Chem. Senses 28: 659–670, 2003 DOI: 10.1093/chemse/bjg053

Does Intranasal Application of Zinc Sulfate Produce Anosmia in the Mouse? An Olfactometric and Anatomical Study

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Abstract

Mice pre-trained in an olfactometer were tested daily on odor detection and discrimination tasks after irrigation of their olfactory epithelium in each naris with 50 μl of 5% zinc sulfate or saline. Anterograde transport of a wheatgerm agglutinin–horseradish peroxidase (WGA-HRP) conjugate from the epithelium to the olfactory bulb was used to assess anatomical connectivity in these and in mice that were used only for histological analyses. One day after treatment, saline controls performed at high levels of accuracy in detecting vapor from solutions of 5–0.01% ethyl acetate and in an odor discrimination task but most $ZnSO_4$-treated mice performed at chance for 5–30 days before showing recovery. Although dense WGA-HRP reaction product was found in the accessory olfactory bulb, there was little or no evidence for axonal transport to glomeruli of the main olfactory bulb in the first 4–8 days after treatment. These results demonstrate that intranasal application of $ZnSO_4$ to mice produces a brief but essentially total disruption of functional connections from the olfactory epithelium to the main olfactory bulb and a corresponding transient anosmia.

Key words: anosmia, axoplasmic transport, mice, odor detection, olfaction, zinc sulfate

Introduction

Subsequent to an initial report by Alberts and Galef (1971), intranasal application of zinc sulfate has been used in a variety of species to destroy the olfactory epithelium and produce anosmia. Indeed, the effectiveness of this method has been so widely accepted that, in some cases, anosmia has been assumed even in the absence of a behavior test for odor detection (Alberts and Galef, 1973; Winans and Powers, 1977; Andine *et al.*, 1995). Nevertheless, there is considerable evidence that this treatment may be far less effective than generally assumed. Anatomical studies with rats and hamsters reveal intranasal application of $ZnSO_4$ may not destroy the entire olfactory epithelium (Winans and Powers, 1977; Johnston, 1992; Slotnick *et al.*, 2000). In behavioral studies, many treated rats and hamsters were clearly not anosmic, and olfactometric studies revealed considerable savings in odor detection and discrimination ability within a few days after treatment with even relatively large volumes of $ZnSO_4$ (Slotnick and Gutman, 1977; Slotnick *et al.*, 2000). These outcomes stand in contrast to many reports in rodents in which similar treatments were claimed to produce a frank but short-lived anosmia (review in Slotnick *et al.*, 2000).

Although similar coordinated anatomical and behavioral studies have not been performed with mice, there are reasons to believe that $ZnSO_4$ treatment may be more effective in the mouse. A number of reports indicate that $ZnSO_4$ produces severe degenerative changes in essentially all of the olfactory epithelium in mice (Matulionis, 1975a,b; Harding *et al.*, 1978; Burd, 1993) and in studies using simple food-finding and similar tests (summarized in Table 1), treated mice have been reported to be anosmic over many days or weeks (e.g. Harding *et al.*, 1978; Drickamer, 1986; Porter *et al.*, 1987). Given the current and rapid advances in the molecular biology of olfaction in the mouse, it becomes increasingly important to develop methods to experimentally manipulate the olfactory system. Intranasal lavage with $ZnSO_4$ may prove useful to this end if it produces a reliable disruption of the olfactory system and anosmia. Unfortunately, most behavioral studies of $ZnSO_4$-induced anosmia in mice have not evaluated the effects of treatment on the olfactory epithelium and the most detailed anatomical studies have not evaluated behavior or have used relatively crude behavioral tests.

Table 1 Behavioral studies employing intranasal irrigation with $ZnSO_4$ in mice to disrupt olfaction

$ZnSO_4$ volume, and conc.	Claim	Test	Duration	Notes	References
Mouse					
0.1 ml, 5%	anosmia	none reported	4 weeks	28% deaths, shrunken olfactory bulbs	Andine *et al.* (1995)
0.05 ml, 5%	anosmia	food-finding task	1 week	not all mice were anosmic	Archunan and Dominic (1990)
0.2 ml, 5%	disruption	odor preference	9 days	did not block ultrasonic calls	Bean (1982b)
0.2ml, 5%	anosmia	odor preference	3 days	treatment did not block aggression	Bean (1982a)
0.1 ml, 5%	anosmia	food-finding task	Weeks	epithelium examined	Burd (1993)
0.015 ml, 5%	anosmia	odor preference	Unclear	illness noted, some deaths	Crusio and van Ableelen (1987)
0.025 ml, 5%	anosmia	odor preference	15 days	some deaths	Drickamer (1986)
0.0025 ml, 4%	anosmia	food-finding task	–	14% were not anosmic	Edwards and Burdge (1973)
0.001–0.002 ml, 5%	anosmia	food-finding task	7–21 days	37% were not anosmic	Edwards *et al.* (1972)
0.05 ml, 5%, multiple treatments	anosmia	food-finding task	5+ days	some mice were not anosmic	Gangrade and Dominic (1983)
0.5 ml, 0.01–1%	anosmia	food-finding task	1 day	normal response to trigeminal stimuli. 0.01% $ZnSO_4$ was ineffective	Hansen *et al.* (1994)
0.05–0.1 ml, 5%	anosmia	food-finding task	6 weeks	little or no effect in 20% of treated mice	Harding *et al.* (1978)
0.1 ml, 1%	somewhat impaired	odor-cued taste avoidance	1–5 days	treatment did not eliminate odor detection	Kimura *et al.* (1991)
0.02 ml, 5%	anosmia	food-finding task	1 day	25% were not anosmic	Maruniak *et al.* (1975)
Spiny mouse 0.02–0.03 ml, 5%	anosmia	food-finding task	1 day	blocked preference for cage mate	Matochik (1988)
Spiny mouse 0.01–0.02 ml, 5%	anosmia	food-finding task	26 days	neonatal mice, growth retarded	Porter *et al.* (1987)
Not reported, 5%	anosmia	odor preference	<10 days	failed to abolish mating in males	Rowe and Smith (1973)
0.015 ml, 5%	anosmia	food-finding task	3 days	13% deaths	Schoots *et al.* (1978)
0.05 ml, 3.5%	anosmia	odor preference	4 days	postpartum infanticide	Seegal and Deneberg (1974)
0.003 ml, 4%	hyposmia	food-finding task	5 days	reduced vocalization to female urine	Sipos *et al.* (1995)
0.05 ml, 7.65%	anosmia	response to odor on swab	7 days	69/86 judged anosmic	Tsuchiya *et al.* (1991)
0.2 ml, 5%	anosmia	odor preference	7 weeks	irregular estrous cycles and reduced pregnancy rate	Vandenbergh (1973)

The purpose of the present study was to re-evaluate the effectiveness of intranasal $ZnSO_4$ treatment in producing anosmia in the mouse. To accomplish this we have replicated in the mouse the anatomical and behavioral methods used in the prior study with rats by Slotnick *et al.* (2000).

