Rapid Classical Conditioning of Odor Response in a Physiological Model for Olfactory Research, the Tiger Salamander

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Abstract

In recent years there have been a number of important advances in the understanding of cellular mechanisms related to olfactory function. Hypotheses regarding the complex relationships among odorant structure, physiological activity and behavioral outcome generated by these findings, however, remain largely untested due to a paucity of psychophysical data on stimulus discrimination in the same experimental species. Comparisons between behavioral and physiological responses are essential for elucidating the critical aspects of stimulus coding in sensory systems. We have developed a method for generating psychophysical data in one of the primary model species used in olfactory research, the tiger salamander, *Ambystoma tigrinum*. These psychophysical experiments are carried out under the same conditions as physiological experiments in our laboratory. Using classical conditioning, individual salamanders are trained over a period of 2–3 h to show skin potential responses to odor and not air. Failure to train using backward pairing demonstrates that the response is not due to sensitization or pseudoconditioning. The conditioned response is mediated by the olfactory pathway, as it is blocked by olfactory nerve section. We show that salamanders detect three odorants that are commonly used stimuli in physiological experiments (butyl alcohol, butyl acetate and amyl acetate), but cannot detect a fourth common experimental odorant, camphor. This method should be a powerful tool for studying olfactory information processing by providing data on discriminability of stimuli used in salamander physiological studies. Chem. Senses 22: 277–286, 1997.

Introduction

Odor stimulation gives rise to distributed patterns of neuronal activity in both the olfactory epithelium and the olfactory bulb (OB), with different odorants eliciting different but overlapping patterns (Kauer and Moulton, 1974; Stewart et al., 1979; Lancet et al., 1982; Mackay-Sim et al., 1982; Kauer et al., 1987; Royet et al., 1987; Edwards et al., 1988; Wilson and Leon, 1988; Cinelli et al., 1995). Recent electrophysiological and molecular biological advances suggest how individual neurons may contribute to these patterns. The emerging view is that each olfactory sensory neuron (OSN), perhaps expressing one or a few receptors proteins (Buck and Axel, 1991; Chess et al., 1994),
is activated by a small range of odorants that share certain aspects of chemical structure (Sato et al., 1994). Activity from different OSN types is processed in the OB, producing temporal patterns of excitation and inhibition in mitral/ tufted output neurons related in some way to the chemical structure of the odorants to which they are responsive (Imamura et al., 1992; Katoh et al., 1993; Yokoi et al., 1995). The mechanisms by which the olfactory pathway translates chemical structure to perceived odor quality through these neuronal responses, however, remains to be elucidated.

Determining how animals discriminate among some chemical structures while perceiving others to be equivalent in odor quality is one of the basic goals of olfactory research. Therefore, any understanding of olfactory coding will be incomplete without knowledge of the animal’s ability to detect and discriminate stimuli used in physiological experiments. In studies of other sensory systems, psychological and physiological data are regularly taken to predict and inform one another. This approach is seldom used in studying olfaction. One such example is the work of Youngentob and colleagues comparing epithelial population responses with psychophysical data in rats (Kent et al., 1995; Youngentob and Kent, 1995). In our laboratory, we have used physiological methods (White and Kauer, 1992, 1993; Kauer et al., 1994) designed to examine hypotheses generated by psychophysical data collected in tiger salamanders (Ambystoma tigrinum) by Mason and colleagues (Mason et al., 1980, 1987; Mason and Stevens, 1981a,b). Directly comparable physiological and psychophysical data are available only for a few compounds in this or any species.

Comprehensive psychophysical data for the tiger salamander would be invaluable because this species has served as one of the major models for olfactory research. Tiger salamanders have been used extensively for studying OSN responses (Getchell and Shepherd, 1978; Baylin, 1979; Masukawa et al., 1985; Anderson and Hamilton, 1987; Kent and Mozell, 1992) and the underlying olfactory transduction mechanisms (Trotier, 1986; Zufall et al., 1991; Firestein et al., 1991, 1993; Lowe and Gold, 1993), and work has recently begun on characterizing receptor proteins which may be involved in transduction in this species (Jesurum et al., 1993; Zhao et al., 1995). Much of what is known about the response properties of OB cells (Kauer, 1974; Kauer and Shepherd, 1977; Hamilton and Kauer, 1989; Wellis and Kauer, 1993), including investigations of activity across OB cell populations (Cinelli and Salzberg, 1992; Cinelli et al., 1995), has come from salamander studies. We have therefore developed a method for generating psychophysical data for tiger salamanders that can be used together with physiological and molecular biological methods to critically examine hypotheses about coding in the olfactory system.

