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Sensitivity and Selectivity of Neurons in the Medial Region of the Olfactory Bulb to Skin Extract from Conspecifics in Crucian Carp, *Carassius carassius*

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Abstract

To examine the functional subdivision of the teleost olfactory bulb, extracellular recordings were made from the posterior part of the medial region of the olfactory bulb in the crucian carp, *Carassius carassius*. Bulbar units classified as type I or type II were frequently and simultaneously encountered at a recording site. Type I units displayed a diphasic action potential (AP) with a relatively small amplitude, a short duration (rise time ~1 ms) and high spontaneous activity (2.5 per s). Type II units exhibited an AP with a rise time of ~1.8 ms and low spontaneous activity (1.5 per s). The AP of this latter unit was nearly always followed by a slow potential, a characteristic diphasic wave with a rise time of ~5 ms. Chemical stimulation of the olfactory organ with a graded series of conspecific skin extract induced an increased firing of the type I units. During the period of increased activity of the type I units, the activity of the type II units was suppressed. Stimulation with nucleotides, amino acids and taurolithocholic acid did not induce firing of the type I units of the posterior part of the medial region of the olfactory bulb. These results indicate that the posterior part of the medial region of the olfactory bulb is both sensitive to and selective for skin extract from conspecifics, which has been shown to be a potent stimulus inducing alarm behaviour. The results of the present study indicate that recording single unit activity from a particular region of the olfactory bulb is a suitable method for isolating pheromones or other chemical signals that induce specific activity in the olfactory system. The projection of the neurons categorized as type II was determined by antidromic activation of their axons by electrical stimulation applied to the medial bundle of the medial olfactory tract. The anatomical basis of the type I and type II units in the fish olfactory bulb is discussed.

Key words: bulbar neurons, mitral cells, olfactory bulb, ruffed cells, selectivity, sensitivity, skin extract

Introduction

Thommesen (1978) first presented the idea that discrimination of odours in teleosts is based upon a topological representation of the sensory neurons onto the olfactory bulb. This concept was confirmed by electrical stimulation of the axons of the mitral cells that form the olfactory tract in living cod. Discrete stimulation of the different tract bundles induced different behaviours interpreted as feeding, alarm and courtship (Døving and Selset, 1980). Ablation of the medial and the lateral parts of the medial olfactory tracts in crucian carp eliminated the alarm reaction (Hamdani *et al.*, 2000) and reduced courtship behaviour (Weltzien *et al.*, 2003), respectively, while ablation of the lateral olfactory tracts significantly reduced the feeding behaviour (Hamdani *et al.*, 2001b). Anatomical studies with a neurological tracer demonstrated that the olfactory receptor neurons (ORNs) with intermediate dendritic lengths, bearing microvilli, participated in feeding behaviours (Hamdani *et al.*, 2001a) and those with long dendrites and bearing cilia were correlated with the alarm reaction (Hamdani and Døving, 2002). Additionally, recent studies using other techniques have revealed that morphologically different ORNs in rainbow trout and zebrafish are stimulated specifically by different stimuli: microvillous ORNs are stimulated by amino acids (Sato and Suzuki, 2001; Lipschitz and Michel, 2002) and ciliated ORNs by pheromones (Sato and Suzuki, 2001).

The ideas expressed by Thommesen have been confirmed in different teleosts by surface electrode recordings from the olfactory bulb (Døving *et al.*, 1980; Hara and Zhang, 1996, 1998) and by optical imaging (Friedrich and Korsching, 1997, 1998). Recently, the chemotopy of the teleost olfactory bulb was confirmed by recording activity from single olfactory bulb neurons (Nikonov and Caprio, 2001). Because anatomical studies (Kosaka and Hama, 1979; Kosaka, 1980; Alonso *et al.*, 1987; Arévalo *et al.*, 1991) and electrophysiological methods (Zippel *et al.*, 1999, 2000) have revealed the existence of different types of neurons in the teleost olfactory bulb, it seems obligatory that forthcoming

electrophysiological studies focusing on the activity of single neurons take into account the knowledge of these different neuron types.

Several properties make the teleost olfactory bulb different from that of mammals. The first is the absence of the periglomerular cells. The second is the existence of a particular type of neurons called ruffed cells (RCs). One distinctive character of this neuron is a series of protrusions making a ruff at the initial portion of the axon (Kosaka and Hama, 1979; Kosaka, 1980; Alonso *et al.*, 1987; Arévalo *et al.*, 1991). RCs, which constitute a significant proportion of the mitral cell region, make few contacts with other neurons and do not seem to receive direct inputs from the ORNs. The third property is the mitral cells (MCs), which in teleosts are divided into two types according to the morphology of their somata, the number and extension of their dendritic arborization, the origin of the axons and their location in medial and lateral portions of the olfactory bulb (Alonso *et al.*, 1988).