Materials and methods

Subjects

Forty 18–22 g female Swiss–Webster strain mice purchased from the National Cancer Institute, Bethesda, MD, were housed in groups of four or five in $57 \times 37 \times 35$ cm plastic cages. Mouse chow (Agway) was provided *ad libitum* and all animals were maintained on a water deprivation schedule of three minutes of water access each day. Housing and experimental procedures followed NIH guidelines and were approved by the American University Animal Care Committee.

Olfactometers

Four identical eight-channel Knosys olfactometers (htpp://chemsenses.com) similar to the multiple-channel units described by Bodyak and Slotnick (1999) were used (Figure 1). Odors were generated in 50 ml vertically oriented plastic centrifuge tubes containing 10 ml of odorant material. The tube caps were drilled to accommodate two 4 mm outside

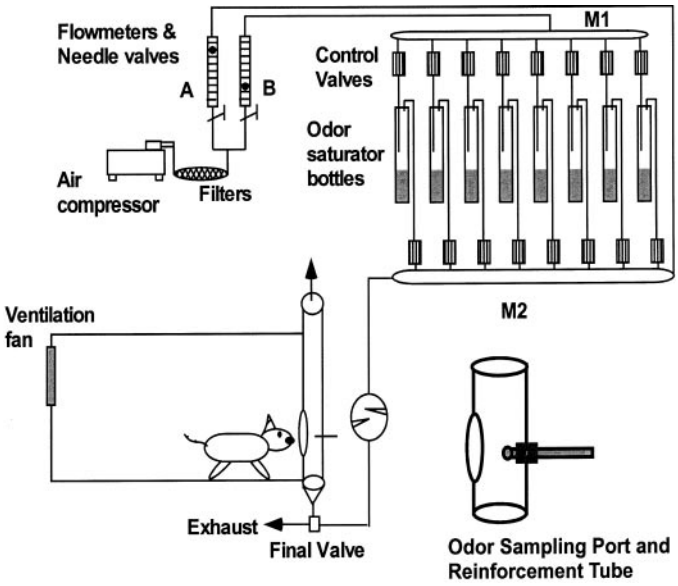

Figure 1 Diagrammatic representation of the odor generator and operant chamber. Carrier flow to the operant chamber was regulated and monitored by needle valve and flowmeter **(A)** while flows for generating odors were regulated and monitored by needle valve and flowmeter **(B)**. As described in the text, the solenoids were normally closed pinch valves that controlled C-flex tubing connectors of the odor saturator bottles.

diameter C-flex tubes. Normally closed pinch valves controlled the air inlet and outlet lines for each odor channel. Operation of the control valves produced a 50 ml/min air stream over the surface of the odorant material. This stream was manifolded to a 1950 ml/min stream of clean carrier air that was connected to the animal sampling port via the common and normally open ports of a three-way pinch valve (Figure 1, final valve).

Odors were diluted in deionized water and concentrations given below are those of the liquid odorant in the saturator tube. Because the 50 ml/min odor stream from the saturator tube was manifolded with the 1950 ml/min stream of clean air before being introduced to the sampling port, the concentration of odor stimuli experienced by the mouse at the sampling port was ~2.5% of the concentration of the headspace above the liquid odorant.

The test chamber was similar to that described by Bodyak and Slotnick (1999). However, in this study, the water delivery tube was located within the odor sampling tube (Figure 1). Licks on the water delivery tube were detected by a touch-sensitive circuit (Field and Slotnick, 1987). All training and testing procedures were automated and controlled by personal computers via digital interfaces. Control programs were written in QBASIC.

Behavior procedures

Initial training

Prior to treatment, mice were trained using standard operant procedures to insert their snouts into the odor sampling port and lick the reinforcement tube for 0.002 ml of water. Once reliable responding was obtained, a discrete trials procedure was initiated in which a snout insertion produced a 2.5 s sample from a 5% solution of ethyl acetate (EA). Reinforcement was contingent upon the mouse keeping its snout in the odor sampling tube and responding on the water delivery tube in the last 2 s of stimulus presentation (response interval). A criterion response was defined as detecting a lick response in each 0.5 s period of this 2 s response interval. Completion of a trial initiated a 5 s intertrial interval.

The last stage of initial training introduced the final valve. At the beginning of a trial, both the final valve and the stimulus control valves operated. This resulted in switching the airflow from the odor sampling port to an exhaust line. When the final valve relaxed, airflow containing the odor stimulus was presented to the sampling port. The odor control valves were closed 2.5 s later. Reinforcement was contingent upon the mouse keeping its snout in the odor sampling port during the final valve period and then meeting the response criterion in the presence of the stimulus. In this session, final valve operation time was increased over 150 trials from 0.1 to 1.2 s.

Odor detection and discrimination tasks

Upon completion of initial training, mice were trained on an odor detection task. Five percent EA served as the S+ stimulus and water served as the S– stimulus. S+ and S– trials were presented in a random order with the restriction that one type of trial was not presented more than three times consecutively and that there were an equal number of S+ and S– presentations in each block of 20 trials. Meeting the response criterion on S+ trials was scored as a hit and not meeting this criterion on S– trials was scored as a correct rejection. Not meeting the response criterion on S+ trials was scored as a miss and meeting the criterion on S– trials was scored as a false alarm. Misses and false alarms constituted errors and were used in calculating percent correct responding in each block of 20 trials. Each mouse was trained to a criterion of 90% correct responding on this task and then given 200 additional (over training) trials. Mice were then trained to criterion in separate sessions using 1, 0.1 and 0.01% aqueous concentrations of EA as the S+ stimulus and water as the S– stimulus.

Next, each mouse was trained to criterion on a two-odor discrimination. The S+ stimulus was a 50% aqueous dilution of Johnson & Johnson cinnamon-flavored mouthwash, and the S– stimulus was a 25% aqueous dilution of Johnson & Johnson bubble gum flavored mouthwash. The concentrations of these odors were judged to be approximately equally intense by human observers.

