processing throughout vertebrates. The odorant information is first received by olfactory receptor neurons in the olfactory epithelium, and the olfactory bulbar neural circuit then processes this information. The olfactory bulb consists of morphologically and functionally distinct neuronal types arranged in different layers, and these neurons contribute to the processing of olfactory information (Shepherd et al. 2004).

Characteristically, the mitral cell, which is the principle neuron in the olfactory bulb, forms reciprocal dendro-dendritic synapses with the granule cells, which are interneurons in the olfactory bulb (Rall and Shepherd 1968). These interactions are considered to be important for determining the temporal firing pattern of the mitral cells, influencing the odorant coding of olfactory bulbar neural circuits (Friedrich et al. 2004; Schoppa 2006; Tabor et al. 2008).

The teleosts are now widely used as excellent model animals for investigating the mechanisms underlying the processing of odorant information in the olfactory system (Byrd and Brunjes 1995; Friedrich et al. 2004; Friedrich and Korsching 1998; 1997; Friedrich and Laurent 2001; Fuller et al. 2006; Hamdani el and Doving 2007; Hanson et al. 1998; Hasegawa et al. 1994; Ichikawa 1976; Kawai et al. 2010; Kawai et al. 2009a; Kawai et al. 2009b; Oka 1983; 1980; Satou 1990; Satou et al. 1983; Satou et al. 2006; Satou and Ueda 1978). Previous studies using the goldfish and the carp have established the morphological and electrophysiological bases for the study of teleost olfactory bulbs (Hanson et al. 1998; Hasegawa et al. 1994; Ichikawa 1976; Oka 1983; 1980; Satou 1990; Satou et al. 1983; Satou et al. 2006; Satou and Ueda 1978). These authors have shown that the basic structure of the goldfish olfactory bulb is similar to that of the
mammalian olfactory bulb, and the odorant stimulation evokes synchronized rhythmic
discharges in the olfactory bulbar neurons. Furthermore, recent studies using zebrafish,
which is closely related to goldfish, have advanced this knowledge (Friedrich et al.
2004; Friedrich and Korsching 1998; 1997; Friedrich and Laurent 2001; Fuller et al.
2006). For example, it has been suggested in zebrafish that the information about each
specific odorant is coded by the dynamic representation of temporal firing patterning of
mitral cells (Friedrich and Laurent 2001).

The olfactory bulb contains a number of intrinsic dopaminergic neurons
throughout vertebrates. Dopamine (DA) is considered to be a neuromodulator in various
regions of the brain as well as in the peripheral sensory organs (Ben-Jonathan and
Hnasko 2001; Ennis et al. 2001; Hsia et al. 1999; Serguera et al. 2008; Umino and
Dowling 1991; Wachowiak and Cohen 1999), and their function in the olfactory bulb
has partially been studied in mammalian species. The DA neurons in the rodent
olfactory bulb have been categorized as belonging to the periglomerular cells that send
their dendrites to the “glomeruli”, which are spherical neuropil formed by the
intertwined axon terminals of the olfactory receptor neurons and the mitral cell
dendrites (Kosaka and Kosaka 2005). In the glomeruli, DA has been reported to cause
depression of synaptic inputs from the olfactory receptor neurons to the olfactory bulb
(Ennis et al. 2001), suggesting that it has an inhibitory role in the processing of
olfactory information in mammals. On the other hand, the morphology and distribution
of DA neurons reported in the teleost olfactory bulb appear to be different from those of
mammalian counterparts. For example, in zebrafish, the cell bodies and fibers of DA
neurons are distributed in the mitral cell layer (Byrd and Brunjes 1995). However, the
function of teleost olfactory bulbar DA neurons has not been studied so far, and,
therefore, their function still remains unknown.

In the present study, we examined the neuromodulatory effect of DA on the teleost olfactory bulbar neural circuits using the goldfish, which is suitable for the electrophysiological analysis of the olfactory bulb. DA suppressed the synaptic transmissions from the mitral to granule cells in the goldfish olfactory bulb, while DA did not influence the synaptic transmissions from the olfactory nerve terminals to the olfactory bulbar neurons as in mammals. The present results suggest that the dopaminergic suppression of synaptic transmission in the reciprocal synapses in the olfactory bulb has an important role in the negative regulation of olfactory responsiveness.
**Materials and Methods**

Adult goldfish (*Carassius auratus*) were purchased from a local dealer in Tokyo. They were kept in 60 litter aquaria containing ~15 fish at room temperature before use in the experiments. All procedures were performed in accordance with the guidelines of the Physiological Society of Japan and the University of Tokyo for the Use and Care of Experimental Animals.

### Labeling the olfactory nerve terminals

After the animals were anesthetized by immersion in 0.02 % 3-aminobenzoic acid ethyl ester (MS-222; Sigma, St Louis, MO), crystals of biocytin were applied onto the olfactory epithelium so that the olfactory nerve terminals were labeled by anterograde axonal transport. After the animals were kept in a tank for 5h, they were used for the immunohistochemistry as described below.

