RECOVERY OF STRUCTURE AND FUNCTION FOLLOWING TRANSECTION OF THE PRIMARY Olfactory NERVES IN Pigeons

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Preliminary experimentation on ring doves to ascertain whether they might regulate any aspects of their reproductive behavior in terms of olfactory cues was vitiated by the discovery that their sectioned olfactory nerves had apparently regenerated. Concurrent work on frogs has shown that the olfactory receptors degenerate after axotomy and are replaced by new ones. This phenomenon was studied in pigeons. All transected nerves were found to be healed. Electrical recording from the regenerated nerves revealed apparently normal receptor function and, indirectly, autonomic reflex responsiveness. Previously untrained pigeons learned an olfactory discrimination after reconstitution of the peripheral olfactory system using a conditioned suppression procedure. The olfactory nerves of trained pigeons were sectioned and the behavioral response recovered within 15–52 days. The grosses sizes of primary olfactory nerves and olfactory bulbs were frequently much less than those of controls, but on the ultrastructural level there was no recognizable morphological deficiency in the receptor cellular organelles or terminal synaptic contacts in bulbar glomeruli. Recent results by other workers indicate that pigeons utilize olfactory cues in homing performance.

For many years there has been considerable controversy about the role played by olfaction in the behavior of birds (see Fink, 1965; Neuhaus, 1963; Tucker & Smith, 1975; Walter, 1943; Wenzel, 1971, for reviews). Since Audubon (1826) published his “account of the habits of the turkey buzzard, particularly with the view of exploding the opinion generally entertained of its extraordinary power of smelling,” the belief has been widely held that all birds have a poor, if any, sense of smell.

In the past decade, however, evidence has been accumulating that indicates at least some birds have a well-developed olfactory capacity. The comprehensive report of Bang and Cobb (1968) gives details of the olfactory anatomy of 108 species of birds. Although the olfactory bulbs vary in size with respect to the cerebrum in different species, essentially the same anatomy is present in all of the species they examined. Tucker (1965) recorded from a twig of the olfactory nerve in 14 species of birds and found a strong response to such odors as amyl acetate. Henton (1969), Michelsen (1959), Wenzel (1967), and Wenzel and Salzman (1968) have all shown that birds can be trained to make olfactory discriminations in a variety of laboratory settings.

Wenzel (1967) pointed out that “the general query—can birds smell?—must be resolved into three formal questions for investigation: (1) are birds capable of perceiving olfactory stimuli; (2) do birds naturally regulate any aspects of their behavior in terms of olfactory cues; and (3) can birds learn to regulate certain aspects of their behavior in terms of olfactory cues? [p. 203].” The evidence for a positive answer to Questions 1 and 3 is available. Addressing Question 2 led to the present research.

In October 1970, the late Daniel S. Lehm- man visited The Florida State University and lectured on the life cycle of the ring dove. A vigorous discussion ensued concerning a possible role for olfaction in the repro-
ducive behavior in these birds. On November 2, 1970 one of us (J. C. Smith) wrote to Daniel Lehman:

I am very excited about your careful observations on the life cycle of the dove and I am enthusiastic about the possibility of investigating the role of olfaction (if any) in this cycle.

I would suggest that you send me six or eight birds so that we can work out the surgery. Cutting the primary olfactory nerves in the pigeon has been a routine operation for our laboratory for many years. We have developed several instruments for this operation which may have to be changed for the ring dove. First, I will sacrifice several birds and get a clear picture of their olfactory anatomy. Next, we will have to work out appropriate anesthesia levels. We have used Equithesin for the pigeons at the suggestion of Dr. Bernice Wenzel. Do you have a routine technique for anesthetizing the ring dove? Once we have a routine operation we should be ready for a larger group of animals.

If you conspire, I will follow this procedure:

1. Subject an equal number of male and female birds to surgery.
2. The primary olfactory nerves would be exposed in all the subjects, but only half of the birds would have both nerves sectioned. The remaining half of the doves would serve as surgical controls.
3. In all subjects the wound opening would be packed with sterile Gelfoam and the skin sutured. There would be no distinguishing characteristics of the wound which would differentiate between sham operated and anosmic animals.
4. The doves would be returned to you in pairs of the following types: (1) both male and female anosmic, (2) both male and female sham operated, (3) male anosmic and female sham operated, (4) female anosmic and male sham operated.
5. After you finish the behavioral tests, you could return the animals to us for perfusion and histology. It is possible that we could arrange for some subsequent histology, but I would plan on only gross verification of the sections.

As you can see from Cobb's article, the anatomy of the olfactory system is a fact. Tucker's work indicates that the system is functional and Manton's work shows (at least for the pigeon) that the bird can detect odors and make both intensity and quality discriminations. The fact that we don't know is if in any way the bird uses olfaction in its behavior. I agree with you that it is doubtful if these "grain eaters" use it in feeding. It seems to me that the elaborate behavior patterns which you described provide the best possible way to test any role of olfaction. It will be most useful to know the effects if there are any. Therefore, any consistent outcome of these proposed experiments will be meaningful.

On November 3, 1970, a day after Smith wrote his letter, Dr. Lehrman wrote:

As you see, I am taking seriously your proposal that you should cut the olfactory nerve in some doves and let me look at their reproductive cycle.