Finally, each mouse was given a 60- or 100-trial test on a control task in which water served as both the S+ and S– stimuli. All mice performed at chance (40–65% correct responding) on this test.

Post-zinc sulfate behavioral testing

Behavioral tests were resumed 1 day after treatment with $ZnSO_4$ or saline. In each test, mice were given 100 trials in which 5% EA served as the S+ stimulus and water served as the S– stimulus. On the day in which response accuracy of 90% in a block of 20 trials was achieved, mice were also tested on the 1% EA detection task and, providing they achieved criterion performance, on the lower EA concentrations. Regardless of performance on the EA detection tasks, each mouse was also tested on the two-odor discrimination task. Except for two mice that were tested every third day, mice were tested on the 5% EA task each day after treatment. As described below, mice were administered wheatgerm agglutinin–horseradish peroxidase conjugate (WGA-HRP) on the day they met the 90% accuracy criterion on the 5% EA detection task.

Zinc sulfate application

One or two days after completing pretreatment training, anesthetized mice received intranasal administration of 5% $ZnSO_4$ (Sigma) or saline using a modification of the method described by Harding *et al.* (1978). Five minutes after applying one or two drops of 2% Xylocaine (Astra) to the external nares, the mouse was placed in a conical holder that exposed the snout. Each nostril was injected with 50 µl of a sterile 5% solution of $ZnS0_4$ (n = 17) or with 50 ul of saline (n = 2). The injections were made by forcefully expelling the contents of a syringe through a blunted 4 mm long 25G needle inserted ~3 mm into the naris. The first injection was made into the right naris. The left naris was injected 2 h later. Immediately after intranasal $ZnSO_4$ irrigation, the mouse was held with its head down for several seconds to minimize spread of the solution to the oral cavity. Two additional control mice received no injections.

One or two hours after completing the post-treatment behavior tests, each naris was injected with 10 µl of an equal parts mixture of 1% WGA-HRP (E-Y Labs) and 1% DMSO (Sigma). Twenty-four hours later mice were deeply anesthetized and killed by perfusion with saline followed by mixed aldehydes according to the method of Mesulam (1978).

Fifty-micron-thick frozen sections through the olfactory bulbs were cut and every other section was processed with TMB and H_2O_2 using the method of Mesulam (1978) to demonstrate anterograde transport of WGA-HRP reaction product in olfactory bulb glomeruli. Sections were mounted on gelatin-coated slides, lightly stained with thionin, dehydrated through cold alcohols, cleared in xylene and coverslipped using Permount.

Each section was photocopied at 40× using a Bell and Howell microfiche reader/printer and examined microscopically at 40× and 400× using both brightfield and polarized light optics by one of the authors (KM) who was blind with regard to the behavioral results. The reaction product in each glomerulus was rated on a three-point scale. A score of zero was assigned if no reaction product could be detected in the glomerulus. A score of 1 was assigned if the glomerulus contained only a light or moderate sprinkling of reaction product and a score of 2 was assigned if it contained dense reaction product that could be easily discerned in bright field and was characteristic of that found in control mice. The scores of identified glomeruli were noted on the corresponding microfiche photocopies. The anatomical results of each case was summarized by plotting glomerular regions containing reaction product on a standard set of eight representative frontal sections through the mouse olfactory bulbs.

Anatomy-only mice

Nineteen mice maintained on *ad libitum* food and water were treated with 50 µl of saline (n = 4) or $ZnSO_4$ as described above and 1 h (n = 1), 6 h (n = 1), 4 days (each saline-treated and four $ZnSO_4$-treated mice), 8 days (n = 4), 12 days (n = 3) or 16 days (n = 4) later were treated with WGA-HRP, and killed by perfusion 24 h later. The olfactory bulbs were processed as described above. For both the behavior tested and anatomy-only mice, survival time is defined by the number of days between $ZnSO_4$ treatment and WGA-HRP application.

Results

Anatomy-only mice

All glomeruli in the four control mice were filled with dense or moderately dense reaction product (Figure 2). In contrast, many glomeruli in the main olfactory bulb in $ZnSO_4$-treated mice had no detectable reaction product or only very light reaction product. The number of glomeruli with any reaction product varied with survival time: no reaction product was detected in any glomeruli of the 1 h and 6 h mice while the 4, 8, 12 and 16 day survival mice had, on average, 6, 17.5, 24.7 and 64.3% glomeruli with detectable reaction product. However, only very light reaction product was observed in the 4, 8 and 12 day survival mice (e.g. Figure 3) while many glomeruli in the 16 day survival mice contained dense reaction product (Figure 4 and Table 2). Although, as inspection of Table 2 shows, there was variability in the number of glomeruli with reaction product within each of these survival groups, the Pearson product moment correlation between the glomerular score and survival time across all anatomy-only mice was 0.80. The correlation between the mean glomerular scores for the 4, 8, 12 and 16 day groups, with survival time was higher (0.93). Thus, sometime between 1 and 16 days after $ZnSO_4$ treatment there was gradual but considerable recovery of input from the olfactory epithelium as indexed by anterograde transport of WGA-HRP to olfactory bulb glomeruli. As shown in Figure 3, very light reaction product in many glomeruli was not evident with brightfield optics but could be seen clearly with polarized light. Interestingly, in virtually all cases, dense reaction product was observed in the accessory olfactory bulb (Figure 5).

