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In goldfish the discriminative ability for odours persists after reduction of the olfactory epithelium, and rapidly returns after olfactory nerve axotomy and crossing bulbs

Hans Peter Zippel

Physiologisches Institut der Universität, Humboldtallee 23, 37073 Göttingen, Germany (hpz@neuro-physiol.med.uni-goettingen.de)

Goldfish are ideal vertebrates for the study of regeneration within the peripheral and the central olfactory system. The present behavioural investigations studied the effects of bilateral lesions on the animals' ability to qualitatively discriminate two amino acids (10^{-6} M) and their performance in two more difficult tasks: (i) rewarded amino acid applied in a lower concentration, and (ii) rewarded stimulus contaminated. A 50 and 85% reduction of the olfactory epithelium resulted in no recordable behavioural deficit. After axotomy of olfactory nerves and lateral olfactory tractotomy, fishes were anosmic for seven to ten days. Following replacement of sensory cells in the epithelium, and after regeneration of olfactory tract fibres a full functional recovery, i.e. a highly specific regeneration, was recorded. After three surgical modifications of the olfactory bulbs' position, (i) crossing olfactory tracts and bulbs, (ii) crossing tracts and turning bulbs, and (iii) turning bulbs upside down, a full functional recovery was recorded for amino-acid discrimination in a similar concentration. A permanent, and similar slight deficit was, however, found during application of different concentrations, and of contaminated stimuli when medial lateral halves of the bulb were in 'incorrect' position (i) and (iii), or olfactory bulbs were positioned in the vicinity of the contralateral epithelium (i) and (ii).

Keywords: goldfish; plasticity; olfactory epithelium; olfactory bulb

1. INTRODUCTION

Goldfish are ideal vertebrates for the study of regeneration within the peripheral and central olfactory system (Zippel 1993). In the present behavioural investigations, the effect of bilateral reduction of the olfactory epithelium after dissecting 50 and 85% of the olfactory lamellae was studied. Functional behavioural recovery was investigated after bilateral intercranial transection of the olfactory nerve placing the olfactory bulbs in a different position, and placing the bulbs in the vicinity of the contralateral epithelium after crossing the olfactory tracts. Operation behavioural tests were made (i) on the animals' ability to qualitatively discriminate two amino acids in olfactory concentrations (10^{-6} to 10^{-7} M), in which one had been rewarded pre-operatively; (ii) to examine the discriminative ability in difficult tasks, i.e. concentration differences and contamination of the rewarded stimulus in intact and in operated specimens at various time intervals after operation, and (iii) for investigation of projection from the olfactory mucosa to the glomeruli at the surface of the olfactory bulb using Di-I studies.

2. MATERIAL AND METHODS

Goldfish (*Carassius auratus*) were fully anaesthetized with tricaine methanesulphonate MS-222; (3-aminobenzoic acid ethyl ester, A-5040 Sigma (Munich, Germany); 300 mg l^{-1} tap water), and the cranium over the olfactory bulbs and tracts was removed. Lesions for the behavioural experiments were made bilaterally.

Details of the standardized apparatus for shock-free training are described elsewhere (Zippel *et al.* 1981, 1993*a*,*b*). During the entire pre- and post-operative tests and training periods, goldfish were kept in groups of two in separate single aquaria (130 cm × 30 cm × 20 cm) under a 12 L:12 D cycle at *ca.* 20 °C. For feeding, testing, and training purposes, opaque plastic funnels were suspended at each end of the tank. The tips of the funnels were perforated with holes through which the food reward (Tubifex worms) was delivered following training. The training stimuli were amino acids, which before training only induce slight behavioural responses in concentrations below 10^{-5} M, i.e. were neither spontaneously preferred nor actively avoided by the goldfish (Zippel *et al.* 1993*b*; Von Rekowski *et al.* 1994). By the end of a habituation period, a complex behavioural repertoire had developed and the following behavioural patterns were recorded: (i) funnel orientation, the time (s) spent by the animals in the immediate vicinity of the funnels; (ii) funnel biting, the number of bites at each of the funnels may be interpreted as a specifically orientated food expectation; and (iii) excursive returns, the number of continuous swimming movements away from the vicinity of one funnel in the direction of the other and then back to the initial funnel which may indicate odour-adaptive reactions.