Given the histological features of the neurons within the teleost olfactory bulb mentioned above, it is rewarding to see that by suitable electrophysiological methods one can observe distinctions between the different neurons (Zippel *et al.*, 1999, 2000). However, at present no histo-physiological combined study has been made to confirm the anatomical identity of the RCs. Yet, in the present study we confirm the duality of the appearance of neuron activity from single units and the specificity of the activity induced by skin extract at the medial part of the bulb. We will advocate the possibilities of using electrophysiological recordings as a bioassay for isolating pheromones or other biological active agents important for the teleost olfactory system.

Materials and Methods

Crucian carp, *Carassius carassius* L. (20–35 g body wt), were caught in a small lake (Tjernsrud) just outside Oslo city, Norway, and were transported to the aquaria facilities at the Department of Biology where they were fed three times a week. Fish were initially anaesthetized with benzocaine (45 mg/l) and immobilized by i.p. injection of Saffan (24 mg/kg; Schering-Plough Animal Health, Welwyn Garden City, UK). To avoid any unforeseen movement during the experiment, fish were wrapped in a wet cloth and fixed by two steel rods, which fastened to the upper parts of the orbital bones, taking care not to damage the olfactory epithelium. Fish were continuously irrigated through the mouth and over the gills by pond water during the experiments.

Surgery

The skull above the olfactory tracts and the right olfactory bulb was removed under a stereomicroscope. The mesenchymal tissue around the olfactory tract was aspirated by gentle sponging and the anterior part of the brain cavity was filled with paraffin oil. The medial bundle of the medial olfactory tract (mMOT) was separated from the rest of the olfactory tract, care being taken to avoid rupture of the blood vessels running along the olfactory tract. The fish remained in good condition for at least 8 h after surgery, as judged by the blood flow and the nervous activity recorded.

Recordings

Extracellular recordings from single (or a few) units in the posterior part of the medial region of the olfactory bulb were performed with microelectrodes made from tungsten wire (125 μm) prepared as described by Hubel (1957). The position of the electrode was adjusted by an electrical micromanipulator (SD Instruments MC 1000), and connected to an amplifier (Grass P55). The bandwidth was adjusted to 0.3–3 kHz. A notch filter of 50 Hz was activated. The reference electrode was positioned on the border of the brain cavity. Signals from the amplifier were displayed on an oscilloscope (Tektronix 565; Portland, OR) and made audible with an audio monitor. The nervous activity was also recorded on a PC (Dell OptiPlex GX1p) via an analogue to digital converter (μ1401; CED, Cambridge, UK) for later analysis and display. The nervous activity was stored on a PC with the aid of a software program (Spike 2, version 4.04; CED).

Electrical stimulation of the mMOT

The projection of the RCs to the olfactory tracts is still uncertain. For this reason, in some experiments (n = 6) of the present study the mMOT was mounted onto a pair of platinium wires connected to an electronic pulse generator that gave electric pulses with variable duration and intensity (Grass SD9).

Chemical stimulation

The olfactory organ ipsilateral to the recording site (the right side) was exposed to a continuous flow of artificial pond water (APW): 2.9×10^{-2} g/l NaCl, 3.7×10^{-3} g/l KCl, 5.8×10^{-2} g/l $CaCl_2$, 1.6×10^{-2} g/l $NaHCO_3$. The flow could be interrupted by a series of miniature valves to give exposure to solutions of different compositions prepared in APW. This part of the study was divided into two subparts according to the aims addressed. The first one, where the olfactory organ was stimulated by conspecific skin extract made up at different dilutions, addressed the sensitivity of the medial part of the olfactory bulb to skin extract. In the second one, the olfactory organ was exposed, besides the skin extract, to a series of potent stimuli of fish olfactory neurons made at 10^{-4} M: a mixture of L-arginine, L-methionine and L-alanine; a single amino acid (L-alanine); the nucleotides adenosine 5′-triphosphate (ATP) and inosine 5′-triphosphate (ITP); taurolithocholic acid (TAUR).

Most of the experiments were performed in the following way. The stimulation series was tested from the lowest to the highest concentration of skin extract. The olfactory epithelium was not stimulated for a second time until the

spontaneous activity returned to the pre-stimulus level. The stimulus was injected into the anterior naris of the olfactory organ through a polyethylene tube at a flow of 0.3 ml/min with minimal mechanical stimulation of the olfactory receptor cells. Inspections of the arrival of stimulant at the outlet of the tubing revealed that the onset of the increased activity of the bulbar cells occurred in conjunction with the arrival of the stimulus at the olfactory epithelium. These responses were termed excitatory.