What I propose is that I send you 20 male and 20 female doves, in, say 4 batches of 10 birds each. You should lesion half the birds, and do sham operations on the other half. You number the birds—males 1 through 20, females 1 through 20, and tell me the numbers you have assigned (with reference to the leg band numbers you will find on the birds when you get them). We will then breed the birds in pairs as you designated. Make sure that, within each pair, both members have the same treatment. You could send me the identification code telling which birds are lesioned and which sham-operated, in a sealed envelope, which we would not open until after we had collected the data. We would collect simple data indicating efficiency of the various stages of the reproductive cycle, as I outlined to you in our conversation.

I should first send you a few birds just for you to play with, and familiarize yourself with the anatomy, and let me know whether it seems practical for you to go ahead with the problem. You ought to have on hand some seed mixture of a kind made for domestic pigeons. Probably any pet store can provide a pigeon mixture, preferably a "no-corn" mixture, since the corn grains are a bit too large for this type of dove. The birds can be kept very easily in double-width rat cages, of the kind that I saw on your Hoetge racks. I don't remember whether you had double-width cages, but it would not hurt the birds to be kept for a few days in single-width cages.

Smith's and Lehrman's letters crossed in the mail. After a short time the experiment was initiated with 8 birds. On January 15, the following information was sent to Dr. Lehrman:

Over the holidays we were able to modify the headholders and other surgical tools and have found the operation generally to be quite routine. In fact, it has been so routine that we successfully performed surgery on 6 of the original 8 birds. It seems to me that it would be a waste to sacrifice these animals now in order to practice perfusing the brains. I called your lab yesterday and suggested that we send these 6 animals back to you so that you can make a preliminary test. I requested that he send another 6 or 8 birds to us and we will continue perfecting the operation and also work out the technique for post-mortem examinations.

In keeping with the original plan, we sectioned the nerves in both the male and female and sham operated the male and female in another
pair. I would suggest the following matches: female $7791$ to be paired with male $7791$; female $7541$ with male $7771$; and female $7107$ with male $7064$.

On July 14, 1971, Dr. Lehrman made his report:

Following are the data on the reproduction of the birds you operated on:

<table>
<thead>
<tr>
<th>First Breeding</th>
<th>Pair</th>
<th>Time (days) to 1st egg</th>
<th>Duration of incubation period</th>
<th>No. of young</th>
</tr>
</thead>
<tbody>
<tr>
<td>$7190$ f</td>
<td>7</td>
<td>14</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>$7791$ m</td>
<td>7</td>
<td>15</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>$7541$ f</td>
<td>8</td>
<td>14</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>$7771$ m</td>
<td>8</td>
<td>14</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Second Breeding</th>
<th>Pair</th>
<th>Time (days) to 1st egg</th>
<th>Duration of incubation period</th>
<th>No. of young</th>
<th>Time (days) in isolation between 1st and 2nd breedings</th>
</tr>
</thead>
<tbody>
<tr>
<td>$7190$ f</td>
<td>6</td>
<td>15</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>$7791$ m</td>
<td>9</td>
<td>14</td>
<td>2</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>$7541$ f</td>
<td>9</td>
<td>14</td>
<td>2</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>$7771$ m</td>
<td>9</td>
<td>14</td>
<td>2</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

"Time to 1st egg" means the number of days from the time the birds are introduced into the cage until the 1st egg is laid.

These three pairs are sufficiently homogeneous so that I would not be encouraged about seeking further differences. If you want to do more operations, I would be willing to test more birds. A negative report on this experiment would probably be useful, since the question is constantly being raised when I talk about these birds.

Or do you see any signs of a positive result?

On July 29, 1971, the following information was sent to Dr. Lehrman:

I was glad to get your letter of July 14 and to see the data from the doves. I agree with you that there is no sign of a positive result. The following table lists our preparation of these birds:

<table>
<thead>
<tr>
<th>Pair</th>
<th>Gender</th>
<th>Sectioned</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>$7100$</td>
<td>Female</td>
<td>Sectioned</td>
<td>Dec. 31, 1970</td>
</tr>
<tr>
<td>$7101$</td>
<td>Male</td>
<td>Sectioned</td>
<td>Dec. 4, 1970</td>
</tr>
<tr>
<td>$7771$</td>
<td>Male</td>
<td>Sham</td>
<td>Dec. 31, 1970</td>
</tr>
<tr>
<td>$7541$</td>
<td>Female</td>
<td>Sham</td>
<td>Dec. 31, 1970</td>
</tr>
<tr>
<td>$7107$</td>
<td>Female</td>
<td>Sectioned</td>
<td>Jan. 1, 1971</td>
</tr>
<tr>
<td>$7064$</td>
<td>Male</td>
<td>Sectioned</td>
<td>Jan. 1, 1971</td>
</tr>
</tbody>
</table>

I would like to suggest the following procedure. Dr. Pasquale Graziaedi, of our Biology Department, is a renowned expert on the anatomy of taste and olfaction. He has been very excited recently about several lines of evidence of regeneration in the primary olfactory nerve. He is convinced that the results of our experiment are due to regeneration and not a lack of olfactory input. I don't know the dates of the first and second breedings, but Graziaedi is convinced that the regeneration is a very rapid process. When I sectioned the nerves, I merely cut and did not remove a segment. Before you and I spend much more time on this, I would like to give Graziaedi the original birds for histological examinations to see if the primary olfactory nerve is intact.

It was not until the following February (1972) that the birds were returned to Florida State University. Without knowledge of the prior treatment condition of the individual birds, 2 of us (PG and RD) sacrificed the 6 doves and prepared the tissue for structural and ultrastructural examination.