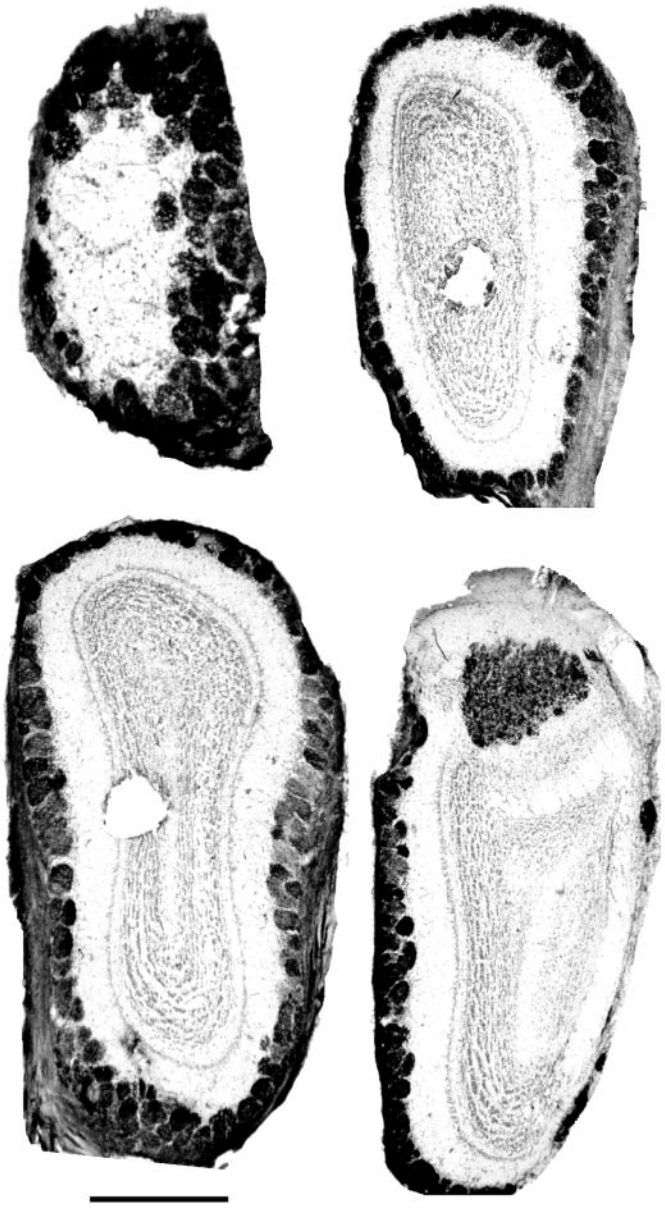

Figure 2 Photomicrographs of frontal sections through the olfactory bulb of a control mouse showing dense HRP-WGA reaction product in the olfactory nerve layer and glomeruli. Note dense reaction product in glomeruli of the accessory olfactory bulb (lower right section). In each section, dorsal is to the top and medial is to the left. Scale bar represents 1 mm.

Figure 4 shows diagrammatically the location of glomeruli with light, moderate and dense reaction product in selected mice of the 4, 8, 12 and 16 day survival groups, and in most of these cases at least some glomeruli in the anterior ventral bulb contained reaction. Reaction product was also found on the medial wall of the bulb in two of the four 4 day and each of the 8, 12 and 16 day mice. All but three mice (two in the 4 day group and one in the 8 day group) had moderate or dense reaction in essentially all glomeruli of the accessory olfactory bulb.

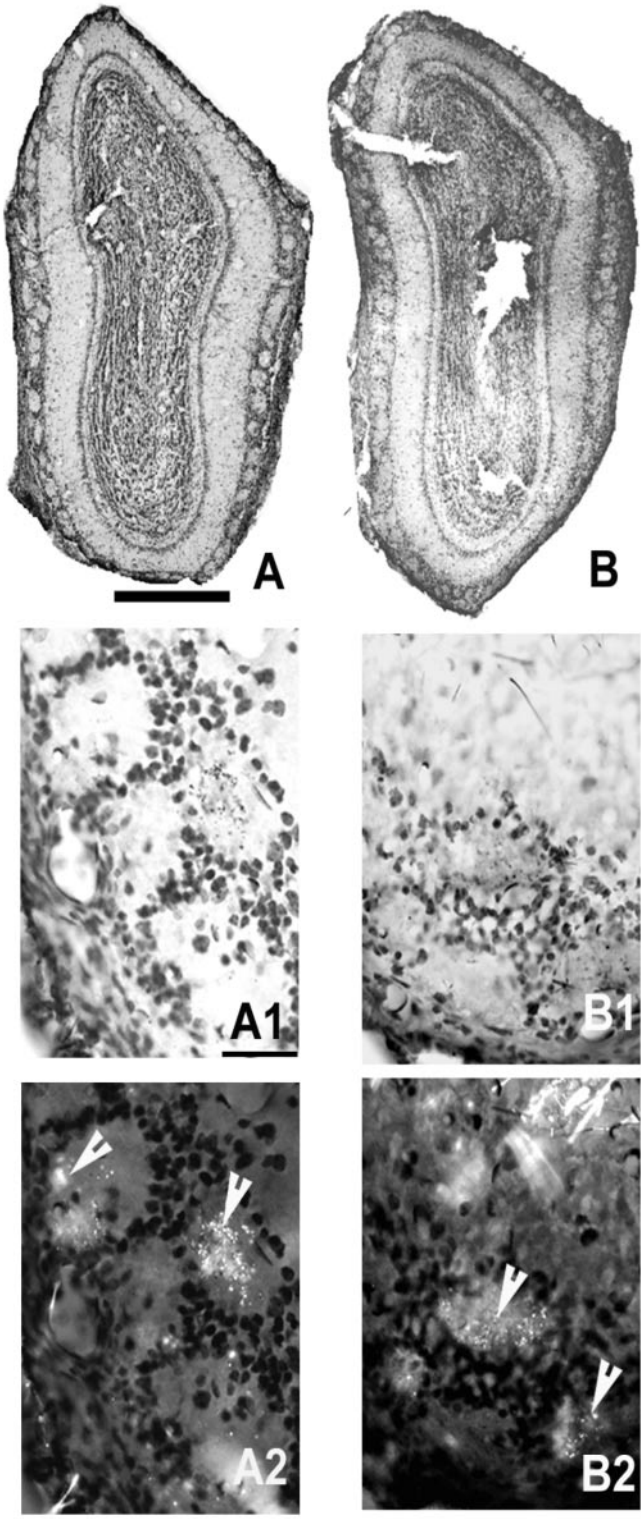

Figure 3 Photomicrographs through a frontal section of olfactory bulbs of $ZnSO_4$-treated 4 day survival **(A)** and 12 day survival mice **(B)**. No glomeruli in these mice contained dense reaction product. Light reaction product within glomeruli was not easily discernible in bright field at higher magnification (A1, B1) but was visible using polarized light optics (arrows in A2, B2). In sections (A) and (B) dorsal is to the top and medial is to the left. Scale bar under (A) represents 1 mm for (A, B). Scale bar under (A1) represents 50 μm for (A1, A2, B1, B2).