### Immunohistochemistry

The animals were anesthetized by immersion in 0.02 % MS-222, and they were perfused from the heart with 0.75 % NaC1, followed by 4 % paraformaldehyde (PFA) in 0.1 M PBS. The olfactory bulb was dissected out and fixed in the same fixative overnight at 4 °C. Serial frontal sections were cut on a cryostat at 30 μm. The sections were incubated with rabbit antiserum against tyrosine hydroxylase (TH) (Chemicon, Temecula, CA; gift of Dr. M. Ichikawa, Tokyo Metropolitan Institute for Neuroscience), the rate-limiting enzyme in the biosynthesis of DA and has been used for a DA neuron marker, diluted 1 : 2500 in a blocking solution for 10 - 14h at room temperature. For observation of the
distributions of TH immunoreactive (=DA) cell bodies and fibers in the olfactory bulb, the sections were incubated in biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 2 h at room temperature. Then the sections were incubated in ABC kit (Vector Laboratories, Burlingame, CA) and were reacted with 3,3'-Diaminobenzidine. They were counterstained with cresyl violet (Merck, Darmstadt, Germany).

For observation of the relationships between TH immunoreactive fibers and the olfactory nerve terminals, the sections of biocytin labeled olfactory bulbs were incubated in AlexaFluor555-conjugated goat anti-rabbit IgG (diluted 1 : 200 in 0.1M PBST; Invitrogen, Carlsbad, CA) and AlexaFluor488-conjugated streptavidin (diluted 1 : 500 in 0.1M PBST; Invitrogen, Carlsbad, CA) for 3 h at room temperature after the first antibody reaction against TH. A Zeiss LSM710 confocal laser scanning system (Carl Zeiss, Oberkochen, Germany) was used for collection of the fluorescence images.

**In vitro field potential recording from the olfactory bulb**

The detailed procedure for recording has been described in Kawai et al. (2010). Briefly, after the goldfish was anesthetized on ice and decapitated, the brain was dissected out. The olfactory tract was hooked on bipolar silver-wire electrodes for stimulation, and a glass recording electrode (tip diameter \( \approx 50 \mu m \)) filled with 0.1 % agar in 2 M NaCl was inserted to the depth of about 350 - 450 \( \mu m \) in the olfactory bulb. Glass recording electrodes were made of borosilicate glass (G-1.5, Narishige, Tokyo, Japan) using a Brown-Flaming micropipette puller (P-97; Sutter Instruments, Navato, CA). The olfactory bulb was perfused at a rate of 1.5 – 2.0 ml/ min with an
artificial cerebrospinal fluid (ACSF) containing (mM): 125 NaCl, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 2.4 CaCl$_2$, 1.3 MgCl$_2$, 26 NaHCO$_3$ and 10 glucose (pH 7.4). The ACSF was bubbled with a mixture of 95 % O$_2$ and 5 % CO$_2$ throughout the experiments. For the electrical stimulation, electrical shocks were delivered using an electronic stimulator (SEN-3301, Nihon Kohden, Tokyo, Japan) through an isolation unit (SS-201J, Nihon Kohden, Tokyo, Japan). Electrical stimuli of 0.1 – 0.2 ms pulses at 0.5 – 1 mA were delivered every 1 min. We also conducted paired-pulse stimulations with a 150 ms interval to determine whether the effect of DA arises from presynaptic or postsynaptic mechanisms (Kawai et al. 2010; Manabe et al. 1993; Satou et al. 2006; Zucker 1989). The field potential responses were amplified by an AC amplifier (AVH-11, Nihon Kohden, Tokyo, Japan) with 0.08 Hz – 3 kHz cut-off frequencies, and the data were sampled at 6 kHz using the Digidata 1322A and pCLAMP8.2 software (Molecular Devices, Sunnyvale, CA).

**Odorant stimulation and in vitro recordings of the synchronized activity from the olfactory bulb**

After the animals were anesthetized on ice, the olfactory epithelium was exposed by removing the tissue covering the nasal capsule. The animals were decapitated, and skulls over the brains were carefully removed using a dental drill. Then, all the structures caudal to the telencephalon were removed, and an intact preparation of the olfactory system was obtained. To examine the modulatory effect of DA on odorant response in the olfactory bulb, we applied odorant stimulation to the olfactory epithelium. We used NaCl as an odorant stimulus because this chemical substance has generally been recognized as an odorant in the fish olfactory
system. The application of NaCl is known to efficiently evoke synchronized olfactory response in the olfactory bulb whose frequency is almost the same as the other odorant stimulation (food-extract) in the goldfish olfactory bulb (Hasegawa et al. 1994; Satou 1990; Satou and Ueda 1978). Throughout the experiments, we applied continuous flow (1.5–2 ml/min) of deionized water (maintained at room temperature) to the olfactory mucosa. For each recording, the odorant was applied for 5 sec using an electronically controlled solenoid valve at every 3 min intervals.

A glass electrode filled with 0.1 % agar in 2 M NaCl was inserted to the depth of 350 - 450 µm from the dorsal surface of the olfactory bulb. Using a gravity-fed system, the olfactory bulb was perfused at a rate of 1.5 – 2.0 ml/ min with ACSF containing (mM): 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2.4 CaCl₂, 1.3 MgCl₂, 26 NaHCO₃ and 10 glucose (pH 7.4). The ACSF was bubbled with a mixture of 95 % O₂ and 5 % CO₂.