Preparation of skin extract

Crucian carp were killed by decapitation and skin was taken from the sides of the fish. Total weight was ~2 g. The skin samples were placed in 100 ml of distilled water and homogenized in a blender. The homogenate was filtered through glass wool. The amount of dry material in the filtrate was ~1.6 g/l. The filtrate was frozen immediately and fresh concentration series were made before each experiment at dilution steps of 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 0.5×10^{-2} and 10^{-2} from stock solution.

Results

Extracellular recordings were made from 175 recording sites in 135 electrode penetrations of the olfactory bulb in 75 fish. When the microelectrode was advanced into the posterior part of the medial region of the olfactory bulb (the poserior medial quadrant), unitary discharges of different amplitudes were regularly observed. The appearance of the spikes was diphasic, with the initial deflection either positive or negative.

The amplitudes of the spikes depend upon the relation between the localization of the recording electrode and the cell structure. Bulbar unit activity was usually encountered at a depth of between 150 and 300 μm corresponding to the MCs layer. Electrode penetration was usually stabilized to a depth which allowed high amplitude of the spikes. Two distinct types of unit activity could be distinguished at one particular recording site based on their shapes and sizes. Type I units were characterized by a diphasic action potential (AP) of short duration (rise time ~1 ms). Such units correspond presumably to MCs (Figure 1A,B). Type II units displayed an AP with long duration (rise time ~1.8 ms) (Figure 1C,D). The AP of this latter unit was nearly always followed by a slow potential (SP), characteristic diphasic wave with a rise time of ~5 ms. The delay between AP and SP (measured as indicated in Figure 1C) varied between 8 and 8.5 ms. Both types of units were activated by electrical stimulation of the mMOT, and the conduction velocities calculated for different APs recorded from type II units varied between 0.34 and 0.55 m/s (n = 6) at room temperature (Figure 2). The appearance of the AP and the following SP indicated a particular type of unit. Zippel *et al.* (1999) proposed that these units represent the activity of the so-called RCs. However, because of the absence of a correlative histological identification of the units recorded, we categorize these units as type I and type II in this study.

Spontaneous activity

The range of spontaneous activity of the bulbar neurons varied greatly from 0.01 to 4 per s. The mean spontaneous frequency varied for each particular unit within a recording session without any obvious external cause. In all the recordings made from the bulb, the mean spontaneous spike frequencies of the two types of units were different. In general, the activity of type I units was higher than that of type II units and reached a mean of ~2.5 per s. The range of spontaneous activity of type II units varied greatly during all experiments, with a mean of ~1.5 per s. The characteristic pattern of discharge displayed by a given type I cell usually remained constant for observation periods of several hours.

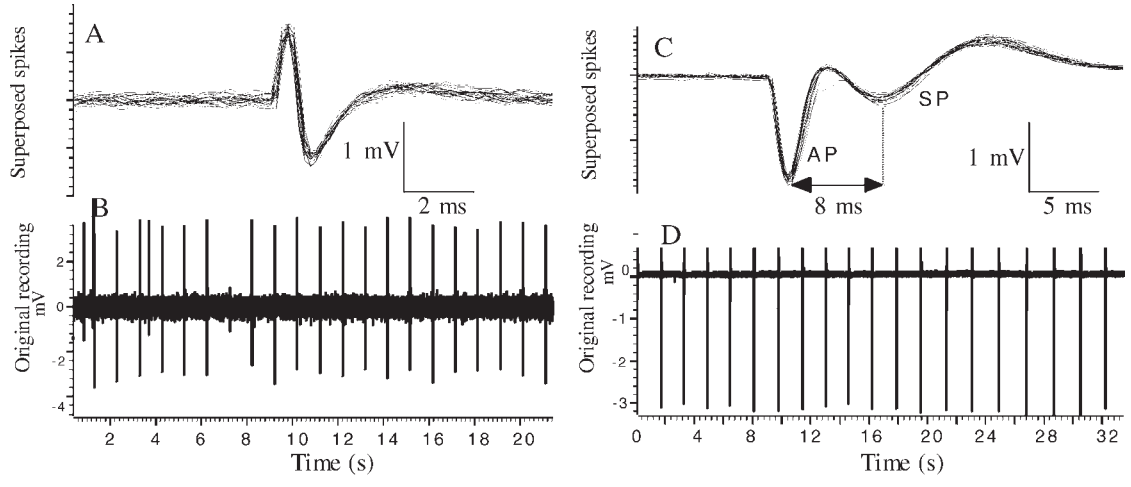

Figure 1 An example of the spontaneous activity of single units recorded from the posterior part of the medial region of the olfactory bulb. The upper traces **(A, C)** show superimpositions of the spikes from a type I unit **(B)** and a type II unit **(D)**. AP, action potential; SP, slow potential.

