Recovery of Olfactory Function After Bilateral Bulbectomy

Abstract. *Mice were trained to discriminate between scented and unscented air. After olfactory bulbs were removed, discrimination was lost, but returned with the formation of synaptic connections between regenerated primary olfactory neurons and the cortex of the forebrain. The acquisition of a second olfactory-mediated task by long-term bulbectomized mice and controls was indistinguishable. The results emphasize the plasticity of the nervous system, correlate the presence of neural connections between olfactory mucosa and forebrain with the recovery of olfactory function, suggest that olfactory-mediated memory resides at least in part outside the olfactory bulbs, and demonstrate that the bulbs are not required for the acquisition of olfactory tasks.*

Unlike other central neurons in adult mammals, the primary olfactory neurons are replaced on a regular basis (*1*). This turnover process is made possible by a stem cell population within the olfactory mucosa which divides and differentiates, and is accelerated in response to physical or chemical injury (*2*). Section of the primary olfactory nerves results in retrograde degeneration of these neurons while the olfactory bulb and epithelium remain intact. Coincident with the sectioning of the primary olfactory nerves in mice (*3*) and pigeons (*4*), olfactory-mediated behaviors and the ability of these neurons to synthesize and transport the neuron-specific chemical marker carnosine are lost (*3*). Olfactory behavior returns within 21 days after the sectioning of the olfactory nerves in mice, however, and recovery of carnosine synthesis and transport parallels behavior (*5*).

Graziadei *et al.* (*6*) have reported that after unilateral ablation of the olfactory bulb, primary neurons undergo retrograde degeneration, which peaks at 8 days. By 30 days, replacement neurons became evident, and in the absence of normal target tissue, their axons migrated through the lamina cribrosa and contacted the protruding forebrain forming "glomeruli-like structures." The number of migrating axons increased between 60 and 120 days, when the experiment was terminated. The possibility of functional recovery of olfactory behaviors after bilateral bulbectomy was not addressed by Graziadei *et al.*, but is the focus of our investigation. Three related questions were tested. (i) What is the time course for the connection of primary olfactory neurons to the forebrain as measured by the transport of carnosine? (ii) Is a previously learned olfactory aversion reestablished after bulbectomy? (iii) Can bulbectomized mice acquire a new olfactory behavior after the newly regenerated sensory system is established? Each question is concerned with function and each is of future medical importance given the possibility that other neural structures can be encouraged to regenerate in a similar fashion following proximal central nervous system damage.

Fifty mature female Swiss Webster mice were randomly divided into two equal groups. Members of the first group were taught an odor aversion to amyl acetate (1:1000 in distilled water) by being placed in a glass bell jar, five at a time, for 5 minutes followed by an intraperitoneal injection (2 percent of body weight) of 0.15*M* lithium chloride, which resulted in malaise that began within 15 minutes and continued for approximately 30 minutes as judged by lethargy (*7*). The animals were immediately returned to the jar after the lithium chloride injection for an additional 25 minutes. The training was repeated on a second day. Members of the second group were trained according to the same procedures, but their aversion was to 1-butanol (1:250 in distilled water).

On two consecutive days after odor aversion training, animals were individually placed in a behavioral test chamber for one 3-minute session per day. The chamber consisted of a 30.5-cm length of 4.4-cm (inside diameter) clear Plexiglas tubing with an odor input port at each end and two exhaust ports in the middle. One 5-liter mixing flask was connected to each input port, and air was pulled through the chamber at 2.4 liter/min by a vacuum applied to the exhaust ports. Group 1 experienced the odor of amyl acetate (500 ml in one flask) through one input port and unscented air (500 ml of distilled water in the other flask) through the second port. Group 2 experienced the odor of 1-butanol versus air. The designation of scented air to input ports was randomized daily, and the Plexiglas chamber was completely submersed in distilled water and dried after the testing of each mouse.

The time spent in each half of the test chamber was visually monitored by the experimenter. Location of the animal, either left or right half of the chamber, was based on the position of the nose. A hand-held silent microswitch fed into one channel of a microcomputer (PET) equipped with a printer (Commodore) and an interphase (Cooper Electronics). The computer accumulated the total time spent on each side of the chamber to the nearest 0.1 second and monitored the 3-minute session duration. At the conclusion of a session, microswitch input was frozen, and the computer alerted the experimenter with a short, low-intensity tone (*8*).

Immediately after these two behavioral test days, the olfactory bulbs of both groups of mice were bilaterally removed under Metofane anesthesia (methoxyflurane; Pitman-Moore). Bilateral trephine holes (1.5-mm carbide burr) were positioned 3 mm rostral to bregma, exposing the bulbs. Each bulb was aspirated with a glass pipette under low suction. Care was taken to avoid forebrain damage, and, if such damage was suspected or incomplete bulb removal was likely, the mouse was discarded and replaced. After bulb removal the incision was packed with Gelfoam and closed. The efficiency of surgery and the presence of connections between primary fibers and forebrain were monitored at the light microscope level in mice chosen at random at various times throughout the study.