The odorant response of the olfactory bulb was amplified using a conventional AC amplifier (AVH-11, Nihon Kohden, Tokyo, Japan) with 0.08 Hz – 3 kHz cut-off frequencies, and the data were sampled at 10 kHz using the Digidata 1322A and pCLAMP8.2 software (Molecular Devices, Sunnyvale, CA). The recorded odorant responses were digitally filtered between 4 and 40 Hz and were used for the analysis. When we examined the effect of DA on the spontaneous activity of the olfactory bulbar neural circuits, we extracted traces in 10 s time window before and during DA treatment. When we examined the effect of DA on the strength of odorant responses, the traces in 10 s time window including the onset and offset of the odorant response were extracted. These traces were full-wave rectified around the mean level of the response using the DataView 6.3 software (W.J. Heitler,
Whole-Cell Recordings from the olfactory bulb slices in response to the olfactory nerve stimulation

After the animals were anesthetized on ice, the olfactory bulb was dissected out and was placed in an ACSF containing (mM): 150 NaCl, 3 KCl, 10 HEPES, 2.4 CaCl$_2$, and 1.3 MgCl$_2$ (pH 7.4). The meningeal membrane was gently removed, and the olfactory bulb was embedded in 3.6% agarose/ACSF and glued onto the baseplate of Vibratome 3000 (Vibratome, St Louis, MO). Then 420 μm thick horizontal sections were cut in the modified ACSF containing (mM): 150 Choline-Cl, 3 KCl, 10 HEPES, 2.4 CaCl$_2$, and 1.3 MgCl$_2$ (pH adjusted to 7.4 with NaOH). The slices were immediately transferred to an ACSF and were stored at room temperature for 1 hr before recording.

Conventional whole cell patch-clamp recordings were performed from the neurons in the mitral cell layer using a PC-501A amplifier (Warner Instruments, Hamden, CT). During the experiment, we selected large cells that are 10-20 μm in diameter, because the previous morphological study suggested that it is the size of the mitral cells (Oka 1983). The recording pipettes were made of borosilicate glass (G-1.5, Narishige, Tokyo, Japan) using a Brown-Flaming micropipette puller (P-97; Sutter Instruments, Navato, CA). The pipette resistance was 4 – 10 MΩ. The internal solution contained (in mM): 110 K-gluconate, 40 HEPES, 3 MgCl$_2$, 0.3 EGTA, 2 Na-ATP, 0.3 Na-GTP, and 5 QX-314 (pH adjusted to 7.2 with KOH). The
internal solution also contained 0.2 % biocytin for the identification of the cell. The
membrane potential was held at -60 mV.

For the stimulation, the bipolar electrode was placed on the olfactory nerve
layer. The electrical stimuli of 0.1 – 0.2 ms pulses at 0.5 – 1 mA were delivered
every 15 sec using an electronic stimulator (SEN-3301, Nihon Kohden, Tokyo,
Japan) through an isolation unit (SS-302J, Nihon Kohden, Tokyo, Japan).

Electrical signals were low-pass filtered at 1 kHz and sampled at 10 kHz using
the Digidata 1322A and pCLAMP8.2 software (Molecular Devices). The liquid
junction potential between the internal solution and the ACSF was estimated to be
within 5 mV and was not corrected.

For the identification of the recorded cell, the preparation was incubated by
shaking in AlexaFluor488 conjugated streptavidin (diluted 1 : 500 in 0.1M PBST;
Invitrogen, Carlsbad, CA) for 5h at room temperature. A Zeiss LSM710 confocal
laser scanning system (Carl Zeiss, Oberkochen, Germany) was used for collection of
the fluorescence images.

Statistical analyses

Statistical analyses were performed with Kyplot software (version 3, Kyence, Tokyo,
Japan). We examined whether it met the equal variance conditions for every analysis.
If so, we used Student’s t-test for statistics, and if not, we used Welch’s t-test. All the
data in this study are represented as means ± s.e.m. For the multiple comparison
(Fig. 6E, Fig. 8E), we used Dunnett's test.

Drugs
(-)-Quinpirole, a dopamine D2-like agonist, and gabazine, a GABA<sub>A</sub> receptor antagonist, were purchased from Sigma (St Louis, MO). DA and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were obtained from Tocris (Bristol, UK). CNQX was first dissolved in dimethyl sulfoxide (DMSO) to 20 mM and then diluted in ACSF.

**Results**

*Distributions of DA neurons in the goldfish olfactory bulbar neural circuits*

As has been reported in the other teleost species (Byrd and Brunjes 1995), we observed abundant TH immunoreactive fibers and cell bodies in the goldfish olfactory bulb (Fig. 1A). TH immunoreactive cell bodies were distributed mainly in the mitral cell layer (Fig. 1B, E), and most of them were bipolar neurons and had spherical cell bodies (8 - 12 μm in diameter). They were distinct from the mitral cells in their size (10 - 20 μm in diameter) and morphology (Oka 1983). Furthermore, the internal cell layer also contained a small number of TH immunoreactive neurons (Fig. 1C, E). Notably, we could observe dense staining in the glomerular layer (Fig. 1B, the area between the dotted lines), possibly reflecting the population of fine processes that originate from the dendrites of DA neurons.

To examine the relationship of these TH immunoreactive processes with the olfactory nerve terminals, we labeled the olfactory nerve terminals by anterograde transport of biocytin from the olfactory epithelium (Fig. 1D). We found that TH immunoreactive processes extensively surround the olfactory nerve terminals (Fig. 1D, the area between the dotted lines), suggesting the existence of some
interactions between the TH immunoreactive neurons and the olfactory nerve terminals. A large population of TH cell bodies was distributed in the MCL, which is located in the zone indicated in Fig. 1E.