### Methods

#### General methods

For all experiments, salamanders were immobilized with d-tubocurarine (0.7 mg/100 g body wt) injected into the dorsal lymph sac in accordance with approved procedures. Animals were loosely covered with a wet tissue and placed on a foam pad to keep them moist. Surface electrodes for recording skin potential were coated with electrode paste and taped to the fore- and hindlimb on one side of the animal, with the ground electrode placed on the contralateral hindlimb. Two additional electrodes were placed ~6 cm apart rostro-caudally on one side of the tail for delivery of a mild electric shock.

The mouth was sealed around a small tube using denture adhesive, and one naris was blocked with petroleum jelly. Vacuum on the tube in the mouth drew a constant stream of air (25 ml/min) into the olfactory sac through the open external naris, across the olfactory epithelium and out through the internal naris. Stimuli were delivered using an air dilution olfactometer (Kauer and Shepherd, 1977) with the nozzle positioned 3–5 mm from the open external naris.

Stimulus delivery and data collection during training and testing were controlled by computer. In training trials, a 3 s pulse of odor was delivered followed by a weak electric shock that occurred 0–500 ms after the termination of the odor pulse. Air was delivered in separate trials followed by no shock. Odor and air trials were given in a random order, with a 1:2 ratio of odor to air trials. The inter-trial interval was ~2 min in duration. The skin potential response to a DC pulse habituates rapidly with repeated presentation. Habituation was slowed by varying the intensity and duration of the shock throughout training. Intensity ranged from 0.1 to 2.0 mA, and duration ranged from 100 to 200 ms. Due to individual differences in the rate of habituation, the responses of some salamanders to the shock habituated before association with the odor was learned. This occurred in ~30% of the animals tested and these animals were dropped from the experiment.
Skin potential measurements were amplified with a DC amplifier, filtered with a 2 s time constant, and digitized at 20 Hz and stored. Each trial was 20 s in duration: 10 s pre-stimulus, and 10 s post-stimulus, which began at the onset of the 3 s stimulus pulse (Figure 1B). The median voltage value for the pre-stimulus period was determined from the digitized data. The difference from that median was calculated for each sampled time point, and those differences were summed for a measure of area under the curve for the pre-stimulus and for the post-stimulus time periods (shaded areas in Figure 1A). The skin potential response to odor or air was calculated by the ratio: (post - pre)/pre, where 'pre' and 'post' refer to the areas under the curve for pre- and post-stimulus periods.

Prior to training, six responses to odor (without shock) and six to air were recorded. During training, one odor (without shock) and one air trial were recorded after every five odor/shock training trials. The mean pre-training air and odor scores were compared to the post-training scores for air and odor measured after 20 or 25 (depending on the experiment) odor shock/pairs.

### Experiment 1
This experiment was designed to determine whether animals could be classically conditioned to respond differentially to four different odorants versus air: amyl acetate at $4 \times 10^{-2}$ of saturated vapor ($1.1 \times 10^{-5}$ M), butyl acetate at $4 \times 10^{-2}$ of saturated vapor ($4.3 \times 10^{-5}$ M), butyl alcohol at $4 \times 10^{-2}$ of saturated vapor ($1.5 \times 10^{-5}$ M) and camphor at $10^{-1}$ of saturated vapor ($4.4 \times 10^{-6}$ M). These stimuli were selected because Mason and colleagues had previously used them, at concentrations ranging from $10^{-6}$ to $10^{-4}$ M, in their conditioned avoidance paradigm with tiger salamanders (Mason et al., 1980; Mason and Stevens, 1981a,b). This experiment sought to determine whether the two methods yield comparable results. Camphor was chosen because Mason and Stevens’ data indicated that tiger salamanders showed no avoidance behavior to the odorant, though they did not specifically test discrimination between camphor and air (Mason et al., 1980). Our own preliminary data indicated that salamanders did not detect camphor at $4 \times 10^{-2}$ of saturation, and a higher concentration was selected. We chose the alcohol and acetates because they are, and have been, used in electrophysiological experiments in this laboratory (e.g. White and Kauer, 1992, 1993).