Effect of chemical stimulation

General effect of skin extract

Figure 3 shows a typical recording from the posterior part of the medial region of the olfactory bulb when stimulating the olfactory organ with skin extract. A remarkable increase in the spontaneous activity of the bulbar units was noticed, i.e. the mean frequency of a unit categorized as type I increased from 0.03 to 15 per s. It is pertinent to note that the response was seen to begin somewhat later than the opening of the miniature valve. This was due to the delay between the opening of the valve and the arrival of the stimulus at the olfactory epithelium. The pre- and the inter-stimulus activities were characterized by a low frequency of the type I unit (mean value 0.03 per s) and a slightly higher frequency of the type II unit (mean value 0.2 per s). During the period of increased activity of the type I unit, which usually persisted after the end of the stimulus, the type II unit was silent. These types of responses were the most frequently observed effects evoked by skin extract.

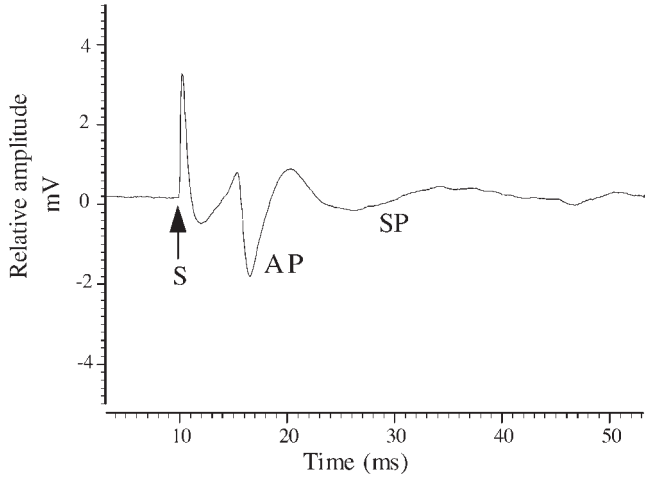

Figure 2 Action potential from a type II unit when stimulating the medial bundle of the medial olfactory tract with electric pulses. Conduction velocity of 0.35 m/s. S, electric pulse; AP, action potential; SP, slow potential.

Sensitivity

Because the recordings showed that only neurons categorized as type I in the medial part of the olfactory bulb reacted to skin extract with increased firing rate, recordings with stimulation of different dilutions of skin extract have been done only from this type of unit. Fifty-three recording sessions were made from the posterior part of the medial region of the bulb in 25 different fishes when stimulating the olfactory organ with a series of increasing concentrations of skin extract (10^{-6},10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} dilutions from stock solution) and a mixture of amino acids (L-arginine, L-methionine and L-alanine) (10^{-4} M). In all recordings, the activated neurons (type I units) responded with increasing frequencies to increasing concentrations of skin extract. No reaction to a high concentration of a mixture of amino acids was noticed (Table 1 and Figure 4).

Effect of L-alanine

Figure 5 shows the effect of stimulation of the olfactory organ with L-alanine on the activity of single units in the posterior part of the medial region of the olfactory bulb. Three consecutive stimuli were applied: a 10^{-2} dilution of skin extract (from stock), 10^{-4} M L-alanine and a 0.5×10^{-2} dilution of skin extract (from stock). As seen, a type II unit