Effects of DA on the synaptic transmission from mitral to granule cells

To examine the action of DA on the goldfish olfactory bulbar neural circuits electrophysiologically, we first focused on the synaptic transmission from the mitral to granule cells, which is believed to be important for the olfactory information processing throughout vertebrates (Abraham et al. 2010; Rall and Shepherd 1968; Satou 1990; Shepherd and Brayton 1979; Yokoi et al. 1995). We examined the effect of DA on the C2 wave, a component of electrically-evoked field potentials in the goldfish olfactory bulb. In the previous studies, the depth profiles of these field potentials revealed that the C2 wave reverses their polarity in the mitral cell layer where most of the mitral to granule cell synapses exist. The C2 waves are always large and positive in the internal cell layer (Satou 1990). These results indicated that the extracellular currents flow from the internal cell layer to the mitral cell layer during the period of C2 wave, supporting that the C2 wave reflects the mitral to granule synaptic transmission. (Fujita et al. 1984; Kawai et al. 2010; Satou 1990; Satou et al. 1983; Satou et al. 2006). Therefore, we inserted our recording electrode to the depth of about 350 - 450 μm from the dorsal surface of the olfactory bulb to place the electrode in the granule cell layer (Fig. 1E) and recorded reproducible field potential waveforms (Fig. 2A, B). As we reported previously (Kawai et al. 2010), the C2 component was drastically attenuated by the application of CNQX, an AMPA/kainate receptor antagonist, supporting that this component reflects the
excitatory synaptic inputs from the mitral to granule cells. On the other hand, the C2 component is not derived from the inhibitory synaptic inputs, because it is not sensitive to GABA<sub>A</sub> receptor antagonist. We found that the application of DA (50 μM) significantly decreased the amplitudes of C2 wave component (decreased by 47.4 % ± 8.2 %, before 10min vs during 10min application of DA, n = 6; Fig. 2 C - E). The amplitude started to decrease within 1 min after the application of DA, and saturated about 5 min afterward. Clearly, the effects of DA started to recover immediately after the washout. Furthermore, their effects were dose-dependent with an EC<sub>50</sub> of 18.4 μM (Fig. 2 F).

To determine whether the DA-induced decrease in C2 is derived from presynaptic or postsynaptic mechanisms at the mitral to granule cell synapse, we performed paired-pulse olfactory tract stimulations and measured changes in the ratio of these consecutive C2 waves (Kawai et al. 2010; Manabe et al. 1993; Satou et al. 2006; Zucker 1989). As indicated in Fig. 3, the application of 50 μM DA significantly increased the normalized second C2 wave amplitude and thus altered the paired-pulse ratio (increased by 18.9 % ± 5.0 %, before 10 min vs during 10 min application of DA, n = 6). The time course of the changes in paired-pulse ratio was similar to that of the C2 wave amplitude (Fig. 3B).

Quinpirole mimics the effect of DA on the synaptic transmissions from the mitral to granule cells

To investigate the mechanisms underlying the action of DA on the synaptic transmissions from the mitral to granule cells, we then examined if (-)-quinpirole, a D2-like agonist, mimics the effect of DA on the C2 wave amplitude as well as on
the paired-pulse ratio. As indicated in Fig. 4, the application of (-)-quinpirole (0.3 µM) gradually suppressed the amplitude of C2 wave (decreased by 42.7 % ± 4.9 %, at 10min from the start of application of (-)-quinpirole, n = 6; Fig. 4A, B), although it was difficult to observe the recovery after the washout of (-)-quinpirole. 0.3 µM (-)-quinpirole also increased the paired-pulse ratio (increased by 19.4 % ± 6.9 %, at 10min from the start of application of (-)-quinpirole, n = 6; Fig. 5) as observed in the application of DA, suggesting that DA show the suppressive effect on the synaptic transmissions via the dopamine D2-like receptor.

DA application suppresses the activities of olfactory bulbar neural circuits

We then examined the functional role of DA in the goldfish olfactory bulb. We found that the bath application of DA (50 µM) suppressed the spontaneous activities of the olfactory bulbar neural circuits when no chemical stimulation was applied to the olfactory epithelium (Fig. 6A, E black bar; decreased by 37.7 ± 5.2 %, n = 6). The effect of DA was reversible (Fig. 6B). The power spectrum of the spontaneous olfactory bulbar activity also suggested that DA suppressed the spontaneous activities (Fig. 6C). Suppression of the spontaneous olfactory bulbar neuronal activity was also evoked by (-)-quinpirole (0.3 µM; Fig. 6D, E gray bar; 17.4 ± 3.0%, n = 6).

Next, we examined the effect of DA or (-)-quinpirole on the odorant response in olfactory bulb. After the odorant information is transmitted from the olfactory epithelium to the olfactory bulb, oscillatory responses are generally evoked in the olfactory bulb throughout vertebrates (Adrian 1950; Friedrich et al. 2004; Hasegawa...
The reciprocal synapses are considered as a crucial microcircuit for generating these oscillatory activities. We therefore examined whether DA, which drastically suppressed the reciprocal synapses in the present study, affects the strength of these odorant-induced oscillatory activity. In the present study, the oscillatory activities were evoked by a strong chemical stimulation (1M NaCl) of the olfactory epithelium (Fig. 7A, B). By evaluating the frequencies at which the peaks of the power spectra were observed, we determined that the average frequency of the odorant response was 12.5 ± 0.50 Hz (n=11; Fig. 7B). The odorant-induced oscillatory activities were abolished by the application of 50μM gabazine, a GABA<sub>A</sub> receptor antagonist (n=3, Fig. 7A, B), supporting the notion that the reciprocal synapses in the olfactory bulbar neural circuits contribute to the synchronized activities of a population of olfactory bulbar neurons in the odorant information processing. The spontaneous olfactory bulbar activities were least affected by the application of gabazine (Fig. 7 C, D), suggesting that the reciprocal synapse is not important for the spontaneous activities.