Conditioning in this experiment included 25 trials of odor paired with shock, and ~50 air trials with no shock, with two exceptions. Two salamanders trained rapidly to butyl acetate and were only given 20 odor/shock pairs (plus ~40 air trials). The air and odor trials collected after 20 odor/shock pairs were used as the “post-training” measure for butyl acetate analyses. After training, responses were extinguished with a series of odor trials with no shock. Every fifth extinction trial was collected, along with a paired air trial.

Nine salamanders were used in this experiment: five were trained with two odorants and the remaining four were trained with all four odorants. Animals were trained with two different odorants per day, with training days at least 1 week apart. On a given day, salamanders were trained with butyl acetate and camphor, or with amyl acetate and butyl alcohol.

To determine whether conditioning resulted in a differential response to odor versus air, the data for each odorant were log transformed and analyzed separately in repeated-measures ANOVAs with stimulus (air versus odor) and condition (pre-training versus post-training) as the repeating factors. t-tests were used for planned comparisons of pre- and post-training scores.

### Experiment 2
This experiment was designed to determine whether the response to odorants was a learned association between odorant and shock, by comparing the effectiveness of backward and forward pairing of conditioned and unconditioned stimuli. Butyl acetate at $4 \times 10^{-2}$ of saturated vapor ($4.3 \times 10^{-5}$ M) was used for both forward and backward pairing. The time-course of the forward-paired odor/shock trials is described in the general methods. For backward-paired training trials, the shock occurred 7 s after the trial began, with odorant onset at 10 s. Thus, the odor occurred at the same time in backward- and forward-paired trials, but the shock occurred 3 s prior to odorant onset in backward-paired training, and 3 s after odorant onset in forward-paired training.

Conditioning in this experiment included 20 trials of odor paired with shock, and ~40 air trials with no shock for each condition (backward pairing and forward pairing). Four salamanders were tested. For both forward and backward pairing, animals received odor/shock (or shock/odor) and air trials in a ratio of 1:2. Backward- and forward-paired training were both completed on the same day, always in the order: pre-training trials, backward pairing and then forward pairing. Extinction trials were not conducted. Odor and air responses for pre-training baseline, after backward
Experiment 3

This experiment was designed to examine whether an intact olfactory nerve (ON) is necessary for conditioning of a response to odorants, i.e. whether the conditioning is mediated by the olfactory system as opposed to some other sensory modality. Salamanders received unilateral ON sections after being anesthetized with ketamine (10 mg/100 g body wt, injected i.p.) and immobilized with d-tubocurarine (0.7 mg/100 g body wt, injected into the dorsal lymph sac). One OB was exposed by reflecting the dorsal skin of the head and removing the cartilage overlying the forebrain on one side, using additional topical anesthesia (lidocaine HCl) at the incision sites in accordance with approved procedures. The bulb was gently retracted with a glass probe, exposing the ON, and the nerve was completely cut under visual control with fine surgical scissors. The skin was then repositioned over the cranium and sutured in place. Animals were allowed to recover 1–2 weeks before testing. All appeared healthy and ate normally after recovery.

Salamanders received 20 trials of odor paired with shock, and ~40 air trials with no shock on each side (sectioned and intact). Conditioning on both intact and ON-sectioned sides was completed on the same day. If no learning occurred during training on the intact side, the animal was eliminated from the experiment. Animals therefore served as their own controls. Eight salamanders were eliminated for this reason; four animals were successfully trained on the intact side and served as subjects. The proportion of animals that did not train on the intact side was higher for this experiment than for experiment 1, possibly due to trauma to the control side during surgery.