At the onset of each session, funnel orientation, funnel biting, and excursive returns were recorded for 3 min in the absence of

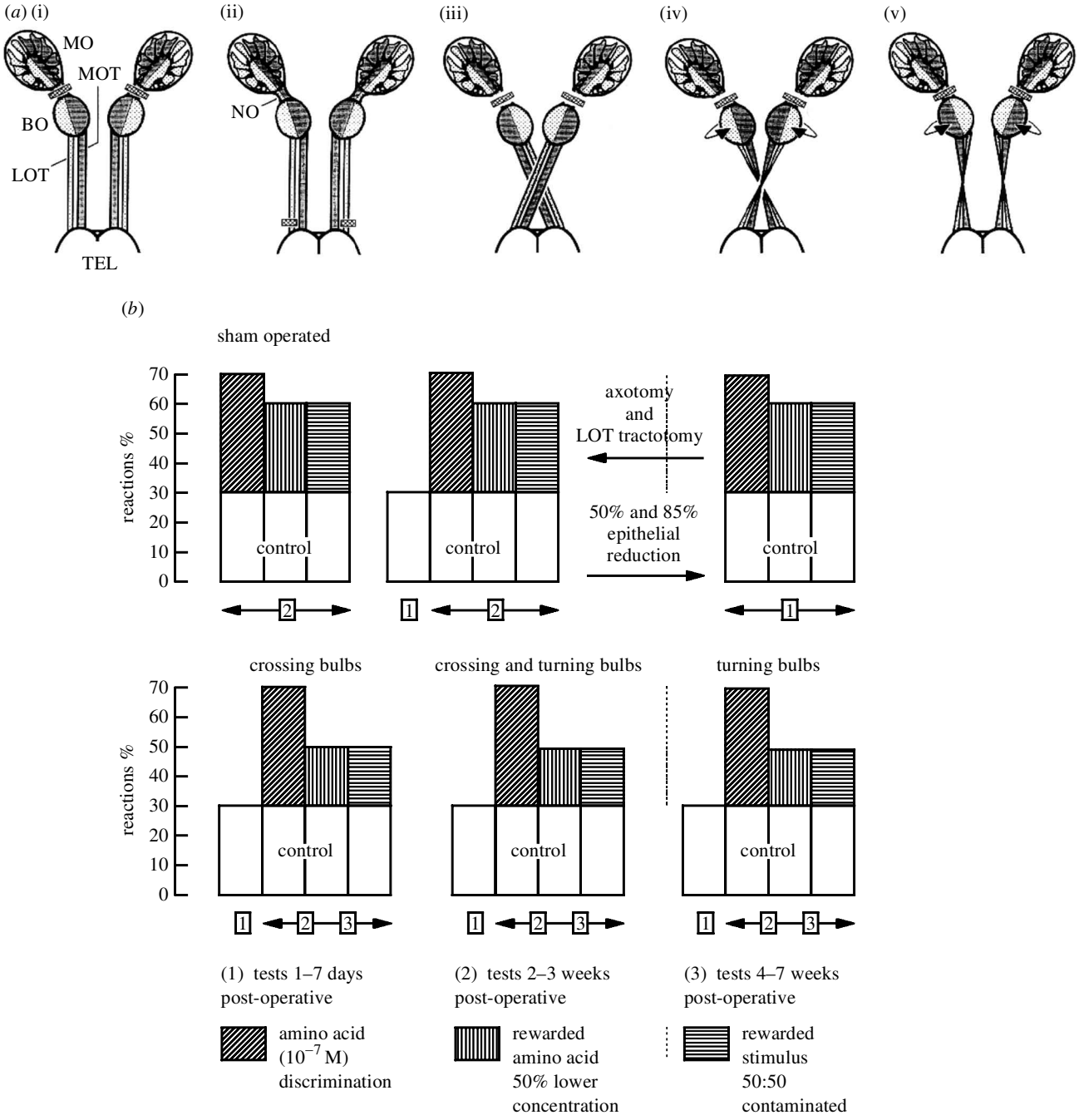

Figure 1. (*a*) A schematic presentation of operations. (i) Receptor axotomy: bilateral intracranial dissection of the olfactory nerve. (ii) Tractotomy tractus olfactorius lateralis (LOT): bilateral dissection of the lateral olfactory tract. (iii), (iv), (v) Receptor axotomy and (iii) crossing tracts: bilateral axotomy of the olfactory nerve, crossing olfactory tracts and bulbs (i.e. mediolateral halves of the contralateral bulbs in 'incorrect' position to the epithelium; connections to the contralateral telencephalic hemispheres). (iv) Crossing and turning bulbs: the mediolateral halves of the contralateral bulbs in the 'correct' position, however, in a dorsoventrally exchanged position to the epithelia; connection via the olfactory tract to the contralateral telencephalon. (v) Turning bulbs: the olfactory bulbs are turned upside down; the mediolateral halves of olfactory bulbs are in the 'incorrect' position to the epithelium; connection via the olfactory tract to the ipsilateral telencephalon. BO, bulbus olfactorius; MO, mucosa olfactoria; MOT, tractus olfactorius medialis; NO, nervus olfactorius; TEL, telencephalon. (*b*) A summary presentation of the detailed behavioural recordings after operation. For the control, *ca.* 30% of behavioural responses (open columns, mean values for funnel orientation, funnel biting, and excursive actions) were recorded before stimulus application at the lesser preferred funnel (i.e. 70% at the spontaneously preferred funnel). Reactions are given as percentage mean values for behavioural responses. The total amount (open plus shaded parts of colums) of reactions is the