An additional 50 mature female mice were bilaterally bulbectomized and studied to determine the magnitude of olfactory neuron connections to the forebrain

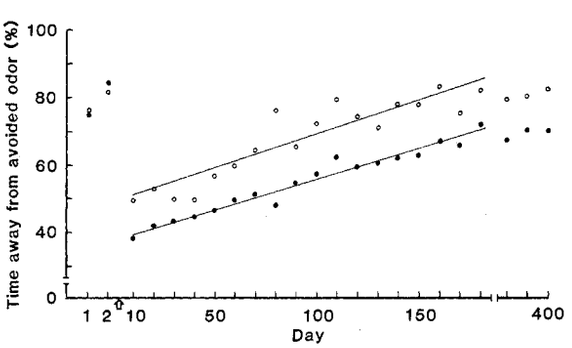

Fig. 1. Mean percentage of time spent away from avoided odor before and after bilateral bulbectomy in two groups of mice trained to avoid either amyl acetate (●) or 1-butanol (○). The arrow indicates the time of surgery. The range of standard deviations for the amyl acetate group was 3.9 to 18.5 percent and, for the 1-butanol group, 6.5 to 17.6 percent. Each line is a least-squares line of best fit for days 10 to 180. Amyl acetate: $Y = 0.175X + 37.59$, $r = +.97$; 1-butanol: $Y = 0.194X + 48.64$, $r = +.90$.

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by the measurement of carnosine transport. Carnosine (β-alanyl-L-histidine) is a specific marker for olfactory neurons (*9*). The procedure entailed the in vivo labeling of the physiological pool of carnosine by the intranasal application of tritiated β-alanine (28.0 mCi/m*M*), a carnosine precursor, through the external nares (*5*). These mice were killed by cervical dislocation 17 hours later; the epithelium and forebrain were dissected on ice, weighed, and individually homogenized with 2 ml of 70 percent ethanol. Supernatant (1 ml) was applied to a 0.5 by 2.0 cm column of AG 50 × 8 (200 to 400 mesh) in the α-picoline form (*10, 11*). The column was eluted with water (4 ml), 0.1*M* α-picoline (4 ml), and 1*M* NH_4OH (4 ml). Carnosine elutes in the 1*M* NH_4OH fraction, while unreacted β-alanine was eluted in the α-picoline fraction. Visual inspection of the forebrain region of mice taken for biochemical samples indicated less than 5 percent of the animals possessed any remnants of bulbar tissue.

The results of behavioral testing are presented in Fig. 1. The two test sessions before bulbectomy indicated that those animals with trained aversions to amyl acetate spent 81 percent of their time away from the odor side of the chamber, while the 1-butanol group spent 78 percent of their time away from the odor. After bilateral bulbectomy, members of both groups crossed the chamber more often and spent approximately equivalent time in each half. During the period after surgery the animals began to recover the odor aversion. By day 100 after surgery for the 1-butanol group and day 150 for the amyl acetate group time spent away from the odor side was not significantly different from that on the 2 days before surgery [paired $t(11) = 0.19$ and $t(13) = 1.64$, respectively, $P > .10$].

Specificity of the odor aversions was tested 190 and 400 days after surgery by reversing the scented air; the group with the aversion to amyl acetate was tested with 1-butanol paired with nonscented air, and the group with the aversion to 1-butanol was tested with amyl acetate. The results indicated each group spent approximately equal time in each half of the chamber, which suggests no generalization of the conditioned odor aversion to an unfamiliar odor.

To further test for functional recovery, beginning 410 days after surgery, each mouse from the behavioral test groups was trained to locate buried food pellets. This behavioral task has previously been utilized in our laboratory and described in earlier reports (*5, 12*). Their performance was compared with a group of intact mice of equivalent age. The results indicated that the bulbectomized mice could acquire the pellet-finding task at a rate that did not differ statistically from that of the control group [$t(38) = 0.54$, $P > .10$]. Half of each of these groups was then intranasally irrigated with 0.2 ml of 0.17*M* zinc sulfate, the other half with an equal volume of normal saline. The application of zinc sulfate destroys the olfactory neuroepithelium by coagulation necrosis; the effect lasts for at least 6 weeks in mice (*11*). After a 4-day recovery period, all animals were again tested on the food-pellet task for six consecutive days (two 3-minute trials per day). The animals treated with zinc sulfate did not locate the pellet during any of the trials. The saline-irrigated control animals were unaffected and demonstrated a continued ability to locate the buried pellet (Mann-Whitney *U* tests, $n_1 = 8$, $n_2 = 12$, $U = 0$, $P < .001$; $n_1 = 14$, $n_2 = 14$, $U = 0$, $P < .001$).