Two of the four experimental animals were trained in the following order: pre-training trials on the intact side, intact side training, pre-training trials on the sectioned side, sectioned side training. Several trials were run on the intact side following sectioned side training to determine whether animals had remained trained, or could be retrained, on the intact side. The two remaining salamanders received conditioning on the sectioned side first. Butyl acetate at 4 × 10^{-5} M as the odorant.
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By pairing odor stimulus presentations with tail shocks, salamanders could be trained to respond differentially to odor versus air. In untrained animals, tail shock elicits a rapid fluctuation of skin potential of up to ~5 mV (see Figure 1A). After 15–25 training trials in which an odor stimulus was followed by shock, the subjects began to respond to odor stimulation alone with a change in skin potential comparable in amplitude to that elicited by the shock (Figure 2). Trials in which air was delivered without shock ensured that the animals were conditioned to the odor itself rather than to other cues associated with stimulus delivery (such as the sound of solenoid valves in the olfactometer). Animals often became conditioned to both air and odor early in the training (after 5–10 odor/shock pairings), with differential response to air and odor arising later (see mean air response at trial 5 in Figure 3A,B).

Conditioned responses to the odor stimulus could be extinguished by presenting a series of odor trials without shock. Training and extinction of a response to one odorant could be completed in 2–3 h, which allowed for the testing of a single animal with two different odorants in 1 day. Not all salamanders could be trained: some animals habituated to the shock or showed no conditioning after 25 odor/shock pairs.

Experiment 1: conditioning to odorants
After training, salamanders gave a conditioned response to three of the odorants (butyl acetate, butyl alcohol and amyl acetate), but did not differentiate the fourth (camphor) from air. Data were log-transformed before analysis because the raw data were not normally distributed: some animals gave very large responses to the odor after training, and therefore variance tended to be larger with higher means. Figure 3 shows mean change in response to odor and to air over training for the four chemicals. For three odorants (Figure 3A–C), there was a significant stimulus (air versus odor) by condition (pre-training baseline versus post-training) interaction, indicating that the difference in response to air versus odor was affected by training [butyl acetate: $F(1, 27) = 11.22, P = 0.015$; butyl alcohol: $F(1, 23) = 6.849, P = 0.047$; amyl acetate: $F(1, 23) = 13.356, P = 0.015$]. For these three odorants, the skin potential response to odor alone was significantly different from the response to air after training [butyl acetate: $t(6) = 4.862, P = 0.0015$; butyl...
Salamanders did not learn an association between odor and shock when odor was delivered to the side on which the olfactory nerve had been sectioned. (A) Response to $4.3 \times 10^{-5}$ M butyl acetate (closed circles) versus air (open circles) with stimulus delivered to lesioned side. (B) Response to odor (closed circles) versus air (open circles) with stimulus delivered to intact side. Each point is the mean ± SEM, $n = 4$. Alcohol: $t(5) = 2.392, P = 0.031$; amyl acetate: $t(5) = 4.008, P = 0.005$ but not before training [butyl acetate: $t(6) = 0.795$; butyl alcohol $t(5) = 1.298$; amyl acetate: $t(5) = 0.656$]. Response to camphor did not differ from response to air either before [$t(6) = 0.365$] or after training [$t(6) = 0.282$].

Salamanders appeared to be unable to distinguish camphor from air (Figure 3D). Although some salamanders did not show conditioning to any odors, all animals tested with camphor were successfully trained to respond to at least one other odorant. All seven animals tested with camphor were also trained with butyl acetate on the same day, and four of those seven were trained with amyl acetate and butyl alcohol on a separate day. Thus, all seven subjects could be trained, and the inability to form an association between odor and shock was specific to the use of camphor as the training odorant.

**Experiment 2: forward versus backward pairing of stimuli**

An association was formed between tail-shock and odorant stimulus when tail-shock followed the odor presentation, but not when the shock preceded the odor (Figure 4). A repeated-measures ANOVA with stimulus (odorant or air) and condition (baseline, after backward pairing, after forward pairing) as the repeating factors gave a significant main effect of condition [$F(2,23) = 11.871, P = 0.022$], and a significant stimulus by condition interaction [$F(2,23) = 4.961, P = 0.009$]. $t$-tests showed that there was a significantly greater response to odor than to air after forward pairing [$t(3) = 3.339, P = 0.022$] but not before training [$t(3) = 1.279$] or after backward pairing [$t(3) = 1.757$]. These results indicate that the salamanders’ responses to odorant after training are due to a conditioned association with the shock.