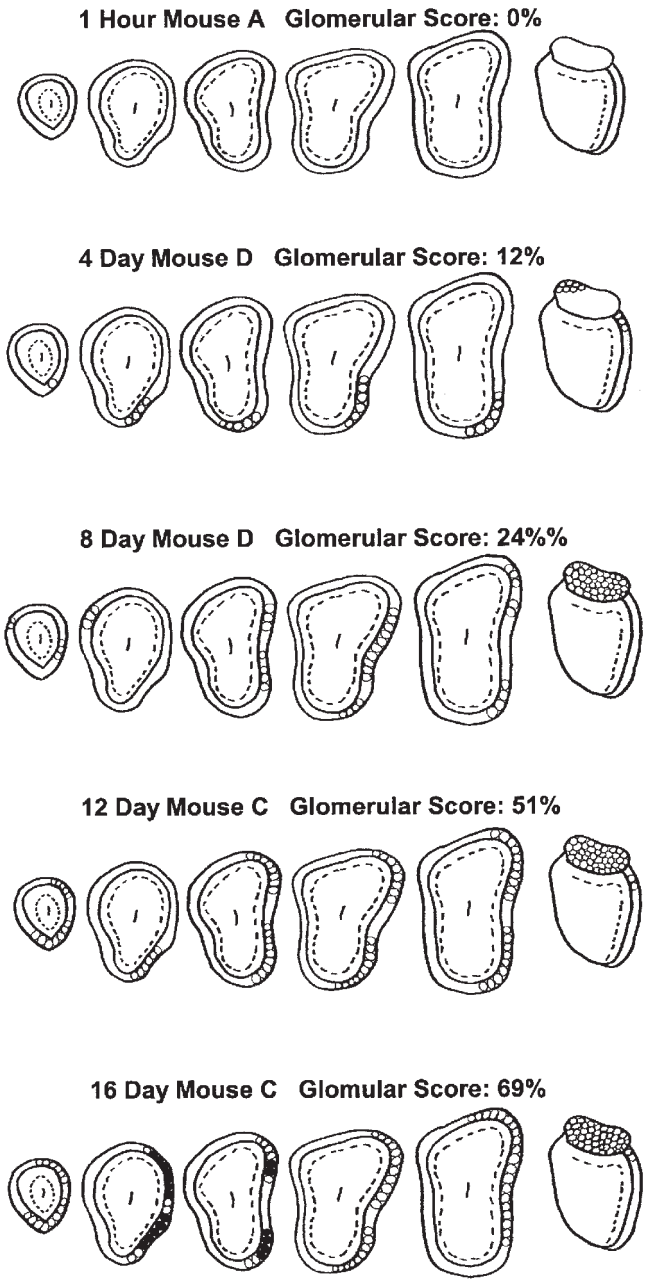

Figure 4 Diagrammatic representations of six frontal sections through the olfactory bulb showing the position of glomeruli containing light to moderate reaction product (open circles) and dense reaction product (solid circles) in representative anatomy-only mice with survival times of 1 h, 4, 8, 12 and 16 days. In each section dorsal is to the top and medial is to the right.

Table 2 Survival time, glomeruli counted and percent of glomeruli with reaction product

Survival time and mouse no.	Total no. of glomeruli observed	Mean no. of glomeruli/ section	% Glomeruli with reaction product		
			Dense reaction product	Light reaction product	With any reaction product
1 h	886	59	0	0	0
6 h	972	65	0	0	0
4 day A	677	45	0	3	3
4 day B	2708	54	0	1	1
4 day C	1181	49	0	8	8
4 day D	1288	52	0	12	12
Mean	1463.5	50.0	0.0	6.0	6.0
8 day A	1377	69	0	5	5
8 day B	1234	59	0	17	17
8 day C	1507	52	0	23	23
8 day D	1912	50	0	24	24
Mean	1507.5	57.5	0.0	17.0	17.0
12 day A	1825	54	0	6	6
12 day C	1656	59	0	51	51
12 day D	2113	53	0	17	17
Mean	1864.7	55.3	0.0	25.0	25.0
16 day A	2008	57	15	23	38
16 day B	399	26	7	43	50
16 day C	1529	42	12	57	69
16 day D	2809	72	99	1	100
Mean	1312.0	49.3	11.3	41.0	52.3

Behavioral results

Pretreatment performance

Acquisition of the pretreatment tasks was rapid: criterion performance of 90% correct responding was achieved in 20–60 trials on the 5, 1, 0.1 and 0.01% EA tasks. This high level of performance on the EA series probably reflects the fact that even the lowest concentration used (0.01%) was well above EA detection threshold for the mouse (Bodyak and Slotnick, 1999). The two-odor discrimination task was also acquired with few errors.

Post-treatment performance

Except for two mice (Z9 and Z11) that were tested every third day, mice were tested on the 5% EA detection task each day after $ZnSO_4$ treatment. The four control mice performed at the 90% criterion level on the first block of trials on each task on their first post-treatment day. However, most $ZnSO_4$-treated mice performed at chance on the 5% EA detection task for at least the first 4 days after treatment. Behavior scores of each mouse are given in Table 3. Figure 6 shows the post-treatment performance and diagrammatic representations of glomerular input of one control and three experimental mice.