Table 1. Tritiated carnosine contents (in counts per minute per animal) of epithelium and forebrain after intranasal irrigation with tritiated β-alanine, for each group of eight mice.

Time (after bulbectomy) (days)	*N*	Epithelium ($\bar{X}$ ± S.E.M.)	Olfactory bulb ($\bar{X}$ ± S.E.M.)	Forebrain ($\bar{X}$ ± S.E.M.)
Control	8	13211 ± 763	11526 ± 507	350 ± 53
5	8	2391 ± 218		370 ± 36
35	8	1598 ± 104		355 ± 51
75	8	3109 ± 263		945 ± 60
410	8	3698 ± 248		1198 ± 65
425 ($ZnSO_4$)	8	237 ± 42		314 ± 53

The tritiated carnosine content of olfactory epithelium, olfactory bulb, and forebrain are presented in Table 1. The control subjects revealed significant content of carnosine in the olfactory epithelium and bulb, with little labeling of the forebrain. After bilateral bulbectomy the incorporation of tritiated β-alanine into carnosine decreased in the epithelium, reaching a minimum 35 days after surgery [$F(4, 35) = 147.46$, $P < .0001$]. However, 75 and 410 days after surgery the concentrations of carnosine in epithelium were significantly higher (Newman-Keuls tests, $P < .05$). Forebrain concentrations showed a similar pattern [$F(4, 35) = 55.24$, $P < .0001$]. The apparent synthesis and transport of carnosine in long-term bulbectomized mice was abolished after zinc sulfate intranasal irrigation.

Our results extend the knowledge of the regenerated olfactory system in several ways. We believe that this is the first demonstration that synaptic contacts between the frontal cortex and newly differentiated neurons from the basal stem cell population are behaviorally functional in bulbectomized animals. This return of olfactory function occurred in the absence of the normal secondary neurons, the mitral cells, and multiple influences from several classes of interneurons located in the olfactory bulb. The fact that an odor aversion task acquired before bulbectomy subsequently reappeared afterward suggests that olfactory memory storage occurs in structures other than, or in addition to, the olfactory bulbs. Also, a new olfactory behavior, in this case in the form of a pellet-finding task, can be acquired by previously bulbectomized mice. The results of the carnosine transport studies indicate that maximum innervation of the forebrain occurs by day 75 or earlier, and suggest that this is a prerequisite for behavioral recovery. Finally, zinc sulfate treatment disrupted the pellet-finding task in bulbectomized animals as well as in intact control mice, which suggests a dependency of this behavior on the regenerated primary olfactory pathway, thus excluding other sensory influences.

The findings by Graziadei *et al.* (*6*) coupled with our data, indicate that considerable functional glomerularization occurs by 120 days after bulbectomy. Unpublished autoradiographic studies from our laboratory using the location of transported [^{3}H]carnosine as a marker indicate that the field of innervation of these neurons is on the ventral portion of the forebrain on the midline (*13*). Behavioral work can thus be initiated at 120 days with confidence concerning the integrity of the system. Subsequent research efforts should be devoted to functional organization on the forebrain and to behavioral specificity, that is, to whether the regenerated olfactory system has the same sensitivity and range of responsiveness to odor stimuli as the nondamaged system.

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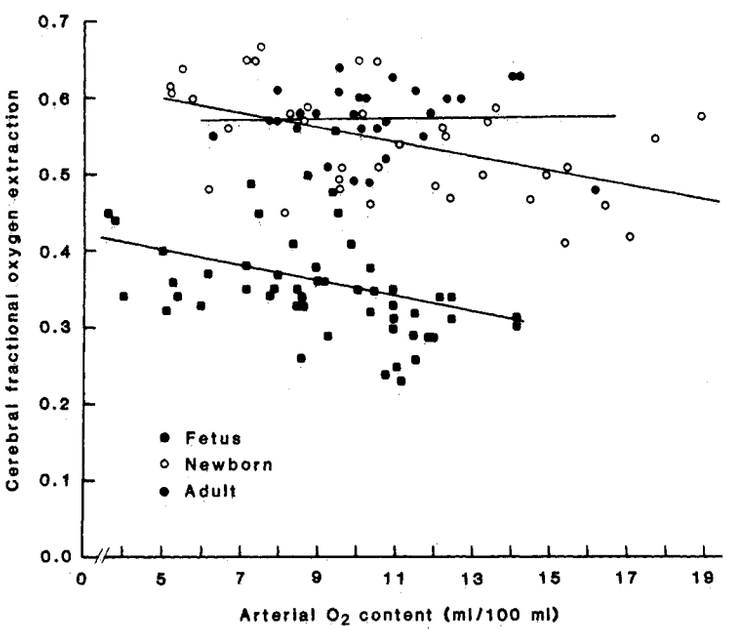
Oxygen Delivery to the Brain Before and After Birth