**Experiment 3: olfactory nerve section**

Olfactory nerve section blocked conditioning of a response to odor when the stimulus was delivered on the lesioned side (Figure 5). A repeated-measures ANOVA with stimulus (odorant or air) and condition (baseline intact side, trained intact side, baseline sectioned side, trained sectioned side) as the repeating factors gave a significant main effect of condition [$F(3,31) = 12.933, P = 0.007$], and a significant condition by stimulus interaction [$F(3,31) = 6.379, P = 0.05$]. With training on the intact side, animals learned an association between the odor and shock, giving a significantly greater response to odor than air after 20 odor/shock pairings [$t(3) = 3.402, P = 0.021$]. On the sectioned side, response to odor did not differ from that to air after training [$t(3) = 1.04$]. The two animals that received training on the intact side first were retested on the intact side after training on the sectioned side to determine whether a response could still be elicited. These subjects gave larger responses to odor than to air either immediately, or after a brief retraining period (3 odor/shock pairings). Thus, the animals could still show skin potential responses, but did not respond to the previously conditioned stimulus when it was presented on the sectioned side, and could not form a new association between odorant and shock in the absence of olfactory input.

**Discussion**

The tiger salamander has been a widely used model for studying the olfactory system for decades. However, there are few psychophysical data available in this species for generating testable hypotheses relating physiological or molecular mechanisms to perceived odor quality. In this study we have developed a rapid method for measuring odor detection in tiger salamanders. Using classical conditioning, we can train animals to respond differentially to odor versus air over the course of several hours. Acquisition of the conditioned response is blocked by olfactory nerve section, demonstrating that the animals are indeed conditioned to olfactory stimuli rather than to other cues such as trigeminal stimulation of their face or auditory cues from the apparatus. Backward pairing results in no conditioning,
indicating that the response is not due to sensitization or pseudoconditioning (Erickson and Walters, 1988).

We have used this method to show discrimination between air and a small set of odorants that are commonly used as stimuli in electrophysiological experiments. This classical conditioning method can potentially be used to screen all compounds used in such experiments to show that they are detectable olfactory stimuli for this species. In the process, we may find other compounds, such as camphor, that do not elicit clear behavioral responses. Such compounds might best be avoided as stimuli in most olfactory experiments, but may be interesting cases to pursue in their own right to determine why the animal is unresponsive.

Several lines of reasoning, taken together, suggest that the salamanders are unresponsive to camphor in this study because they cannot detect it. First, it is unlikely that the behavioral deficit was due to an inability to learn per se. All of the salamanders that we attempted to train to camphor did learn to associate odor and shock using at least one other odorant, in some cases three others. Second, camphor was presented at a concentration that one would expect to be detectable based on the results with other odorants in this paradigm, and also based on camphor concentrations used in electrophysiological studies. Tiger salamander electroolfactogram and mitral cell responses to camphor have been reported for concentrations ranging from \(8.8 \times 10^{-8}\) to \(4.4 \times 10^{-6}\) M (Kauer, 1974; Kauer and Shepherd, 1977; Hamilton and Kauer, 1989). Third, tiger salamanders also failed to respond to camphor at saturated vapor in an entirely different paradigm (Mason et al., 1980). Although Mason and his colleagues did not explicitly test whether salamanders could discriminate between camphor and air, they did report that the salamanders failed to show avoidance responses to camphor when it was paired with a bright light. The animals did, however, learn to associate other odorants with the light (Mason et al., 1980; Mason and Stevens, 1981a,b). Fourth, our data are not consistent with the possibility that camphor has some property, such as aversiveness, which would interfere with learning an association with other stimuli. Tiger salamanders show an unconditioned skin potential response to aversive stimuli—such as the tail shock, a light pinch or a high concentration of an odorant—and our subjects occasionally showed skin potential changes before training to odorants used in these experiments. If camphor was particularly aversive to the salamanders, we would expect to see more unconditioned responses to this compound than to the others we used in experiment 1. If anything, camphor caused fewer of these responses than did other stimuli (as indicated by the low mean and small standard error bars in Figure 3D for trial 0). Salamanders also showed no elevated baseline avoidance of camphor in Mason’s paradigm (Mason et al., 1980).