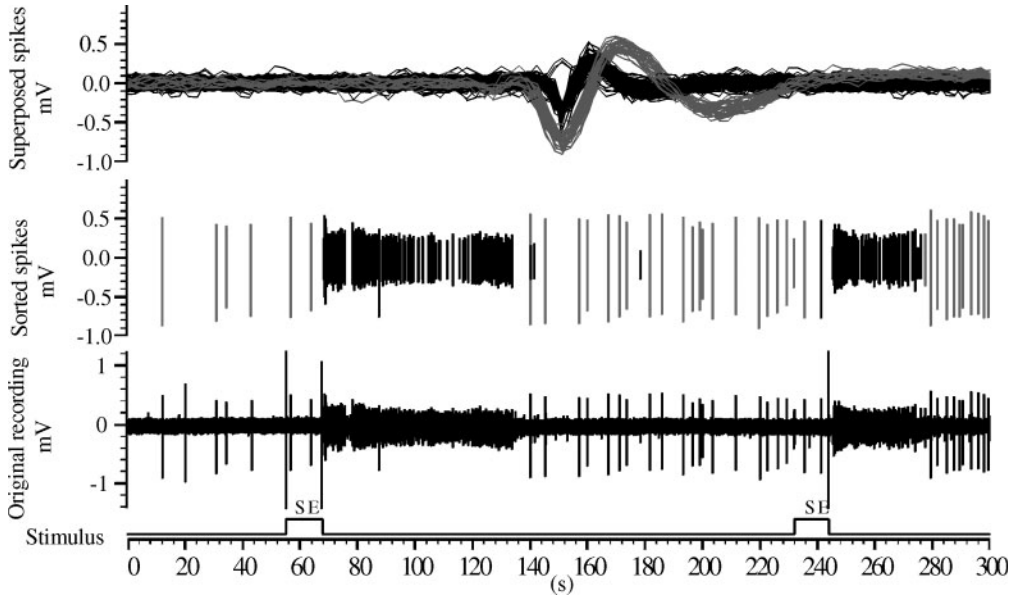

Figure 3 An example of an extracellular recording from the posterior part of the medial region of the right olfactory bulb upon exposure of the right olfactory organ to a 10^{-2} dilution of skin extract (SE) (from stock). The upper trace shows the superimposed spikes from a type II unit (grey) and a type I unit (black).

displayed a spontaneous discharge rate of 1.1 per s and the type I unit was silent. The stimulation with skin extract caused an increase in the activity of the type I unit to 9.5 per s for a dilution of 10^{-2} and to 1.75 per s for a dilution of 0.5×10^{-2} of the stimulus. During firing of the type I unit, the type II unit ceased to fire. Application of L-alanine caused a slight increase in the activity of the type II unit from 1.5 to 4 per s. In a total of 17 observations, it was shown that L-alanine induced an increase in the activity of the units categorized as type II in the posterior part of the medial region of the olfactory bulb.

Table 1 Number of type I units tested, number of type I units excited and the percentage of units responding for each concentration for different concentrations of skin extract (10^{-6}–10^{-2} dilutions from stock solution)

Skin extract conc.	No. of type I units tested	No. of type I units excited	Percentage
10^{-6}	20	5	25
10^{-5}	39	28	71.7
10^{-4}	42	36	85.7
10^{-3}	45	45	100
10^{-2}	26	26	100

Selectivity

The variation in activity observed during chemical stimulation of the olfactory epithelium by skin extract, a mixture of three amino acids (L-arginine, L-methionine and L-alanine), a single amino acid (L-alanine), adenosine 5′-triphosphate, inosine 5′-triphosphate and taurolithocholic acid could be categorized in two classes. Stimulation caused either an increased activity of the bulbar units or an inhibition of

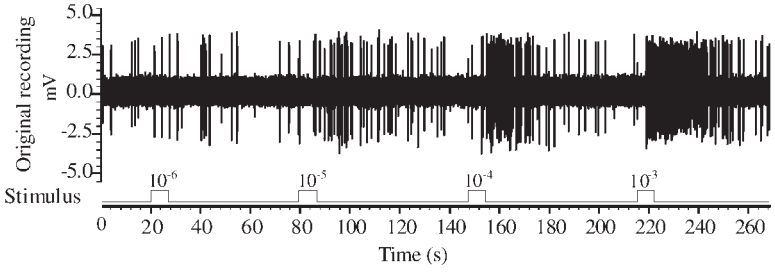

Figure 4 Extracellular recording from the posterior part of the medial region of the right olfactory bulb showing the response of a single type I unit to stimulation with a series of skin extract dilutions (10^{-6}, 10^{-5}, 10^{-4} and 10^{-3}).