Birds 7541 and 7771 (the sham operated subjects) showed no sign of abnormality in the olfactory mucosa, the olfactory nerves and the bulbs at the macroscopic examination. The following notes were made on the other 4 birds. Bird 7791, male, was sacrificed on February 17, 1972. The examination did not show any change from normal with the exception of some scar tissue along the left olfactory nerve proximal to the olfactory mucosa. Both right and left olfactory nerves were, however, continuous between the olfactory mucosa and the olfactory bulbs. Bird 7197, female, was sacrificed on March 1, 1972. The remains of a large hematoma were found to cover the dorsal surfaces of both olfactory bulbs. Both olfactory nerves presented signs of scar tissue. However, the nerves were clearly continuous from their peripheral origin to the bulbs. Their diameters seemed to be smaller than those observed in other doves; however, this assessment is difficult to substantiate. The mucosa of both sides were normal. Bird 7664, male, was sacrificed on March 2, 1972. Both the mucosa and the bulbs were normal. Along the course of both nerves there was abundant scar tissue requiring removal before the continuity of the olfactory nerves could be ascertained. This continuity was observed without doubt. Bird 7190, female, was sacrificed on February 10, 1972. The left nerve looked slightly "shredded" in appear-
ance, but the main bulk was still intact, clearly connecting the mucosa and the bulb.

Following the macroscopic examination of these 6 doves, performed under Nembutal anesthesia, the animals were perfused through the aorta with a cacodylate-buffered solution of paraformaldehyde and glutaraldehyde. Following this procedure appropriate pieces were dissected out and, after postfixation in osmium tetroxide in the same buffer, they were embedded in Araldite for further structural and ultrastructural study. Preliminary examination of the light and electron microscopic preparations of all 6 birds had shown that the olfactory mucosae were normal, with the usual histological components of receptors, and supporting and basal cells. The olfactory nerves did have a population of fine unmyelinated fibers that are typical of the olfactory nerves. The olfactory bulbs showed at the ultrastructural analysis the usual pattern of glomeruli. The fine synaptic structure of these formations was alike in all animals. Clearly, the microscopic and submicroscopic study of the olfactory structures in all 6 doves did not show any qualitative variation from normal.

Sectioning of the olfactory nerves in birds has been a routine process in The Florida State University laboratories for many years. The dissected nerve could be clearly visualized through the dissecting microscope during surgery, so it was unlikely that the nerves were not sectioned in the original operation. A more likely hypothesis was that regeneration had occurred, probably before Dr. Lehrman made any observations (Graziaedi, 1973; Graziaedi & Metealf, 1971). It seemed also probable that all 6 birds were essentially normal at the time the behavior was tested.

The present experimentation was designed to study the function of the bird's olfactory system before and after bilateral sectioning of the olfactory nerves. Using both electrophysiological and behavioral techniques, observations were made of functional recovery following surgery and its relation to structural recovery. The pigeon was selected as the subject because both the electrophysiological (Tucker, 1964, 1965) and behavioral (Henton, 1969; Henton, Smith, & Tucker, 1966, 1969; Shumake, Smith, & Tucker, 1969) procedures had been worked out on this species.

Amyl acetate was chosen as the stimulus odorant because no other odorant has yet been found that has so great a range between olfactory and trigeminal thresholds (Tucker, 1971). Nasal trigeminal threshold concentration was found to be between $10^{-1.2}$ (3.16%) and $10^{-1.0}$ of vapor saturation for both the domestic rabbit and the gopher tortoise by means of electrophysiology (Tucker, 1963a, 1963b). The assumption has been made here that the bird's trigeminal sensitivity will likely be similar. This assumption can be challenged by a study of the presumed nasal trigeminal mediation of behavioral capabilities of the pigeon (Henton et al., 1969), in which birds were readily retrained to discriminate presentation of amyl acetate at 10% saturation after losing the discrimination to 3% when the olfactory nerves were sectioned. Protracted experimentation with different odorants and procedures ensued, during which amyl acetate threshold concentration values gradually declined to apparently stable values that were less than 1 log unit higher than odor thresholds of subjects with intact olfactory nerves. The conclusion was that trigeminal sensitivity to odorants was the source of information to the subjects. Although segments had been extirpated from the nerves of these pigeons and anatomical examination indicated that the nerves were still divided, the more recent experience with Dr. Lehrman's ring doves suggested to us the possibility that some olfactory axons had found their way back through the scar tissue to the olfactory bulbs.

Olfactory structural dynamics have been worked out principally on the frog in this laboratory (Graziaedi, 1973; Graziaedi & DeHan, 1973; Graziaedi & Metealf, 1971) and references to other works on frog as well as other animals will be found in the above citations. The cell bodies of the axons comprising the olfactory nerve are the receptor cells located in the olfactory mucosa. Olfactory axons grow from the periphery toward the brain, unlike regenerating fibers.
of other cranial sensory nerves. The first synapse in this sensory pathway is in the olfactory bulb, a central nervous system structure. A discrete nerve on either side joins the olfactory mucosa in the nasal cavity with the olfactory bulb in the cranial cavity in either frog or pigeon (Figure 6), contrasting with the fila olfactoria transmitted through the foramina of the mammalian cribiform plate. A normal turnover of receptor cells has been demonstrated with electron microscopic autoradiography after systemic injection of tritiated thymidine. The process is accelerated greatly by sectioning the olfactory nerve. All the axotomized receptors die and, of course, the distal axonal segments synapsing with dendrites in the bulbar glomeruli degenerate. A burst of mitotic activity appears in the mucosal basal cell region shortly after the beginning of receptor degeneration. These daughter cells may divide several times and eventually differentiate into new receptors the neurites of which grow back toward the olfactory bulb in the nonneural remnants of the nerve. Another point is that degeneration of all the axons in a nerve does not cause its disappearance, at least not soon. There are many nonneural components in a nerve trunk that persist. The neurites can establish synaptic connections in the bulb in large numbers if the remnant stumps of the nerve at the site of lesion are closely apposed. This process might better be termed renewal or reconstitution, because regeneration in other types of nerve denotes regrowth of the distal axonal portion from a neuron that survives axotomy. Often, neuromas of varying size are seen in experimental material. The reunion of sectioned olfactory nerves has been observed in turtles, frogs, and pigeons. Results obtained so far indicate that the regenerative process is basically similar in these animals and mice (Metcalf, 1974), with temporal differences between warm- and cold-blooded species.