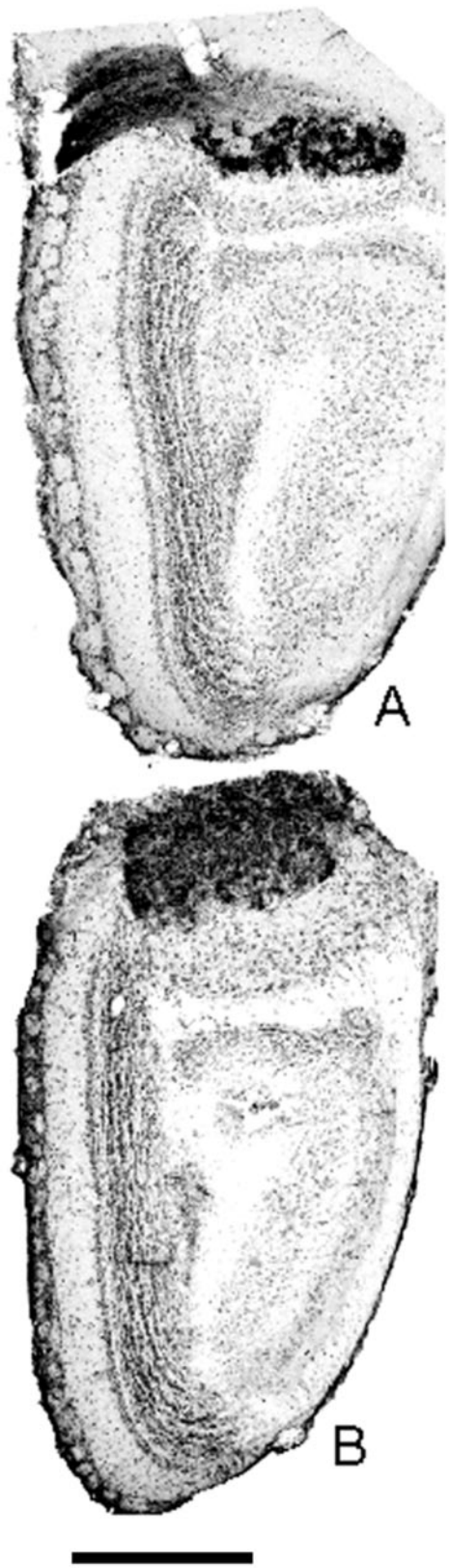

Figure 5 Photomicrographs of frontal olfactory bulb sections from $ZnSO_4$-treated mice. In both the 6 h survival and 8 day survival cases illustrated only glomeruli in the accessory olfactory bulb contained dense reaction product. Scale bar represents 1 mm.

As shown in Table 3, mice achieved 75% accuracy on at least two blocks of trials on the 5% EA detection task, on average, within 8 days after treatment. Seven of these mice also achieved 90% accuracy on the 5% EA detection task on the same day they reached the 75% accuracy level. The remaining 10 mice took 2–12 additional days before reaching the 90% criterion on this task (Table 3).

On the day that a mouse achieved the 90% criterion level on the 5% EA task it was tested on lower concentrations of EA and on the two-odor discrimination problem. Two of these 17 mice failed to reach criterion on the 1% EA detection task. Of the remaining 15 mice, only five achieved criterion on the 0.1% EA detection task and four of these five mice also achieved criterion on the 0.01% EA detection task (Table 3). Twelve of the 17 experimental mice achieved criterion performance on the two-odor discrimination task within a 200 trial session.

Mice that recovered rapidly did not perform better on their recovery day than those that recovered more slowly: product moment correlations between the number of days to achieve accuracy scores of 75% and 90% on the 5% EA problem and the number of errors made in achieving those criteria on that day was 0.12 and –0.17, respectively.

Anterograde transport in behavior-tested mice

WGA-HRP was administered to experimental mice on the day in which they achieved the 90% criterion on the 5% EA detection task. On average, 32% (range 5–85%) of glomeruli in these mice contained detectable reaction product and 22% of these glomeruli were filled with dense reaction product (range, 0–82%). Reaction product tended to occur in several clusters of glomeruli in different regions of the bulb that were separated by areas that contained no detectable reaction product. The pattern of inputs in these cases differed somewhat from that found in the anatomy-only mice. Thus, only 11 of the 16 behavior mice for which histology was available had reaction product in the ventral anterior bulb but, like the anatomy-only cases, all but one mouse (Z27) had reaction product in glomeruli along the medial wall of the bulb. All but two of the mice with glomerular scores of 24% or higher also had reaction product on the dorsal and dorsomedial aspect of the anterior half of the bulb. However, like mice in the anatomy-only study, most (12 out of 16) had moderate or dense reaction product in essentially all glomeruli of the accessory olfactory bulb.

Survival time and anterograde transport scores. In contrast to the results with the anatomy only mice, there was no apparent relationship between survival time and amount of bulbar input. The correlation between survival time and the percent of glomeruli with any detectable reaction in the 16 mice for which data was available was 0.06. There also was no relationship between the number of errors in achieving accuracy of 90% on the 5% EA detection task and percent of glomeruli with detectable reaction product (r = –0.17).

Table 3 Days to achieve accuracy scores of 75 and 90% correct on the 5% ethyl acetate detection task, maximum accuracy score on the ethyl acetate detection tasks and the two-odor discrimination task, and the percent of glomeruli with dense and light WGA-HRP reaction product

Mouse	Days to achieve 75% accuracy	Days to achieve 90% accuracy	Maximal accuracy on each problem					% glomeruli with reaction product		
	5% EA	5% EA	5% EA	1% EA	0.1% EA	0.01% EA	Two-odor discrim.	Dense	Light	Total
9	24	25	95	100	100	95	100	0	25	25
11	21	21	100	90	70	0	65	no histology		
21	6	6	95	95	65	0	100	0	11	11
25	19	30	90	80	0	0	45	16.9	24.9	41.8
26	5	5	95	95	55	0	65	0	22.4	22.4
27	4	7	90	95	65	0	100	0	5	5
28	5	6	100	95	60	0	100	3.4	22.7	26.1
29	6	6	100	95	55	0	100	3.1	21.2	24.3
30	5	10	90	70	0	0	65	0.02	13.8	14
31	1	13	100	95	75	0	50	1.3	33	34.2
32	8	18	90	100	80	0	95	16.5	23.1	39.6
33	6	6	95	90	65	0	95	7	31.3	38.3
36	1	3	100	95	100	100	100	15.1	24	39
37	6	8	100	100	90	95	100	20.2	19.5	39.7
38	4	6	100	100	100	95	100	70	15	85
39	8	8	90	90	70	0	50	0.5	22.4	22.9
40	7	7	90	90	90	95	100	10	30	39.7
Mean	8	10.9	94	79.9	47.6	56.2	84.1	15.9	26.6	31.8
SD	6.7	7.8	6.4	24.9	42.4	44.9	21.6	14.4	14.6	18.2

Amount of glomerular input required for odor detection

In this study, post-treatment tests were continued until mice reached a 90% performance criterion and, thus, in most cases, their ability to detect odors had returned for one or more days before they were treated with WGA-HRP and killed. However, the results of the mouse that had the lowest glomerular score (Z27) and five mice (Z21, Z29, Z33, Z39 and Z40) that reached criterion performance of 75% and 90% on the same day are of particular interest. Of the later five cases, each performed largely at chance on their prior test day. These mice survived for 6–8 days and, on average, had dense reaction product in 4% of their glomeruli (range 0–10%) and light or moderate levels of reaction product in 23% of their glomeruli (range 11–31%). The behaviorally tested mouse with the lowest glomerular score (Z27) had no glomeruli with dense reaction product and, in the 25 sections available, only 74 of 1481 glomeruli (5%) had light or moderate levels of reaction product. This mouse reached the 75% correct criterion on day 4 and the 90% correct criterion on day 7 after ZnSO4 treatment. It was able to detect 1% EA at 95% accuracy but failed on the 0.1% EA detection task. However, it reached the 90% criterion on the two-odor discrimination task.