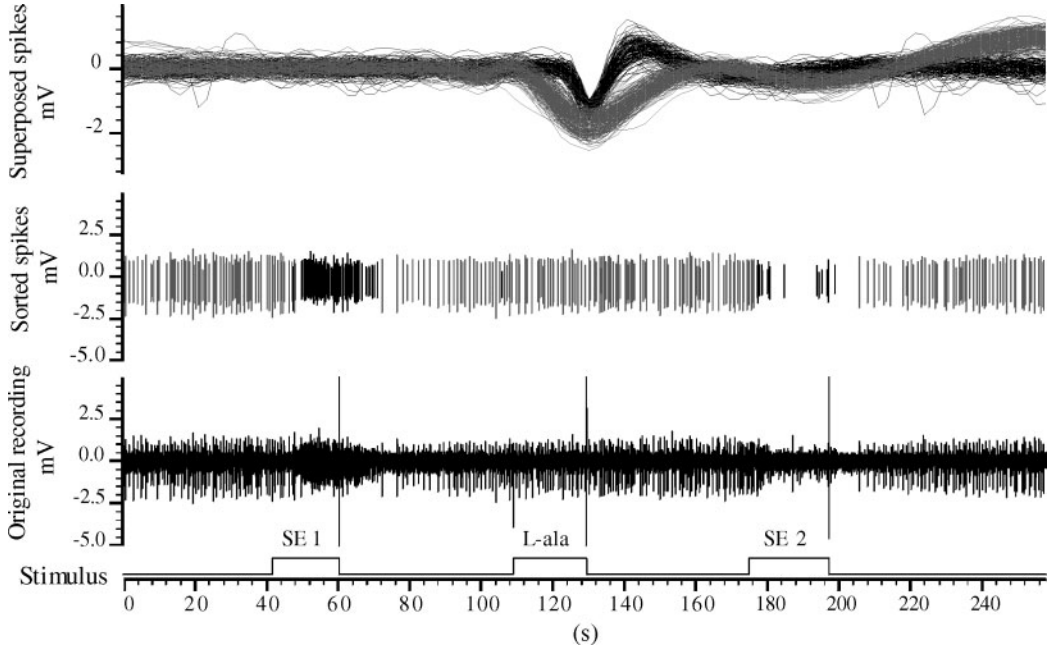

Figure 5 An example of an extracellular recording from the posterior part of the medial region of the right olfactory bulb upon exposure of the right olfactory organ twice to a solution of skin extract (SE 1 = 10^{-2}; SE 2 = 0.5×10^{-2}, dilutions from stock) and to L-alanine 10^{-4} M. Note the interaction between type I and type II units. The upper trace shows the superimposed spikes from a type II unit (grey) and a type I unit (black).

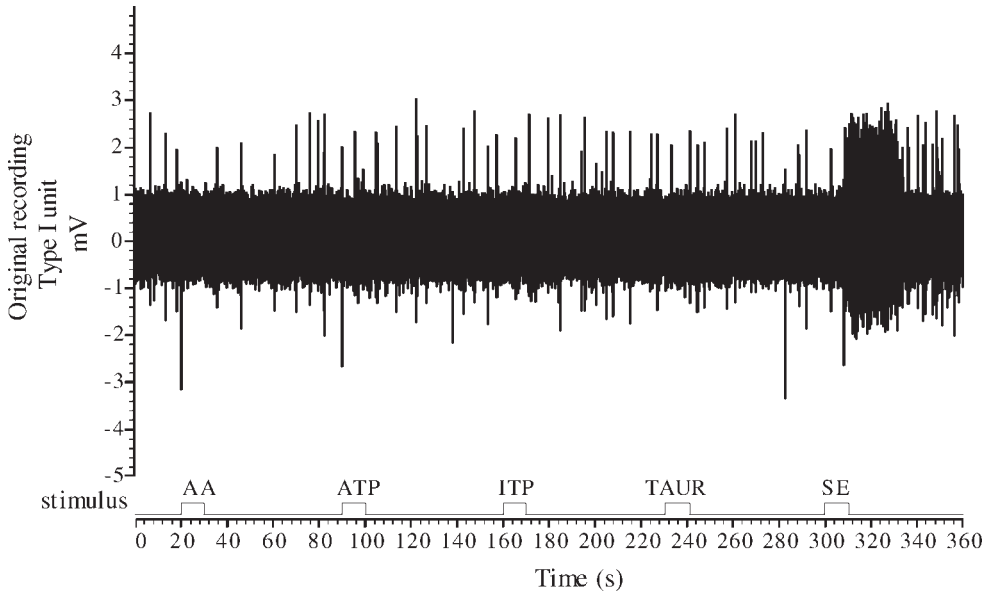

Figure 6 An example of an extracellular recording from the posterior part of the medial region of the right olfactory bulb upon exposure of the right olfactory organ to a 10^{-4} M mixture of amino acids (AA), 10^{-4} M adenosine 5′-triphosphate (ATP), 10^{-4} M inosine 5′-triphosphate (ITP), 10^{-4} M taurolithocholic acid (TAUR) and a 10^{-4} dilution of skin extract (SE), from stock.

their spontaneous discharge. Chemical stimuli were applied at fixed durations and separated by long inter-stimulus intervals in order to ensure the recovery of epithelial receptor neurons.