Studies with human subjects have demonstrated that there are a number of compounds to which some percentage of the normal population shows significantly lower sensitivities, with that percentage varying for different compounds (Amoore, 1977). This phenomenon, known as ‘specific anosmia’, has long been studied with the hope that determining why an individual is insensitive to one particular odorant will provide information about how odor quality is processed. To date there are few reported animal models for specific anosmia (Wysocki et al., 1977; Pourtier and Sicard, 1990; Wang et al., 1993; Griff and Reed, 1995). The inability to detect camphor in the salamander may prove to be an example of a similar phenomenon.

We do not yet know whether only a subpopulation of tiger salamanders is unable to detect camphor, or whether this deficit extends to all individuals in this species. Either case is interesting and may provide insight into how specific odors are in fact detected. The advantage of studying anosmia in tiger salamanders is that electrophysiological and perhaps molecular biological methods can be used to investigate a documented psychophysical phenomenon. The camphor results are particularly surprising because this compound has often been used as a stimulus in electrophysiological recording in tiger salamanders, in part because it elicits large responses (Kauer, 1974; Kauer and Shepherd, 1977; Hamilton and Kauer, 1989). In extracellular and intracellular recordings from OB mitral/tufted cells in tiger salamanders, the types of responses reported for camphor do not differ in an obvious way from responses to other experimental odorants, including amyl acetate, butyl acetate and butyl alcohol; yet those odorants are discriminated from air and camphor is not. Thus, it will be of great interest to determine how the patterns of activity elicited by camphor, either in single cells or across a neural population, do in fact differ from the patterns of response to other odorants. The lack of behavioral response to camphor, which serves as a strong electrophysiological stimulus, emphasizes the need to compare psychophysical data with data obtained using other methods.

In addition to determining which chemicals tiger salamanders can detect, the method reported here can be used to obtain other measures of salamander olfactory function.
ability. For example, detection thresholds can be measured by training with a series of concentrations, and finding the lowest concentration to which responses can be conditioned. Threshold data are important for selecting appropriate odorant concentrations for electrophysiological studies, for examining relationships between the sensitivity of individual neurons, neuronal populations and the whole organism, and for chemical analyses examining binding constants and receptor specificities. Classical conditioning can also be used to determine which odorants tiger salamanders can and cannot discriminate. Animals could be trained by presenting one odorant paired with shock and a second odorant without shock. Differential responses would result only for odorant pairs that salamanders are able to discriminate. Finally, the degree of similarity between discriminable odorants can be demonstrated by conditioning with one odorant and then testing with other odorants. It would be expected that a response conditioned to one odor would be more easily generalized to odors similar to it than to odors that are perceptually quite different. Knowledge about both the discriminability and the perceptual similarity of olfactory stimuli in a model species is critical for determining how response characteristics of individual neurons or neuron populations encode odor quality information. Such studies are ongoing in our laboratory.

Mason and Stevens (1981a,b) have shown that a conditioned avoidance paradigm can be used for measuring detection thresholds as well as discrimination and generalization among odorants in salamanders. The method presented here, however, has several advantages over conditioned avoidance. First, acquisition of the conditioned skin potential response is rapid, taking ~2 h rather than 1–2 weeks, thus the ability of one animal to detect a number of different odorants can be assessed over a period of days rather than months. Second, the actual preparation of the animal closely approximates the conditions used in electrophysiological experiments, i.e. the animal is immobilized and stimulus delivery is essentially identical to the physiological preparation used in our laboratory. Comparisons can therefore be made between studies using different techniques to address questions about the physiological bases of perceptual phenomena such as odorant discrimination, detection thresholds and specific anosmia.

In addition to those advantages, it is possible that the behavioral conditioning can be completed while recording physiological responses in the same animal. This will allow us to examine directly the physiological correlates of olfactory learning on the single cell or neuronal population level, recording extracellularly or with voltage sensitive dyes in animals undergoing conditioning. Further, we can look at effects of experimental treatments on olfactory behavior and physiology simultaneously, examining, for example, whether pharmacological treatments that alter the pattern of response of a single cell or population also affect the perceptual quality of the stimulus. The ability to do experiments such as these will make this psychophysical method a powerful tool in increasing our understanding of odor quality coding. Most importantly, it will be possible to compare physiological and psychophysical responses to the same sets of odorants to test hypotheses about the functional organization of the olfactory system.

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