Abstract. *We studied the relationship between cerebral oxygen consumption and cerebral oxygen delivery (cerebral blood flow × arterial oxygen content) in fetal, newborn, and adult sheep. Relative to the amount of oxygen consumed, cerebral oxygen delivery in the fetus exceeds that in the lamb and adult by 70 percent. This may represent a protective advantage for the fetus or simply a necessary adaptation to the low arterial oxygen pressure in the intrauterine environment.*

A number of physiologic differences distinguish intrauterine from postnatal life. At birth, arterial PO_2 and blood pressure rise, while arterial PCO_2 falls (*1*). Blood pressure continues to rise to adult values. Each of these variables can affect cerebral blood flow (CBF) (*2*), and thus the supply of oxygen and metabolic substrates to the brain. Meanwhile, cerebral O_2 consumption (per gram of brain) rises after birth, then falls with maturation (*2, 3*). As development proceeds, the net result for the quantitative relationship between the brain's requirement for metabolic substrates, on the one hand, and their delivery by arterial blood, on the other, is unknown. In this report we examine only one aspect of this issue: developmental changes in the relationship between cerebral O_2 consumption and cerebral O_2 delivery.

We studied eight fetal sheep in utero at 125 to 135 days of gestation (0.86 to 0.93 of term), nine newborn lambs at 4 to 8 days of age, and five adult sheep. We placed catheters in the brachiocephalic artery and superior sagittal sinus while subjects were anesthetized (*4, 5*). Between 1 and 4 days after surgery, we made four to ten paired measurements of O_2 content in arterial and sagittal sinus blood. In order to compare subjects over a range of arterial O_2 content, the inspired O_2 concentration was varied from 6 to 25 percent by established techniques (*4, 5*). Changes in arterial CO_2 tension were prevented by appropriate modifications of the inspired gas mixture. We measured CBF twice in each animal with the radioactive microsphere technique (*4, 5*). The CBF (milliliters per 100 g per minute) represents flow to all cerebral tissue anterior to the cephalic border of the pons. Cerebral oxygen consumption was calculated according to the Fick principle, by multiplying CBF by the cerebral arteriovenous O_2 difference.

The relationship between cerebral metabolic rate for O_2 ($CMRO_2$) and the total amount of O_2 available to the brain is given by the ratio of $CMRO_2$ (CBF × cerebral arteriovenous O_2 difference) to cerebral O_2 delivery (CBF × arterial O_2 content). This represents the fraction of available O_2 that the



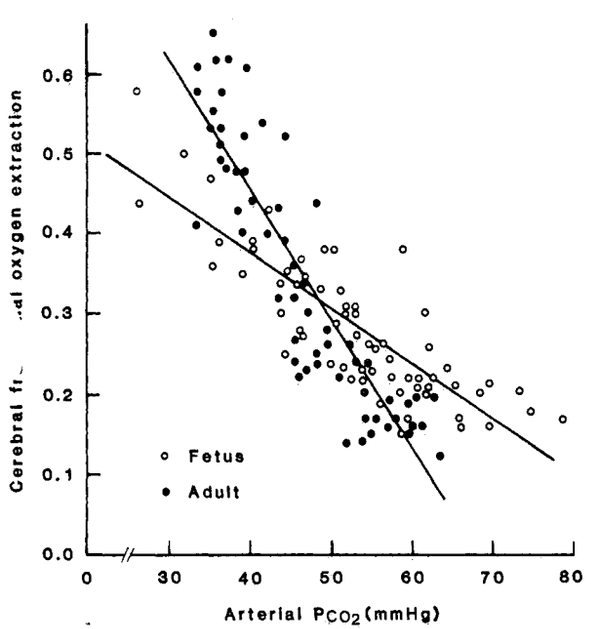


Fig. 1 (left). Relation of cerebral fractional oxygen extraction to arterial O_2 content (milliliters per 100 ml) in fetuses, lambs, and adults as the inspired O_2 concentration was changed. Fetus: $y = -0.01x + 0.45$, $r = -.40$, $P < .01$; lamb: $y = -0.009x + 0.65$, $r = -.49$, $P < .01$; adult: $y = 0.0004x + 0.57$, $r = .02$. Fig. 2 (right). Relation of cerebral fractional oxygen extraction to arterial PCO_2 (mmHg) in fetuses and adults as the inspired CO_2 concentration was changed. Fetus: $y = -0.0068x + .64$, $r = -.84$, $P < .01$; adult: $y = -.0164x + 1.11$, $r = .90$, $P < .01$. The regression coefficients differed significantly ($P < .05$) when compared by a two-tailed t-test for independent means [$t(118) = 8.1$, $P < .05$].

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