Discussion

The present results demonstrate that intranasal application of 50 µl of 5% $ZnSO_4$ per naris produced a dramatic and virtually complete anatomical deafferentation of the main olfactory bulb. The deafferentation of the main olfactory bulb was observed as early as 1 h after treatment and persisted for ~1 week. As expected, during this period, mice appear essentially anosmic. However, recovery occurs both anatomically and behaviorally. An unexpected but potentially important finding was that treatment-induced anosmia occurred despite the fact that intranasal $ZnSO_4$ appeared to have little or no effect on axonal transport from the vomeronasal organ to the accessory olfactory bulb.

Deafferentation and subsequent recovery after treatment with $ZnSO_4$

Recovery of input was closely related to survival time in the anatomy-only mice but not of the behavior-tested mice. The two groups differed with regard to training and water deprivation as the anatomy-only mice were not maintained on a water deprivation schedule and, of course, were not trained in the olfactometer. Interestingly, Schwob *et al.* (1999) found that in rats, those maintained on a food deprivation

schedule had less recovery of inputs following inhalation of methyl bromide. While the reasons for this are unclear, they indicate an interaction between deprivation state and anatomical recovery after damage to the olfactory epithelium. The effects of training to detect odors on such recovery have not been examined and, in the present study, these two variables (deprivation and training) were confounded. An additional variable, adequacy of $ZnSO_4$ treatment, may also play an important role in rate of recovery. Thus, treatment notes for Z36 and Z38, indicated that $ZnSO_4$ injection produced nasal bleeding and that not all of the $ZnSO_4$ volume was injected. These two mice were among the first to show behavioral recovery (Table 3) and both had relatively high glomerular scores.

Previous reports of biochemical measures of disruption of the projection of the olfactory epithelium to the bulb following intranasal irrigation with 5% $ZnSO_4$ solutions (Margolis and Grillo, 1977; Harding *et al.*, 1978) are consistent with the present results. A reduction of >95% in synthesis and transport of the dipeptide carnosine persisted for many weeks following treatment with the same dose of $ZnSO_4$ reported here. These authors also reported variable recovery of the biochemical measures of reinnervation. Factors that could play a role in the efficacy of the intranasal irrigation may include adequacy of treatment, strain and age of the mice as well as nutritional state. Thus, it is essential to routinely confirm the efficacy of the treatment using both anatomical and behavioral measures.

Despite a severe loss in axonal transport to glomeruli of the main olfactory bulb, most treated mice had dense reaction product in most or all glomeruli of the accessory olfactory bulb. No attempt was made to perfuse the vomeronasal organ during $ZnSO_4$ or WGA-HRP treatment but, in most cases, excess WGA-HRP conjugate was expired and seeped into the roof of the mouth where it probably entered the vomeronasal organ (VNO). The absence of reaction product in the 1 h anatomy-only mouse (Figure 4) and small variations in the density of reaction product as illustrated in Figures 5 and 6 are probably attributable to the amount of WGA-HRP that entered the VNO. Perfusion of the VNO was less likely to occur during $ZnSO_4$ treatment because immediately after injection the mouse was held with its head down for some seconds. As indicated in the anatomy-only results, input to the accessory olfactory bulb was undoubtedly present during the period of anosmia in behavior-tested mice. These outcomes indicate that either the accessory olfactory system did not respond to the EA odor used in this study or that any inputs from the accessory olfactory system were unable to drive the behavioral output required by the operant task. In any case, it is unlikely that recovery of function was mediated by inputs to the accessory olfactory bulb. Because the VNO appears to be spared, intranasal treatment with $ZnSO_4$ might prove useful for examining VNO function in the absence of afferent input from the main olfactory epithelium.

Does intranasal injection of $ZnSO_4$ produce a frank anosmia in mice?

Even relatively large volumes of $ZnSO_4$ failed to produce anosmia in rats (Slotnick *et al.*, 2000). Indeed, treated rats were able to detect the vapor from a relatively low (0.01%) concentration of EA and performed well on a two-odor discrimination task within a few days after treatment. In sharp contrast, mice, in the present study failed to reach even the modest level of 75% accuracy on a much stronger concentration of EA for, on average, 8 days after treatment and ~30% of the treated mice remained anosmic or certainly hyposmic for two or more weeks. Thus, intranasal treatment with $ZnSO_4$ is far more effective in mice than in rats. However, there was also considerable variability in the duration of anosmia. This variability is due, in part to adequacy of treatment. Also, $ZnSO_4$ probably does not have a uniform effect across the epithelium and the extent of contact may be less in the recesses of complex folds in the turbinates than in more accessible regions and less where possibly air bubbles or other local conditions may reduce contact of the metallic salt with the epithelium. Nevertheless, it appears that under optimal conditions treatment with 50 µl of 5% $ZnSO_4$ per naris effectively disrupts all essentially all input from the epithelium to the olfactory bulb for some days and generates a somewhat short-lived (5–30 days) anosmia or severe hyposmia in the mouse.

$ZnSO_4$ volume and duration of anosmia

Different volumes and concentrations of $ZnSO_4$ solution have been used in prior studies with mice to produce anosmia and/or to destroy the entire olfactory epithelium. In behavioral studies these vary from 1–2 µl of a 4% solution (Edwards *et al.*, 1972) to 800 µl of a 5% solution (e.g. Rowe and Smith, 1973) injected into each nasal vault. Most often 50–100 µl of 5% solution was used (Harding *et al.*, 1978; Gangrade and Dominic, 1983; Archunan and Dominic, 1990; Burd, 1993; Andine *et al.*, 1995; Chuah *et al.*, 1995). Although the smallest dose used (1–2 µl of 4% $ZnSO_4$: Edwards *et al.*, 1972) was effective in producing 'anosmia' in only some mice, most investigators using volumes of 15–50 µl of a 5% $ZnSO_4$ solution claimed that anosmia was produced in most or all experimental mice (Table 1).

Longer periods of anosmia have been reported for mice treated with 100 µl of $ZnSO_4$. In these studies mice failed in a food-finding task for at 14–60 days after treatment (Harding *et al.*, 1978; Burd, 1993). Although olfactometric tests would have provided a more sensitive measure in those studies, it seems quite reasonable that the larger volume of $ZnSO_4$ would be more effective.