We found that DA or (-)-quinpirole suppressed the oscillatory activities in response to the odorants in the goldfish olfactory bulb. Prior treatment with DA (50μM) or (-)-quinpirole (0.3μM) resulted in a significant decrease in the strength of the odorant response compared with the vehicle treatment (decreased by 31.0 % ± 4.7 % for DA, 14.2 ± 3.6% for (-)-quinpirole, before four trials vs during three trials of application of DA or (-)-quinpirole, n=6 in both; Fig. 8). Thus, these results suggest that DA has a suppressive role in the activities of the olfactory bulbar neurons in response to the odorants just as it suppresses the electrically-evoked field potentials.
DA does not modulate synaptic transmissions from the olfactory nerve terminals to the olfactory bulbar neurons

Because DA suppresses the synaptic inputs from the olfactory nerve terminals to the olfactory bulbar neurons presynaptically in mammals (Ennis et al. 2001; Gutierrez-Mecinas et al. 2005; Hsia et al. 1999), we examined whether similar synaptic modulation in the olfactory bulb exists in the teleost olfactory bulb. We performed conventional whole-cell recordings from the olfactory bulbar neurons in the mitral cell layer and recorded synaptic inputs from the olfactory nerve terminals by stimulating the olfactory nerve layers (Fig. 9 A, B). As expected, excitatory postsynaptic currents (EPSC) originating from the olfactory nerve terminals were observed in the olfactory bulbar neurons, and these responses were abolished by the application of 50 μM CNQX (n = 5, Fig. 9 C, D), an AMPA/kainate receptor antagonist. Then we examined if the application of DA (50μM) alters the amplitude of these glutamatergic synaptic transmissions. However, there was no change in the amplitude of these EPSCs by the application of 50μM DA (Fig. 9E-G; n=5 for 5 min DA application; n=4 for 10min DA application). These results suggest that in the goldfish olfactory bulb, unlike in the mammalian ones, DA does not suppress the glutamatergic synaptic transmissions from the olfactory nerve terminals to the olfactory bulbar neurons.

Discussion

DA neurons are distributed in the mitral cell layer and extend fine processes toward the olfactory nerve terminals
In the goldfish, we found that the somata of TH-immunoreactive neurons are mainly distributed in the mitral cell layer like the zebrafish olfactory bulb (Byrd and Brunjes 1995; Fuller et al. 2006). We could also demonstrate abundant TH-immunoreactive fibers in the mitral cell layer, suggesting that they can potentially affect the mitral cells. Apparently, this distribution patterning of somata and fibers of DA neurons in the olfactory bulb is different from that of mammalian species, in which the olfactory bulbar DA cell bodies surround the glomeruli. This difference in the distribution pattern of DA cell bodies and fibers may underlie the functional difference between teleosts and mammals in the regulation of the olfactory bulbar neural circuits. For example, it is possible that the teleost DA neurons mainly target reciprocal synapses between the mitral and granule cells, while the mammalian DA neurons mainly target synapses at the olfactory nerve terminals. The present results suggest that DA does not suppress the synaptic transmission from the olfactory nerve terminals to the olfactory bulbar neurons in the goldfish olfactory bulb, while in mammals DA does so (Ennis et al. 2001). Thus, the mammalian olfactory bulb may have newly acquired their regulatory roles of DA to suppress synaptic transmissions at the olfactory nerve terminals. The previous report concerning the neuromodulation of DA in the frog olfactory bulb is consistent with this idea (Davison et al. 2004; Duchamp-Viret et al. 1997). In the frog, DA suppresses the synaptic transmission from mitral to granule cells (Davison et al. 2004). Furthermore, according to the results of the Ca\(^{2+}\) imaging experiment (Davison et al. 2004), DA does not appear to suppress the synaptic transmission from the olfactory nerve terminals presynaptically. Their results were further strengthened by the present electrophysiological data, in which we monitored the
synaptic transmissions more directly. Thus, it is likely that there are some differences between the mammals and the nonmammalian vertebrates (amphibians and teleosts) in the mechanisms underlying the suppressive role of DA in olfactory bulb.

On the other hand, there are also similarities between the goldfish and the mammalian DA neurons in the olfactory bulb. That is, the goldfish DA neurons also extend their fine processes toward the olfactory nerve terminals like the mammals, although it is difficult to confirm that these fine processes interact directly with the glomeruli because of the less developed glomerular structure in the teleost olfactory bulb. In the mammalian olfactory bulb, DA neurons are thought to receive olfactory information directly from the intraglomerular region (Kosaka and Kosaka 2008; Philpot et al. 1998) and are involved in the processing of the odorant information. Possibly, DA neurons in the goldfish olfactory bulb may also receive olfactory information directly from the olfactory nerve terminals as in mammals, and thereafter it may be involved in modulating the synaptic interactions between the mitral and the granule cell.