**Experiment 1: Olfactory Nerve Recording**

The purpose of this experiment was to demonstrate that regenerated (renewed) receptors do indeed respond to odorants, and that they conduct action potentials over their axons toward the olfactory bulb. Simple transection of the primary olfactory nerve was used to facilitate regeneration, as is presumed to have occurred in the ring doves.

**Method**

**Subjects.** Four adult pigeons from Palmetto Pigeon Farm, Sumter, North Carolina, were used for electrical recording from healed nerves and 8 normal birds were used as controls. The subjects were kept in individual cages and given free access to food, grit, and water.

**Apparatus.** Either clean or odorized air was brought to the pigeon's nares by a delivery system (olfactometer) similar to that described by Tucker (1963a). A schematic of this apparatus is illustrated in Figure 1. Laboratory compressed air was reduced in pressure to about 7 lb/in², dried with Linde 13X molecular sieve (Union Carbide), cleaned with activated coconut charcoal (Fisher Scientific), and saturated at 20°C and 105 atm. with either distilled water or amyl acetate (Matheson, Coleman and Bell). Odorant and water-saturated air streams were combined in a mixing manifold with the appropriate ratio of flow rates to achieve the desired dilution. Thus, for example, a 3.16% or 10⁻⁴⁴ per unit saturation of amyl acetate concentration was produced in 1 channel by mixing 189 X 1.05 cm.² per min. of air saturated with amyl acetate with a sufficient flow volume of air saturated with distilled water to make a total flow rate of 6,000 cm.² per min. The same flow rate (6 l/min) of air saturated with distilled water was maintained in a second output channel. Both the clean and the odorized air streams were brought to a noninterrupting line transposition switch made of Teflon and anodized aluminum adjacent to the bird. From this point, either air stream was diverted manually to the bird's nares, while the other was simultaneously exhausted to the outdoors.

**Sectioning of nerves.** Pneumatised bone between the eyes was taken out under light anesthesia (Equi-Thesin, 2 ml/kg, im), and the extracranial olfactory nerves and accompanying blood vessels were brought into view by removing small portions of the bony orbital walls in 4 of the birds. A short, shallow incision was then made in the outer and inner sheaths surrounding each nerve to gain access to the nerve bundle. Care being taken not to disturb the adjacent vasculature or ventral portion of the sheath, the nerve was lifted slightly and transected. The cut ends were returned to the sheath and approximated. The bony cavity was lightly packed with Gelfoam and the skin was closed with Michelle clamps.

**Electrical recording.** The electrical recording procedure was the same for all birds. Each olfactory nerve was exposed and 1-2 mm. of a small-diameter nerve twig was dissected free from the large nerve trunk (Tucker, 1965). In the 4 previously operated birds this twig was dissected a few
millimeters proximal to the site of the previous nerve section. The twig was placed across 2 Pt-Ir wire electrodes under mineral oil, and the neural signals were amplified with a Grass P5 preamplifier set for a frequency bandpass of 7-500 Hz (6 db down points). The output of the preamplifier was fed to an audio monitor, a Tektronix 555 oscilloscope, and a Sanborn hot-stylus recorder. The asynchronous neural activity was processed in the recorder unit with a Sanborn 350-1400 amplifier on the no linear mode of operation, for which the output indication is "full wave average." This method of quantifying the neural activity is strictly analogous with that introduced by Beidler (1933), and records of olfactory "raw" and "integrated" neural activity obtained simultaneously have been compared elsewhere (Moulton & Tucker, 1964).

Results

Neural activity was recorded from regions on the bulbar side of the lesion site of the olfactory nerve of the 4 previously operated pigeons in response to olfactory stimulation 189-302 days after transection. These nerves were healthy in appearance, differ-
AMYL ACETATE CONCENTRATION PER UNIT SATURATION

Figure 2. Integrated response of an olfactory nerve to amyl acetate stimulation, which was obtained 302 days after section of the nerve. (The integrator (filter) time constant was small enough to resolve the burst of action potentials occurring during inspiration, thus yielding a moving average of the impulse traffic on this time scale. The gain for recording A was 4 times that in B. For ease of visualization, the first pen deflections for $10^{-1.8}$ and $10^{-1.4}$ amyl acetate presentations in record B have been retouched.)

ing principally in the distribution of capillary blood vessels in the almost transparent tissue. Figure 2 shows the integrator records obtained from a representative bird 302 days after section during the first (record A) and the fifth (record B) hours of continuous recording. The train of peaks of activity recorded during each 30-sec. stimulus presentation reflects successive responses of the population of receptors in the nerve twig to individual inspirations of the anesthetized bird. During recording sessions, ascending concentrations of amyl acetate were presented to the bird’s nares for 30 sec. each, approximately once every 3.5 min.