Effect of amino acids mixture, nucleotides and taurolithocholic acid

Figure 6 shows a typical recording from the posterior part of the medial region of the bulb when stimulating the olfactory organ with a series of stimuli. Note that only skin extract induced firing of type I units, which showed an increase in frequency from 1.5 to 12 per s, while other stimuli did not induce an increase in activity. No type II unit was active during this recording session, excluding the possibility of observing the interactions between these two types of interneurons. This type of response was the most common effect evoked by these stimuli in the medial part of the bulb (Figure 7). Figure 7 also shows that the order in which stimuli were delivered during one recording session had no effect on the activity of the neurons.

Discussion

The results of the present study demonstrate four features of the neurons in the olfactory bulb of crucian carp: (i) only type I units in the medial part of the olfactory bulb react exclusively to conspecific skin extract with increased activity; (ii) this reaction is concentration-dependant, i.e. the higher the concentration the higher the frequency of the response; (iii) the activity of the type II units is inhibited during the increased activity of the type I units; (iv) the type II units do probably project to the olfactory tracts.

Principally, the olfactory system in vertebrate animals possess the same neural circuitry; ORNs expressing a particular receptor converge into restricted areas within the olfactory bulb where they, together with dendrites of MCs, form spherical structures called glomeruli (Ressler *et al.*, 1994; Vassar *et al.*, 1994; Mombaerts *et al.*, 1996). The information received through receptors expressed on ORNs triggers electrical signals travelling towards the brain via synaptic connections in the glomeruli. However, many differences could be noticed in different species. In teleosts, three morphologically different ORNs are scattered throughout the olfactory epithelium: ciliated neurons, microvillous neurons and crypt neurons (Ichikawa and Ueda, 1977; Thommesen, 1983; Hansen *et al.*, 1997; Hansen and Finger, 2000). Recently, we have shown that each morphological type of ORN sends axons to a restricted area in the olfactory bulb. Microvillous neurons project to the MCs that form the lateral olfactory tract (LOT) and participate in feeding behaviour (Hamdani *et al.*, 2001a), whereas the ciliated neurons project to the MCs that form the mMOT and participate in the alarm reaction (Hamdani and Døving, 2002). In the olfactory bulb, the two types of relay neurons (MCs and RCs) lie close to each other in the same layer and make synaptic connections via granule cells. Consequently, when recording nervous activity from the bulb by extracellular means, one should take into account the presence of these two types of neurons, which, although located in the same layer, could be easily distinguished. The distinction between MCs and RCs has been done anatomically in different species (Kosaka and Hama, 1979; Kosaka, 1980; Alonso *et al.*, 1987; Arévalo *et al.*, 1991). Furthermore, Zippel *et al.* (1999, 2000) have suggested a

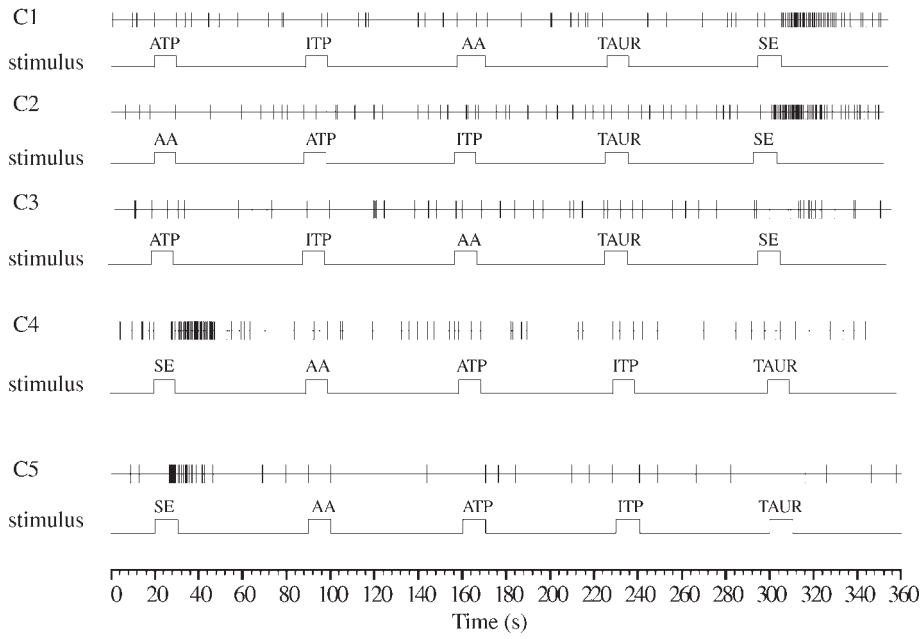

Figure 7 Activity patterns of five different type I units recorded extracellularly from the posterior part of the medial region of the right olfactory bulb when stimulating the right olfactory organ with: amino acids (AA), adenosine 5′-triphosphate (ATP), inosine 5′-triphosphate (ITP), taurolithocholic acid (TAUR) and skin extract (SE). Note that only skin extract induced a noticeable response with a high frequency of impulses in the type I units. C1–C5, type I units 1–5.

correlation between two different APs recorded from the bulb and these anatomically different neurons (MCs and RCs).