Another possibility is that DA suppresses the synaptic transmissions from the mitral cells to the interneurons that exist in the glomerular or mitral cell layers. In the present study, we observed abundant TH positive fibers in the glomerular layer, which may suggest that DA is also released in this region. Although DA did not affect the olfactory inputs to the mitral cells, DA may regulate the synaptic interactions between the mitral cells and the interneurons outside of the internal cell layer. Thus, DA may be widely involved in the processing of olfactory information in the goldfish olfactory bulb.
DA appears to suppress the processing of olfactory information in the goldfish olfactory bulb

In the olfactory bulb, the mitral cells and the granule cells form reciprocal dendro-dendritic synapses, where the mitral cells form excitatory glutamatergic synapses with the dendrites of the granule cells, and the granule cells form GABAergic synapses with the dendrites of the mitral cells (Satou 1990; Shepherd et al. 2004). These reciprocal synapses are believed to be important for evoking the synchronized activity of the olfactory bulbary neurons. Here, the mitral cells undergo synchronized subthreshold oscillations in the membrane potential by receiving synchronized inhibitory inputs from the granule cells (Schoppa 2006). Therefore, the mitral cells tend to fire near the peaks of these oscillations in the membrane potentials, contributing to synchronized activities of the mitral cells in the odorant response (Friedrich et al. 2004).

In this context, the changes in the reciprocal synaptic transmissions may alter these odorant-induced synchronized activities of the olfactory bulb neurons. In the present study, DA suppressed the glutamatergic inputs from mitral to granule cells presynaptically, and it also reduced the strength of the oscillatory odorant response as well as spontaneous activities of the goldfish olfactory bulb. Furthermore, when we blocked the GABAergic synaptic transmission, which is one of the reciprocal synaptic responses, by application of gabazine, the odorant-induced oscillatory activities were completely abolished, although the spontaneous activities were least affected by gabazine. These results support the idea that the modulation of reciprocal synapses affects the strength of the odorant-induced oscillatory activities
of the olfactory bulb. Thus, it is probable that the modulation of the reciprocal
synapses by DA contributes to the reduction of the odorant-induced synchronized
activity of the olfactory bulbar neurons. Therefore, DA may be one of the major
negative neuromodulators for the olfactory responsiveness in the goldfish olfactory
bulb.

Functional role of TH neurons in the goldfish olfactory bulb

It should be important to discuss the basic property of TH neurons
(DA-releasing neurons in the goldfish olfactory bulb). In the mammalian olfactory
bulb, the TH neurons are categorized as a subpopulation of GABAergic
periglomerular cell groups (Kosaka and Kosaka 2008). These neurons appear to
receive excitatory inputs from the olfactory nerve terminals and suppress synaptic
inputs from the olfactory nerve terminals by co-releasing GABA and DA (Maher
and Westbrook 2008). Although there is not enough study that describes whether TH
neurons are GABAergic in teleosts, there is one report showing the colocalization of
GABA and dopamine in the olfactory bulbar neurons of sea lamprey
(Barreiro-Iglesias et al. 2009), a primitive vertebrate species. Thus, TH neurons in
the goldfish olfactory bulb may be GABAergic as well as dopaminergic. In this
context, it is possible that the TH neurons in the goldfish olfactory bulb receive
synaptic inputs from the olfactory nerve terminals and suppress the odorant response
in the olfactory bulb by co-releasing GABA and DA.

The release of DA from TH neuron in olfactory bulb may be modulated by the
physiological state of animals. Although the timing of DA release in the olfactory
bulb has not been studied yet, there are some reports describing changes in the
expression level of TH mRNA in the olfactory bulb (Baker et al. 1993; Dluzen et al. 2002; Hayashi et al. 2004; Serguera et al. 2008; Weltzien et al. 2006), which should result in the alteration of DA synthesis. For example, an interesting mechanism has been reported in the mouse main olfactory bulb as follows (Serguera et al. 2008). In a copulated female mouse, the scent of the urine of an alien male, but not that of the stud male, blocks the pregnancy within 3 days post copulation (the Bruce effect). However, after 3 days post copulation, the number of TH neurons starts to increase in the main olfactory bulb, and the synthesized DA in the TH neurons functions as an active sensory barrier in the olfactory bulb which now avoids the action of such olfactory stimuli (Serguera et al. 2008). Although the Bruce effect is restricted to the mouse, it is possible that DA functions as a sensory barrier also in the goldfish olfactory bulb, depending on their external or internal environments. It has also been reported that the expression level of TH mRNA is altered by hormonal level of the animal. In the European eel, treatment with testosterone increased TH mRNA specifically in the olfactory bulb (Weltzien et al. 2006). This result may suggest that DA in the olfactory bulb may be somehow related to the internal environment of the animals. Centrifugal modulation can also alter the spontaneous activity of DA neurons in the olfactory bulb. For example, the application of acetylcholine, which is a centrifugal neuromodulator mainly from the lateral preoptic area (Halasz and Shepherd 1983), to the mouse olfactory bulb reduced the spontaneous firing of DA neurons by muscarinic activation (Pignatelli and Belluzzi 2008). Thus, the volume of DA released from the olfactory bulbar DA neurons can be controlled in various ways. Overall, these hormonal and centrifugal regulatory mechanisms may allow animals to modulate their olfactory responsiveness appropriately according to their
internal or external environments.
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**Figure legends**