These records show that the previously sectioned nerve of this subject responded to olfactory stimulation and, hence, the receptors did also. Further, various features of the responses were like those observed in the normal birds. At higher concentrations, the olfactory response to the initial inspiration was greater than to subsequent inspirations. While the initial response increased with increasing concentration of the odorant, subsequent responses during the 30-sec. stimuli were often of lower amplitude than analogous responses to lower amyl acetate concentrations. These differences were more noticeable during the fifth (B) than during the first (A) hour of recording. Response amplitudes were about 4 times greater during the fifth hour to the lower concentrations of amyl acetate.

Autonomic reflex responses to odorant stimulation are also reflected in the records of Figure 2. The respiratory stability during presentation of the odorant was related to the time the recordings were made. In the earlier one, respiration occurred at a relatively constant rate during stimulus presentations, even at the higher amyl acetate concentrations. However, during the fifth hour, respiratory frequency decreased during a stimulus presentation, especially at the higher odorant concentrations. Another feature of the later recording was the appearance of artifactual pips during the interstimulus interval. These pips were correlated with the bird’s spontaneous eyelid reflex, which was not present during the first recording (A). The olfactory nerve is medially apposed to the orbital contents, and for this preparation an opening was made in the orbital wall to expose the nerve.
The anatomical results are presented in the section on morphological observations.

Discussion

The renewed olfactory receptors were responsive and the electrical recording results were typical of normal birds (Shibuya & Tucker, 1967; Tucker, 1965). The much larger responses recorded in the second trace of Figure 2 probably represent a reflex change in accessibility of the olfactory organ to odorant and therefore a change in the effective stimulus (Tucker, 1963a, 1963b, 1965). Thus, one can roughly visualize the response-concentration functions and deduce that at the lower narial concentrations, the stimulus at the receptor level was about 10 times greater in B than in A.

Respiratory deceleration appearing at $10^{-2.0}$, and possibly at $10^{-2.5}$, of saturation with amyl acetate strongly suggests that this autonomic response was mediated by the olfactory system (Tucker, 1963a, 1963b, 1965; Tucker & Beidler, 1956a). The appearance of such autonomic responsiveness in birds with reconstituted olfactory systems therefore indicates another type of functional recovery.

In general, the size of the recorded response is affected by receptor adaptation and the quickness of inspiration in addition to intrinsic variation in accessibility. For example, the responses for the last 3 stimulus presentations in Figure 2 reveal a strong increase in adaptation with increase in concentration. However, the accompanying rapid decrease in respiratory frequency also likely caused a decrease in the forcefulness of inspiration, which also contributes to the diminution of responses. The increases in the later inspiratory responses to 10% amyl acetate signal a rebound quickening of inspiratory strength. Momentary breath holding indicated by the record at $10^{-3}$ amyl acetate is not unusual. This concentration of amyl acetate is aversive to humans and is a potent stimulus for nasal trigeminal receptors (Tucker, 1971). Aversive concentrations are probably stimulatory to pharyngeal and laryngeal receptors too, but at reduced levels the nasal and ocular trigeminal receptors are still responsive, with the olfactory receptors achieving the ultimate sensitivity to amyl acetate.

Experiment 2: Postoperative Acquisition of an Olfactory Discrimination

Experiment 1 demonstrated that regenerated olfactory neuroreceptors could respond to normally adequate stimulation at least as early as 189 days following bilateral section. The present experiment sought to determine whether the free-moving, unanesthetized bird with a reconstituted olfactory system could learn an olfactory discrimination.

Method

Subjects. Four adult pigeons served as subjects. The birds were maintained at 70% free-feeding weight and allowed water and grit ad lib in their home cages. They were all implanted with stainless steel pubic electrodes (Aarrin, 1959) prior to training.

Apparatus. The olfactometer used to deliver odorant or clean air to the bird's breathing chamber is described in Experiment 1 (see Figure 1). As shown in Figure 3, the breathing chamber was housed in a modified Lehigh Valley pigeon box (26 × 37 × 33 cm), which was enclosed in turn in a sound-attenuating shell (81 × 81 × 132 cm).

The breathing chamber was a Teflon-lined stainless steel enclosure 16 cm. long and 6.5 cm deep, with funnel-shaped glass intake and exhaust ports above and below. Regularly perforated Teflon disks within the ports served to linearize the flow of air through the chamber. An opening (5.5 × 10.5 cm.) on the face of the chamber permitted the subjects access to the pigeon key and grain hopper mounted on the rear wall. The total air flow into the chamber was 6 l/min at all times. However, air was exhausted from the chamber at 7 l/min to prevent leakage into the pigeon box. “White” masking noise and a 24-r. houselight were always on during training.

Sectioning of nerves. The birds' olfactory nerves were bilaterally sectioned as in Experiment 1.

Behavioral training. Conditioning was begun 145–165 days after surgery. Each subject was initially trained to peck the key in the presence of a constant flow of clean air. Each peck was rewarded by 2-sec. access to grain. The probability of reinforcement was then decreased gradually using a series of random ratio schedules until a high rate of responding was established. To promote stable responding, the pigeon was transferred to a variable interval (VI) schedule of approximately equivalent reinforcement density, and the length of the VI was increased in 30-sec. steps to 90 sec. (VI 90-sec.) during successive 1-hr. sessions. A 2-sec. limited hold (LH 2-sec.) contingency was then imposed to further discourage pausing. If the sub-
ject did not respond within 2 sec after a reinforce-
ment became available, it lost the opportunity to
obtain reward until the next inter-reinforcement
interval had elapsed.