percentage mean values for behavioural responses during stimulus application through the lesser preferred funnel. During the first week after receptor axotomy and lateral tractomy, fishes are anosmic. After a 50 and 85% reduction of olfactory epithelium, fishes behave like sham-operated specimens. Two to three weeks after operation, following replacement of receptor cells after axotomy, and after regeneration of the lateral olfactory tract fibres, a highly specific regeneration was recorded. After modifications of the olfactory bulbs' position and following crossing olfactory tracts, a specific regeneration for amino-acid discrimination was recorded. Responses during application of lower concentrations of the rewarded stimulus, and during application of contaminated (a mixture of 50% of the rewarded plus 50% of the unrewarded stimulus) stimuli, however, were permanently reduced. For details see § 3.

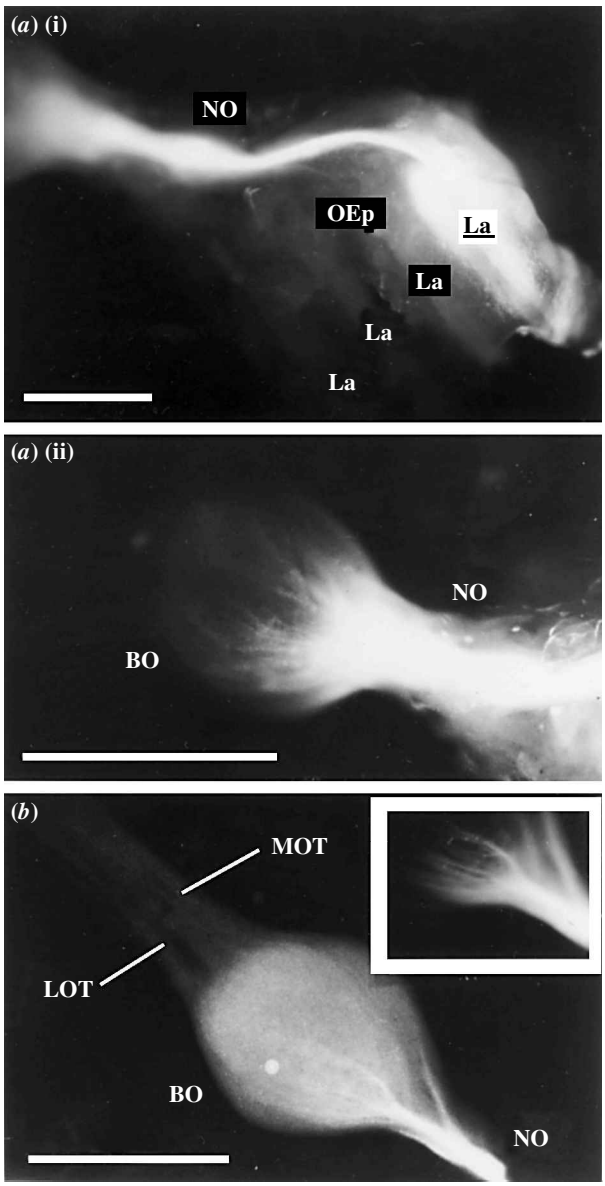

Figure 2. Di-I labelled whole mounts of goldfish. Scale bars, 1 mm. (*a*)(i,ii) Di-I injection to a nasal lamella. Two weeks after injection, (i) the local injection to one nasal lamella (La) can clearly be seen by fluorescence. Three further lamellae in the olfactory epithelium (OEp) can hardly be seen. A narrow bundle of nerve fibres project in the olfactory nerve (NO) to the olfactory bulb. (ii) Taken from the same specimen. At the frontal area of the olfactory bulb (BO) the narrow bundle splits and spreads out over the whole frontal part. (*b*) Di-I injection to a lamella three weeks after injection. Again the narrow bundle splits (see inset picture for more detail) and the fibres terminate across the bulbar surface. No labelling can be seen at the medial (MOT) and lateral (LOT) olfactory substracts projection to telencephalic nuclei.