**Fig. 1.** Distribution of TH immunoreactive cell bodies and fibers in the goldfish olfactory bulbar neural circuits. (A - C) Low power photomicrographs of cross-sections of the goldfish olfactory bulb, immunolabeled for TH antiserum and counterstained with Cresyl Violet. (A) Distribution of TH immunoreactive neurons (arrowheads) in the goldfish olfactory bulb. Scale bar = 100 μm. (B) TH immunoreactive cell bodies (arrowheads) and fibers were abundantly distributed in the mitral cell layer (MCL). Note the dense distribution of immunoreactive fibers in the glomerular layer (the area in the dotted lines). Scale bar = 50 μm. (C) A small number of TH immunoreactive cells (arrowheads) and fibers were found in internal cell layer. Scale bar = 50 μm. (D) Confocal photomicrograph showing interactions between the TH immunoreactive fine processes (magenta, dotted line area) and the biocytin-labeled olfactory nerve (green) terminals. TH immunoreactive fine processes surround the olfactory nerve terminals. Scale bar = 20 μm. ONL, olfactory nerve layer; GL, Glomerular layer; MCL, mitral cell layer; ICL, internal cell layer. (E) Distribution of TH immunopositive cell bodies in the goldfish olfactory bulb (n=3). A large population of TH cell bodies is distributed in the MCL (indicated area by arrow).

**Fig. 2.** DA suppresses the synaptic transmission from the mitral cell to the granule cell. (A) A scheme illustrating the preparation for the *in vitro* field potential recording in the present study. OB, olfactory bulb; OT, olfactory tract; TEL, telencephalic hemisphere. (B) Examples of field potential traces in response to the olfactory tract stimulation (arrowheads) from five different animals. In all the
experiments, we observed large C2 waves (arrows). The vertical and horizontal scale bar = 0.2mV and 10ms, respectively. (C) Traces of field potentials in response to the olfactory tract stimulation (arrowhead) before (black dashed line), during (black solid line), and after (gray line) DA application (50 μM). (D) Time course of the effect of 50 μM DA application on the amplitude of C2 wave (n = 6). The amplitude of the C2 wave is represented as a relative value based on the first response during the baseline trials. The data are indicated as means ± s.e.m. (E) Statistical data for the effects of DA on the C2 wave. The Y-axis indicates % change in the C2 wave amplitude during treatment compared with the baseline. In the control experiments, a vehicle solution was perfused during the treatment. A significant decrease of C2 wave amplitude was observed by 50 μM DA treatment. *** indicates a significant difference (p<0.001) (F) Concentration dependence of the effect of DA on the C2 wave. The number in parentheses represents the number of trials tested for each DA concentration. EC50 values are 18.4 μM

Fig. 3. DA alters the paired-pulse ratio. (A) Superimposed traces of two consecutive C2 wave responses normalized by the first response (P1) amplitude. The stimulation interval was 150 ms. DA (50 μM) increased the amplitude of the normalized second response (P2) (dashed black line, before 50 μM DA application; solid black line, during 50 μM DA application; gray line, after 50 μM DA application). (B) Time course of the effect of 50 μM DA application on the paired-pulse ratio (n=6). The data are represented as relative values based on the ratio of the first responses during the baseline trials, and are indicated as means ± s.e.m. (C) Pair-wise comparisons of
the paired-pulse ratios, before 10min vs during 10min application of DA. (D)

Statistical data for the effects of DA on the paired-pulse ratio. The Y-axis represents % change in paired-pulse ratio during treatment compared with the baseline. In the control experiments, a vehicle solution was perfused during the treatment. The significant increase in the paired-pulse ratio was observed by 50 μM DA treatment. ** indicates a significant difference (p<0.01)

Fig. 4. (-)-quinpirole mimics the effect of DA on the synaptic transmission from the mitral cell to granule cell. (A) Traces of field potentials that reflect the synaptic transmission from the mitral to granule cell before (dashed line) and during (solid line) (-)-quinpirole application (0.3 μM). (B) Time course of the effect of 0.3 μM (-)-quinpirole application on the amplitude of the C2 wave (n = 6). The amplitude of the C2 wave is indicated as a relative value based on the first response during the baseline trials. (-)-quinpirole gradually suppressed the field potentials. The data are indicated as means ± s.e.m. (C) Suppressive effect of (-)-quinpirole in different concentrations on the C2 wave. Numbers in parentheses represent the numbers of trials tested for each (-)-quinpirole concentration.

Fig. 5. 0.3 μM (-)-quinpirole alters the paired-pulse ratio. (A) Superimposed traces of two consecutive C2 wave responses normalized by the first response (P1) amplitude. The stimulation interval was 150 ms. (-)-quinpirole increased the amplitude of the normalized second response (P2) (dashed line, before 0.3 μM (-)-quinpirole application; solid line, during 0.3 μM (-)-quinpirole application). (B) Pair-wise comparisons of the paired-pulse ratio. The averaged values during 10 min
interval before, vs the value at 10 min from the start of the application of
(-)-quinpirole. (C) Time course of the effect of 0.3 μM (-)-quinpirole application on
the paired-pulse ratio (n = 6). The data are represented as relative values based on
the ratio of the first responses during the baseline trials, and are indicated as means ± s.e.m.

Fig. 6. DA and (-)-quinpirole suppress the spontaneous activities of the olfactory
bulbar neural circuits. (A) Spontaneous activities of the olfactory bulbar neural
circuit were suppressed by a 50 μM DA application. Scale bar = 500 ms. (B) A
representative example of the time course of DA-induced changes in spontaneous
activities of the olfactory bulbar neural circuits. (C) A power spectrum showing that
the spontaneous olfactory bulbar neural circuit activity was suppressed by a 50μM
DA application (black trace, ACSF; gray trace, 50μM DA). Averaged spectra from
four (ACSF) or three (DA; 50 μM) trials are shown. (D) Spontaneous activities of
the olfactory bulbar neural circuit were suppressed by a 0.3 μM (-)-quinpirole
application. Scale bar = 500 ms. (E) Statistical comparison of the DA- (n=6) or
(-)-quinpirole- (n=6) induced suppression of spontaneous activities of the olfactory
bulbar neural circuit in comparison with the vehicle application (n=5). * p<0.05;
***, p<0.001.