After 5-10 sessions on the VI-50-sec, LH-2-sec.
schedule, conditioned suppression training was be-
gun. Five odor trials, 5 zero-concentration control
trials, and 5 baseline control trials were presented
in random at 4-min. intervals during each session.
During an odor trial, the normally present clean
air flow was transposed with a stream containing
amyl acetate at 10−5 of saturation, the conditioned
stimulus (CS), for 20 sec. The unconditioned stim-
ulus (US) was a 40-msec. 44-v. ac shock in series
with a 100-kΩ resistor delivered at the end of the
20-sec. CS. To control for nonolfactory cues pro-
duced by the manual switching operation, zero-
concentration control trials were presented during
which the odorized air stream was adjusted for zero
concentration. During baseline control trials, re-
sponding was monitored for 20 sec, but the flow
lines were not switched. To avoid confounding the
effects of the CS with those of positive reinforce-
ment, reinforcements due on the VI schedule were
withheld during each trial and for 20 sec preceding
and following each trial. Twenty seconds after
the end of a trial, reinforcement was made avail-
able for the usual 2-sec. LH period.

The magnitude of the response suppression dur-
ing an odor trial was expressed by the ratio \((A - B)/A\), where \(B\) represents the number of responses
during the 20-sec. trial, and \(A\) is the number of re-
ponses during the immediately preceding 20 sec.
Thus, complete suppression of responding during
a trial would be indicated by a ratio of unity, while
no suppression of responding would be represented
by zero.

A suppression ratio of .7 was used as the crite-

dion for odor detection in this experiment. After a
bird reached a mean suppression ratio of .7 for the
5 odor trials on a given day, the sessions were con-
tinued to show stability of responding. The pigeons
were tested on alternate days for 14-16 sessions and
then tested every sixth day for 3 additional ses-
sions.

Results

Figure 4 illustrates the behavior of 4
pigeons presented with the odorant for the
first time 211-231 days following bilateral
postoperative training sessions

Figure 4. Acquisition of an olfactory discrimination following bilateral section of the olfactory nerves. (On days when odor, zero-concentration, and baseline control trials were presented, each datum point represents the mean of 5 suppression ratios. On intermediate days, when only zero-concentration control trials were administered, each point is the average of 15 suppression ratios. Apparatus failure occurred during those sessions for which no points are shown.)

Section of the olfactory nerves. In general, near-zero suppression ratios during baseline control trials (open triangles) indicate the overall stability of responding on the baseline schedule (VI-90-sec., LH-2-sec.), while marked suppression of responding in the presence of the olfactory stimulus ($10^{-1.5}$ per unit saturation of amyl acetate) during odor trials (dark circles) is revealed by ratios exceeding .7. For ease of visualization, both the 0.0 and .7 values are shown as dashed lines parallel to the x axis. As this figure indicates, 2 of 4 operated birds acquired the olfactory discrimination during a single training session, and 2 after 4 such sessions. Once acquired, performance of the
discrimination remained at criterion levels during subsequent test sessions.

**EXPERIMENT 3: PRE- AND POSTOPERATIVE PERFORMANCE OF AN OLFACTOR Y DISCRIMINATION**

In Experiment 2 it was shown that pigeons readily learned to discriminate between the presence and absence of amyl acetate when training was begun over 200 days following bilateral sectioning of the olfactory nerves. The present experiment was performed to study recovery of the learned behavioral discrimination after olfactory nerve section.

**Method**

The 4 pigeons in this experiment were trained to discriminate the odorant as in Experiment 2, then subjected to bilateral section of the olfactory nerves and retested until they recovered their preoperative discrimination performance. Finally, they were sacrificed and the status of regeneration of the olfactory system was examined.

**Results**

The initial discrimination performance between odorized (dark circles) and clean air (open circles) streams is shown in Figure 5. Bilateral section of the olfactory nerves did not affect stability of the baseline performance (open circles and triangles). However, when tested 4 days after surgery, the birds all failed to suppress responding in the presence of the odorant. During the course of continued testing, all the birds eventually recovered their previous olfactory discriminations (compare the dark- and open-circle functions). Bird 4's performance returned to normal within 3 testing sessions (16 postoperative days). However, the recovery period was considerably longer for Birds 2, 3, and 1 (52, 76, and 82 postoperative days, and 9, 13, and 14 testing sessions, respectively). In each case, once performance returned to normal, it was maintained at that level during subsequent testing.

The anatomical results are presented in the section on morphological observations.

However, it is pertinent to the following discussion that the nerves of each bird were found to be intact.

**Discussion of Behavioral Results**

The major findings of Experiments 2 and 3 were that (a) pigeons with olfactory nerves which had been cut more than 200 days prior to initial training learned to discriminate between amyl acetate and clean air streams, and that (b) normal birds trained to make the same discrimination lost their ability to detect the odorant immediately after olfactory nerve section, but relearned the discrimination within 16 to 82 days following surgery. Experiment 1 revealed that the new receptors in the reconstituted olfactory system are functional, but indicated little about how successful the reorganization in the olfactory bulb might be. The latter experiments show that the regenerated system can mediate a simple behavioral discrimination task.