stimuli. During subsequent qualitative discrimination training (or tests after operation), the 'positive', reinforced, amino-acid stimulus was applied through the spontaneously disliked funnel. Simultaneously another 'negative', non-reinforced (competing) amino acid was applied through the opposite (spontaneously preferred) funnel. Immediately after recording the behavioural patterns for 3 min, the animals were reinforced by the presentation of Tubifex worms through the appropriate funnel (interval reinforcement); for details see Zippel *et al.* (1988, 1993*b*) and Von Rekowski *et al.* (1994).

In each series, eight to ten groups of two goldfish were trained to discriminate different amino acids (10^{-6} M). Following successful discrimination training, the threshold for the discriminative ability (10^{-9} M) was investigated. From detailed behavioural experiments (Von Rekowski *et al.* 1974) it is evident that all L-amino acids investigated are rapidly learned and retained in long-term memory. Significant discriminative positive responses were still recorded when the rewarded stimulus was applied in a 50-fold lower concentration, and were also apparent during contamination of the rewarded stimulus with up to 50% the unrewarded stimulus.

After determination of the behavioural thresholds, different operations were made. First, bilateral receptor axotomy and lateral olfactory tract dissection (figure 1) as controls for functional recovery, because after both operations (after replacement of olfactory receptors and regeneration of lateral olfactory tract fibres) rapid and highly specific behavioural responses were recorded (Zippel *et al.* 1970; Hudson *et al.* 1990; Von Rekowski & Zippel 1993; Zippel 1993). Second, reductions of the olfactory epithelium by dissection of the raphe (50%) plus dissection of lamellea (85%) to investigate behavioural thresholds following minimizing the input to the olfactory bulb, which is also apparent during degeneration of sensory neurons after receptor axotomy (Hansen *et al.* 1999). Third, modifications of the olfactory inputs to telencephalic nuclei by (i) crossing olfactory tracts and olfactory bulbs after receptor axotomy (figure 1*a*(iii))—in this case the olfactory bulb and the contralateral telencephalic nuclei also obtain inputs from the 'incorrect' contralateral epithelial areals; (ii) crossing tracts and turning bulbs (figure 1*a*(iv))—the contralateral telencephalic nuclei obtain inputs from the 'correct' epithelial areals; and (iii) turning bulbs (figure 1*a*(v))—the ipsilateral telencephalic nuclei obtain input from the 'incorrent' epithelial areal.

3. RESULTS AND DISCUSSION

After operation, behavioural tests were made and the data compared with recordings from sham-operated specimens (figure 1*b*). After axotomy and lateral tractotomy, the fishes were anosmic for approximately one week. They did not respond positively when food stimuli (Tubifex worm extracts in concentrations below the taste threshold) were applied.

However, after a 50 and 85% reduction of the surface of olfactory epithelium, amino-acid discrimination and behavioural responses recorded during application of low concentrations of the rewarded stimulus persisted. When a contaminated stimulus was applied, responses remained similar in comparison to the pre-operatively recorded behaviour and to that recorded in sham-operated controls (figure 1*b*).

Two to three weeks after operation, responses of axotomized and lateral tractotomized groups became positive during application of the food stimuli. From subsequent behavioural tests with amino acids, lower concentrations of rewarded stimulus and contaminated stimulus a highly specific functional recovery was found in 'simply' axotomized and laterally tractomized fishes (figure 1*b*).

Following functional recovery after crossing olfactory tracts and bulbs the preference for Tubifex food odour below the taste threshold is as rapid as following axotomy,

and goldfish discriminate amino acids in similar concentrations as they did during the pre-operative training period (figure 1*b*). Discrimination of concentration differences and contaminations, however, is less accurate in comparison to axotomized, lateral tractotomized, and sham-operated specimens. Training sessions four to seven weeks after operation do not yield a significant increase in discriminative ability.

Behavioural deficits with respect to concentration differences and contamination were either the result of contralateral telencephalic projection of olfactory information, or by the post-operative position of the olfactory bulb, i.e. exchanging the medial and lateral halves of the bulb after cutting the olfactory nerves.