Fig. 7. Gabazine (a GABA_A antagonist) abolishes the odorant responses of the
olfactory bulbar neural circuits. (A) Odorant-induced oscillatory activity in the
olfactory bulbar neural circuit (upper trace). Scale bar = 1 s. The inset shows an
expanded trace of the dotted area in the odorant-induced oscillatory activity (Scale bar = 200 ms). The odorant response was abolished by a 50μM gabazine application. (B) A power spectrum showing that the oscillatory activity induced by the odorant stimulation was abolished by a 50μM gabazine application (black trace, ACSF; gray trace, 50μM gabazine). The values from four trials were averaged for the ACSF, while those for three trials were averaged for 50μM gabazine. (C) Spontaneous activity of the olfactory bulbar neural circuits was little affected by the 50μM gabazine application. Scale bar = 500 ms. (D) A power spectrum analysis showing that the spontaneous activity was least affected by the 50μM gabazine application (black, ACSF; gray, 50μM gabazine).

Fig. 8. DA and (-)-quinpirole suppress the odorant responses in the olfactory bulbar neural circuits. (A) Oscillatory activities induced by the odorant stimulation were suppressed by the 50 μM DA application. Scale bar = 1 s. (B) A representative example of the time course of DA-induced changes in the odorant response of the olfactory bulbar neural circuits. (C) A power spectrum showing that the oscillatory activity induced by the odorant stimulation was suppressed by a 50μM DA application (black, ACSF; gray, 50μM dopamine). The values from four trials were averaged for ACSF, while those for three trials were averaged for 50μM DA. (D) The oscillatory activities of the olfactory bulbar neural circuit were suppressed by a 0.3 μM (-)-quinpirole application. Scale bar = 1 s. (E) Statistical comparison of the DA- (n=6) or (-)-quinpirole- (n=6) induced suppression of spontaneous activities of the olfactory bulbar neural circuit in comparison with the vehicle application (n=5).
The Data are indicated as means ± s.e.m. * p<0.05; ***, p<0.001.

**Fig. 9.** Effects of DA on the synaptic transmission from the olfactory nerve terminals to the olfactory bulbar neurons. (A) A schematic illustration of the whole-cell recording experiments. The stimulation electrode was positioned at the olfactory nerve layer, and whole-cell recording was performed from the olfactory bulbar neurons. (B) A representative example of the identified olfactory bulbar neuron which was recorded in a whole-cell recording mode. The morphology of the neuron corresponds well to that of the goldfish mitral cell (Oka 1983). Scale bar = 20 μm. (C) Representative traces showing that 50 μM CNQX, AMPA/kainate receptor antagonist, abolishes the EPSC evoked by the stimulation of olfactory nerve (black, before CNQX application; gray, after CNQX application). (D) Time course of the effect of 50 μM CNQX application on the amplitude of the evoked EPSC (n = 5). Each data point represents the averaged value of four sweeps recorded within 1 min. The amplitudes are represented as relative values based on the first value of the trial. The data are indicated as means ± s.e.m. (E) Representative traces showing the effects of 50 μM DA on the evoked EPSC (black, before DA application; gray, after DA application). DA application showed little effect on the amplitude of EPSC. (F, G) Time course of the effect of 50 μM DA application for 5 min (F, n = 5) or for 10 min (G, n = 4) on the amplitude of the evoked EPSC. Each data point represents the averaged value of four sweeps recorded within 1 min. The amplitudes are indicated as relative values based on the first value of the trial. Data are indicated as means ± s.e.m.
A region of the olfactory bulb (OB) is shown with different layers labeled: ONL (Outer plexiform layer), GL (Granule layer), MCL (Mitral cell layer), and ICL (Internal plexiform layer).

E: A bar graph showing the proportion of TH (tyrosine hydroxylase) neurons at different distances from the surface of the OB (μm). The x-axis represents the distance range, and the y-axis represents the proportion of TH neurons. Bars are presented for distance ranges of 0-100, 100-200, 200-300, 300-400, 400-500, and 500-600 μm, with error bars indicating variability.
The effect of DA, normalized to control. DA (μM) concentrations: 0, 0.1, 1, 10, 100, 1000. The change in field potential amplitude is shown relative to control.

Change in the field potential amplitude (%):
- Control (n=6)
- 50 μM DA (n=6)

The effect of DA, normalized to control, is shown against different concentrations of DA (μM).

Significance:
- *** indicates a statistically significant difference at the 0.001 level.
The effect of Quin, normalized

- A: Graph showing relative amplitude vs. time (min) for different Quin concentrations.
- B: Graph showing the effect of 0.3 μM Quin over time (min).
- C: Bar graph representing the effect of Quin concentrations on the normalized effect of Quin.
A
ACSF

50μM DA

B

Normalized amplitude

0 5 10 15 20 25 30 35
0.2
0.4
0.6
0.8
1
1.2

Time (min)

C

Magnitude

2 6 10 14 18 22 26 30 34 38
0 4 8

Frequency (Hz)

D
ACSF

0.3μM quin

E

Change in the strength of OB activity (%)

Vehicle DA Quin

* 

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