Recovery of behavioral discrimination by the 1 bird 16 days after nerve section seems surprisingly soon, in comparison with the others. Odor testing every sixth day imposed by the experimental regimen was too gross a probe to resolve satisfactorily with this short a recovery time. Obtaining 3 sessions of criterion performance prevented looking for evidence of rapid regeneration, or possibly a continuously surviving remnant of nerve, at the crucial time, for at 36 days the observed continuity of the nerve agreed with parallel morphological findings. An alternative suggestion that the 1 bird might have been unusually quick to utilize other sensory cues seems of dubious value.

The suggestion has been made that, in the absence of olfactory neural input, the trigeminal nerve of pigeons may come to mediate a behavioral discrimination between clean air and air odorized with amyl acetate below the concentration used in these experiments (Henton et al., 1969). This point was discussed in the introduction and need not be repeated. However, use of a lower concentration would reduce the possibility of equivocation. The vomeronasal system is beyond consideration as a possible source of nonolfactory responses to odorant that.
Figure 5. Performance of an olfactory discrimination before and after bilateral section of the olfactory nerves. (On days when odor, zero-concentration control, and baseline control trials were presented, each datum point represents the mean of 6 suppression ratios. On days when only zero-concentration control trials were given, each datum point is the average of 15 suppression ratios. Birds 3 and 4 were treated with a broad spectrum antibiotic (Tylan) and vitamins (Avitron) for an undiagnosed infection on those days for which no data points are shown.)
migh cue the lesioned pigeons, because it only appears transiently during embryonic development (Tucker, 1971).

In summary, the rapid acquisition of the behavioral discrimination in birds with regenerated olfactory systems, the loss of the discrimination in trained birds upon section of the olfactory nerves, the finding of healthy nerves after return of the discrimination, and the supportive electrophysiological results strongly suggest that the regenerated olfactory system mediated the behavioral capability.

**Morphological Observations**

During the other experiment, 12 additional pigeons were subjected to unilateral olfactory nerve section and examined at various times later. Data were collected also from the 4 birds of Experiment 3, 2 from Experiment 1, and 4 untreated pigeons. A schematic diagram of the pigeon's olfactory anatomy is shown in Figure 6 with the site of olfactory nerve section indicated.

The birds were sacrificed under Nembutal anesthesia by means of intravascular perfusion of buffered aldehydes (Graziadei, 1973). Following perfusion, each animal was examined macroscopically during the dissection procedure for securing pieces of olfactory mucosa, olfactory nerves, and olfactory bulbs. Following the gross examination, adequately trimmed pieces of these 3 structures were prepared and embedded in Araldite. From these blocks both thick (1 μ) and thin (800 A) sections for light microscopy (LM) and transmission electron microscopy (TEM), respectively, were obtained and stained appropriately.

In the unilaterally operated pigeons the severed olfactory nerve regained its anatomical continuity by the 25th day. Specimens exhibited considerable variation in the amount of scar tissue and neuroma formation. The regenerated nerve and the homolateral bulb were smaller than those of the control side and the reduction in size persisted at the 6-mo. period. A rough estimate gave values of 20%–30% decrease in size on the operated side.

At the LM level the unilaterally operated birds showed 5 to 15 days after section of the nerve a massive degeneration of the olfactory receptors and their axons and extensive degeneration of their terminals in the bulbar glomeruli. These observations were confirmed at the TEM level. Regenerating olfactory receptors were detectable after the second week and some small unmyelinated axons had crossed the original surgical gap of the 25-day nerve. By 7 wk, axons were numerous, although smaller than those of the control side, and signs of degenerative activity were still present in the bulb.

The observation of the olfactory mucosa, olfactory nerve, and olfactory bulb by means of LM and TEM yielded "normal" morphological patterns by 10 wk. after the nerve section. TEM observations confirmed that all along the course of the nerve unmyelinated axons, similar to those of the control nerve, are the constituents of what has been interpreted as the regenerated ol-
factory nerve (Figures 7 and 8). In the olfactory bulb the glomerular pattern of synapses showed in the operated side little variation from the control side (Figures 9 and 10).

The study of the 6 birds used for electrophysiology and behavior provided observations in close agreement with those made on the unilaterally sectioned birds. Gross examination showed that in each of the birds both olfactory nerves were continuous, the experimental gap being bridged by what appeared to be, under the dissection microscope, a normal nerve. Bird 4 of Experiment 3 (Figure 5) had an abscess surrounding the olfactory structures; there was a neuroma and the left bulb and nerve were smaller than those of the other side. Figure 11 shows an example of the TEM confirmation of appropriate axons crossing the pre-existing surgical gap in the olfactory nerve of 1 of the subjects from Experiment 1.

From these morphological observations in pigeons, which correspond closely to similar ones performed previously in frogs (Graziadei, 1973; Graziadei & DeHan, 1973), we came to the conclusion that the severance of the olfactory nerves induces fast degeneration of all the existing mature receptor neuron perikarya. The pattern of glomerular degeneration after 15 days has been shown before (Wenzel & Salzman, 1968). Following this phenomenon, basal cells divide and differentiate into fully mature receptor neurons that send their axons to the olfactory bulb, where their terminals reconnect synaptically with the postsynaptic elements of the glomeruli. This anatomical reconnection of neural elements seems to be in good agreement with the physiological and behavioral results.