In the present study, recordings from both bulbar neurons (type I and II neurons) were made either separately or simultaneously in the posterior part of the medial region of the olfactory bulb. We made recordings from this part of the bulb because previous studies have shown that this part contains neurons that project to the mMOT (Satou *et al.*, 1979; Dubois-Dauphin *et al.*, 1980). In accordance with previous findings, our recordings show two distinct types of nervous activity. One type of unit had a relatively fast diphasic AP. The other had a longer diphasic AP of long duration and was followed by a SP. The delay between the peak of the AP and the peak of the SP was ~8 ms. Zippel *et al.* (Zippel *et al.*, 1999) have made recordings from the goldfish olfactory bulb and suggested that this type of unit most probably originates from RCs. The RCs make synaptic contacts with a large number of granule cells. It is conceivable that the SP is the summed potential of the activity of a population of granule cells induced by the particular unit from which one is recording [see also (Zippel *et al.*, 1999, 2000)]. According to this suggestion, the amplitude of the slow wave potential depends on the number of granule cells activated. It is a pertinent observation that the amplitude of the peak of the SP varied more than the peak amplitude of the AP. The fact that at a given recording site the amplitude of the AP followed by a SP was always larger than that of the AP from a MC might reflect that the RCs are larger than the MCs.

One of the objectives of the present study was to obtain information on the projection of the type II cells from the bulb to the olfactory tracts. For that reason, electrical stimulation of the mMOT was made in some recordings. To ascertain that a neuron projected to mMOT one should ideally use a collision test. However, in the present study this was impractical because several units were encountered at a time and isolation of a particular unit was difficult. Nevertheless, stimulation of the mMOT with electric pulses evoked distinct recruitment of nervous units so that those with the lowest firing threshold appeared first and displayed the highest conduction velocities. The delay between the electrical shock and the appearance of a particular unit was constant, indicating antidromic invasion and not activation via synaptic input. These units were most probably MCs. The conduction velocities of the APs of the type II units, probably RCs, indicate that these cells had both myelinated and unmylineated axons in the mMOT. These findings are in accordance with anatomical studies as both myelinated (Alonso *et al.*, 1987) and unmyelinated (Kosaka, 1980) axons have been described to originate from the RCs. The projection of the RCs to the olfactory tract has not been demonstrated in previous studies and raises questions of the functional significance of these cells and their central projections.

In our recordings of the nervous activity in the olfactory bulb we encountered only one type of unit (type I) whose activity increased its firing rate upon stimulation of the olfactory epithelium with skin extract, the type I units. The firing rate of these cells increased with increasing concen-

tration of the skin extract. Thus, our results imply that the medial part of the bulb reacts specifically to skin extract. No other odorants used in the present study generated an increased firing rate of the neurons in this part of the bulb. These results are in accordance with other studies using other methods suggesting that the olfactory bulb is divided into different functional zones (Friedrich and Korsching, 1997, 1998; Nikonov and Caprio, 2001). It should be noted that during the firing period of the type I units, the type II units were silent, and *vice versa*, suggesting functional coupling between these relay neurons, possibly via granule cells. A specific chemical stimulation of the olfactory epithelium results in a stimulation of specific ORNs that project to MCs in a delimited zone of the olfactory bulb. The activated MCs stimulate granule cells, which in turn inhibit the RCs in the vicinity. But because the RCs do not receive direct inputs from the ORNs, it is still unclear how stimulation of the RCs induces inhibition of the MCs. Zippel *et al.* (Zippel *et al.*, 2000) suggested that it is the inhibition of the MCs that decreases the inhibition of the RCs via granule cells and consequently induces the activation of the RCs.

The skin extract is a blend of various substances. Chemical analysis of skin extract pointed out a content of amino acids of 56.6 μmol/g freeze dried extract (Saglio and Fauconneau, 1985). A comparable concentration in our skin extract would mean that the highest concentration of amino acids used in our experiments would be ~0.56 μM. There were no responses in the medial olfactory bulb to our amino acid mixture at 100 μM, indicating the specificity of the neurons in the posterior part of the medial region of the olfactory bulb.

Finally, based on the results provided by the present and other investigations demonstrating a division of the olfactory bulb into different functional zones, one should be convinced that the method used in this study offers an advantage for using it as a bioassay for isolating pheromones or other biological active agents that are important for the fish olfactory system.

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