However, larger volumes of $ZnSO_4$ may increase illness and mortality. In preliminary studies in this laboratory, only two of eight anesthetized mice treated with 100 µl of 5% $ZnSO_4$ survived. Andine *et al.* (1995), Harding and Margolis (1976) and Margolis *et al.* (1974), the only investigators reporting on survival in 100 µl-treated mice, noted mortality

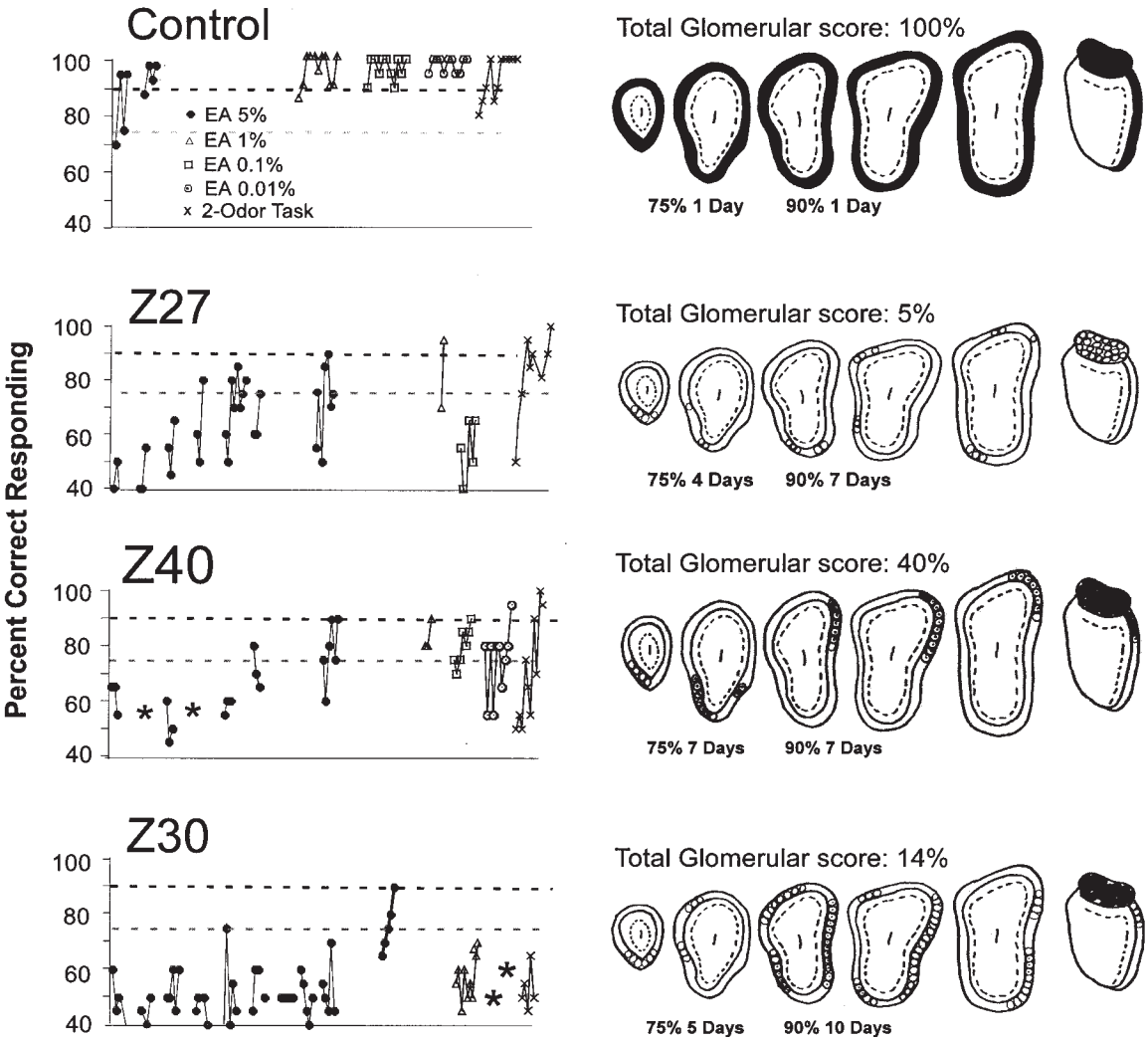

Figure 6 Post-treatment behavior scores (left) and diagrammatic representations of the olfactory bulb sections (right) showing the location of glomeruli with dense reaction product (solid circles) and light reaction product (open circles) for one control mouse and three experimental mice. In each section dorsal is to the top and medial is to the right.

rates of ~20–30%. Also, Harding *et al.* (1978) reported that 100 µl-treated mice had much lower than normal food intake for the first 3–4 days. Mice in the Andine *et al.* (1995) and Margolis *et al.* (1974) studies were anesthetized before being treated with $ZnSO_4$ but better survival and recovery from $ZnSO_4$ treatment occurs in unanesthetized mice (F.L. Margolis, unpublished observations). In any case, a treatment that produces high mortality rates or illness may be acceptable in a 'test after only' experimental design but would certainly be contraindicated in studies that involved considerable effort in pretreatment training.

Of course, modifications in administration might reduce the high mortality rate associated with larger volumes of $ZnSO_4$. In preliminary studies, it was found that with only a brief interval between 50 µl injections of the two nasal vaults, mice had difficulty in breathing and exhibited tremors for some minutes. However, when injections were separated by 2 h these problems were not encountered. Because the effects of even 50 µl of $ZnSO_4$ last for at least several days, the survival rate of mice given 100 µl volumes of $ZnSO_4$ might be increased by injections that were separated by several hours.

In summary, the present results demonstrate that treatment of mice with a volume of $ZnSO_4$ that is compatible with a high survival rate produces severe to complete disruption of transport from the olfactory epithelium to the olfactory bulb and a consequent frank anosmia as measured olfactometrically in most mice for ~1 week. While these outcomes confirm prior reports that treatment with $ZnSO_4$ produces anosmia, there is considerable variability in the duration of anosmia and in the extent of bulbar input once olfactory function returns. Finally, there appears to be a significant species difference between mice and rats in the efficacy of this treatment.

Acknowledgements

Supported in part by NIH DC03112 (F.L.M.) and NIMH 6118 (B.S.).

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Accepted June 30, 2003