In summary, the functional regeneration of olfactory nerves is highly specific after axotomy (when the bulbs were left in their original position), after lateral tractotomy, and following reduction of the olfactory epithelium. Functional recovery is less specific after crossing tracts and turning the contralateral bulbs in a 'normal' lateromedial position.

Olfactory receptor cells are unique for a number of reasons. The cell bodies are located in the peripheral epithelium, and can be replaced continously during the lifetime of the animals. The unmyelinated olfactory axons are ensheathed by Schwann cells in an unusual manner; many (20–50) axons may be enclosed within a single mesaxon by a single Schwann cell. This kind of relationship between Schwann cell and axon is generally seen in developing embryonic nerves. In the olfactory nerve, however, it persists into adulthood. Continous production and persistence of the juvenile relationship between Schwann cell and axon are probably the main prerequisite for the fact that after axotomy of the olfactory nerve new receptor neurons, which develop by mitotic processes from basal cells, can grow dendritic sensory endings and can grow axons which synapse in the olfactory bulbs' glomerular layer. This unique replacement of post-operatively degenerated receptor neurons was experimentally shown in the whole vertebrate kingdom. The axonal growth is obviously guided to the respective 'correct' glomeruli or telencephalic nuclei. After receptor axotomy and lateral tractotomy, the pre-operatively learned behaviour is present without an additional reward. After surgical modulation of the olfactory bulbs' position, the information transmitted to the telencephalic hemispheres is obviously sufficiently precise for discriminating learned amino acids. However, for concentration differences and contaminated stimuli there is a slight deficit. Exchanging the lateral and medial halves of the olfactory bulb also results in behavioural deficits in discrimination of stimulus concentration differences and contaminations.

The fact that all the various surgical modifications of olfactory bulbar positions only slightly reduced the olfactory discriminative ability was unexpected: projection of the olfactory bulbs' neuronal activity to contralateral telencephalic nuclei did not result in a significant reduction of behavioural responses. The presentation of the olfactory bulbs in various dorsoventral and lateromedial variations for the outgrowing epithelial axons also did not result in a significant reduction of behaviour. From the fact that the pre-operatively learnt discriminative ability was, in comparison to simply axotomized and sham-operated fishes, only slightly reduced, it is obvious that the regrowing axons from the replaced sensory neurons mainly project to glomeruli connected with bulbar relay neurons responsible for stimulus discrimination and transmission to respective telencephalic nuclei.

Growth cones are obviously guided by a number of mechanisms: contact-mediated attraction, chemo-attraction, contact-mediated inhibition and chemo-repulsion. The mechanisms are mediated by various families of guidance molecules, including neural cell adhesion molecules of the immunuglobulin superfamily, netrins, and membrane-bound inhibitors, all of which appear to be highly specific in their activity on certain growth cones. We are just beginning to gain insights into the function of these and other molecules in the developing and regenerating neuron system.

From anatomical experiments (Di-I staining) it is evident that projections from single folds of the olfactory epithelium are not localized to specific bulbar areas, neither in intact specimens (figure 2) nor following regeneration of the olfactory nerve. Goldfish ranging from 12 to 15 cm in body length were deeply anaesthetized with tricaine methanesulphonate, sacrificed by decapitation and immersion fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) after a brief irrigation of the nasal sacs with the same fixative. Specimens were left in the fixative at least over night. Crystals of the fluorescent carbocyanine dye Di-I (1,1′-dioctadecyl-3,3,3′-tetrametyl-indocarbocyanine perchlorate; Molecular Probes, Leiden, The Netherlands) were applied to the nasal lamellae. The site of Di-I application was covered with 2% agarose and stored in 4% paraformaldehyde, Di-I was allowed to spread for two to four weeks at room temperature. Labelled specimens were studied as whole mounts. Labelling was viewed in an epifluorescence microscope with a rhodamine filter set.

In figure 2*a*(i) two weeks after Di-I injection to a single lamella a part of the olfactory epithelium is shown. The Di-I stained nerve bundle can easily be seen projecting to the olfactory bulb via the olfactory nerve. When the stained bundle reaches the bulbar surface (figure 2*a*(ii) from the same specimen) it broadens and splits into many single fibres which are distributed over the whole frontal part of the olfactory bulb. After an incubation time of three weeks (figure 2*b*) it is evident that the whole surface of the olfactory bulb is innervated by fibres from a single lamella following Di-I injection.

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