**Discussion**

Our belief that the olfactory nerves of Dr. Lehrman's ring doves grew back is substantiated by the present results obtained on pigeons. All the sectioned nerves were
rejoined when examined at sacrifice 25 or more days later. However, some bore neuromas of varying size at the sites of lesion. Some were visibly smaller than were normal nerves, as was also true of some of the olfactory bulbs. Nerves and bulbs in frogs were found to be 20%–30% smaller than contralateral controls in frogs 40 days after neural transection (Graziadei & DeHan, 1973). Evidently, there were variations in the successful reconstitution of the peripheral olfactory system.

The propensity of pigeon olfactory nerves to regenerate was discovered independently by Benvenuti, Fiaschi, Fiore, and Papi (1973b, cf. Method section), which necessitated reoperation upon all their experimental birds. The regeneration was confirmed at the ultrastructural level (Bedini, Fiaschi, Fiore, & Lanfranchi, 1971), which is necessary to demonstrate unequivocally the tiny olfactory axons. Undoubtedly, in the original experimentation on the ring doves, substantial segments of the olfactory nerves should have been resected to try to ensure permanent anosmia.

Some pilot experimentation on pigeons revealed that the hook used to lift the nerve sometimes split it, thus raising the specter of small nerves and bulbs merely being accidental remnants that had survived continuously. Therefore, great pains were taken to visualize the whole of the nerve and to section it completely, even at the expense of a clean cut that would be expected to facilitate reconnection. Nevertheless, many of the olfactory nerves and bulbs approached normality in size, independently of their contralateral neighbors.

The reconstituted peripheral olfactory system was functional as judged by electrophysiology. Receptor responses seen on the oscilloscope, and heard, were like those characteristic of other animals (Beidler & Tucker, 1955; Moulton & Tucker, 1964) and birds (Shibuya & Tucker, 1967;
Tucker, 1965). Bedini et al. (1971) showed that the receptor axons conducted impulses across the original lesion site by stimulating the nerve electrically on one side and recording the compound action potential on the other. Receptor responses to amyl acetate increased with concentration similar to those reported for other species of bird (Shibuya & Tucker, 1967; Tucker, 1965). Receptor adaptation also increased normally with odorant concentration. Autonomic reflex responses, i.e., changes in depth and frequency of respiration, were observed in the urethane anesthetic state. These autonomic responses are thought to be mediated by the olfactory system (Tucker, 1965), if the trigeminal threshold concentration of amyl acetate for the pigeon is above $10^{-1.5}$ vapor saturation as was found for the rabbit and gopher tortoise (Tucker, 1963a, 1963b). Reflex modulation of sympathetic efferent activity to the nasal cavity was observed for amyl acetate between olfactory and nasal trigeminal thresholds (Tucker & Beidler, 1956b) and electrical stimulation of the cervical sympathetic nerve altered accessibility of the olfactory organ to the inspired air stream (Tucker, 1963a, 1963b; Tucker & Beidler, 1956a).

Behavioral capability was exhibited by all the experimental birds. The acquisition of olfactory discrimination by the naive birds in Experiment 2 was consistent with results obtained in normal pigeons previously (Henton, 1969; Henton et al., 1966, 1969; Shumake et al., 1969). A wide range of recovery time was exhibited by the trained birds in Experiment 3 subsequent to section of the olfactory nerves. The shortest time of 16 days contrasts with the first observation by means of electron microscopy of receptor axons traversing the lesion site in 25 days. However, further ultrastructural observations may reveal a similar wide
range in recovery time among subjects. The nerves of the birds in Experiment 2 had been sectioned more than 200 days before the start of odor training and the longest time for recovery in Experiment 3 was 82 days.

The basis for behavioral recovery was most likely the regrowth of receptor axons to the olfactory bulb and the establishment of synaptic connections in the olfactory glomeruli. All of the anatomical elements serving in the chain of events from peripheral receptor activation by odorant to the initiation of secondary neural response in the olfactory bulb were shown to be present at the time of sacrifice by means of electron microscopy. The events of retrograde degeneration of mature receptor neurons after axotomy, proliferation and differentiation of olfactory mucosal basal cells into new receptors and reestablishment of glomerular synaptic contacts have been documented in the frog (Grassiadei, 1973; Grassiadei & De-Han, 1973; Grassiadei & Metcalf, 1971). These events in the warm-blooded pigeon are faster, but otherwise seem to be quite similar.

Whether there are definite receptor types and by inference labeled addresses to which they must connect in the olfactory bulb is still an unanswered question. The recovery of behavioral function in these experiments indicates that at least a required amount of order was imposed on the reorganization of this system of numerous elements. The methods of physiology and anatomy cannot prove this.

A role of olfaction in natural behavior is suggested by the results of Benvenuti et al. (1973a, 1973b) and Papi, Fiore, Fiaschi, and Benvenuti (1973). Homing pigeons learn to home. They appear to do this by orienting to visual landmarks and to naturally occurring odors which become airborne. Turkey vultures have also been shown to orient to odor plumes (Stager, 1967). The role of olfaction in the mating behavior of the ring dove is yet an unanswered question.
REFERENCES

Audubon, J. J. Account of the habits of the turkey buzzard (Vultur aura) particularly with the view of exploding the opinion generally entertained of its extraordinary power of smelling. Edinburgh New Philosophical Journal, 1826, 2, 172-184.


Metcalfe, J. F. The olfactory epithelium—a model


Tucker, D., & Beidler, L. M. Efferent impulses to nasal area. Federation Proceedings, 1966, 15, 613. (b)


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