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Influence of Olfactory Bulbectomy and the Serotonergic System Upon Intermale Aggression and Maternal Behavior in the Mouse[1]

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NECKERS, L., M. X. ZARROW, M. MYERS AND V. H. DENENBERG. *Influence of olfactory bulbectomy and the serotonergic system upon intermale aggression and maternal behavior in the mouse.* PHARMAC. BIOCHEM. BEHAV. 3(4) 545–550, 1975. – Biochemical parameters in the brains of olfactory bulbectomized male and female mice were studied in two experiments, followed by three experiments in which 5-HTP was injected into bulbectomized males and females to try to block abnormal behaviors. In Experiment 1 bulbectomized male and female mice had significantly less tryptophan hydroxylase in their brains than did sham controls. Neither 5-hydroxtryptophan decarboxylase nor tyrosine hydroxylase activity was affected. In Experiment 2 the rate of synthesis of 5-HT was significantly less in bulbectomized males and females. Since bulbectomy leads to increased pup killing by female mice, the objective of Experiments 3 and 4 was to see whether the injection of 5-HTP into bulbectomized females could block this behavior. The incidence of pup killing was not influenced, but in both studies the latency to kill was significantly prolonged. Olfactory bulbectomy eliminates aggressive behavior in male mice, and the purpose of Experiment 5 was to determine whether 5-HTP treatment could restore normal levels of aggression. No significant effect was found. The data suggest that a dual mechanism is needed to explain the behavioral abnormalities seen in the two sexes; the mechanism in the female appears to be serotonergic while that in the male is still unknown.

Aggression Pup killing Serotonin Maternal behavior 5-hydroxytryptophan

RECENT investigations of several species have found that olfactory bulb removal interfers with normal behavior. Male sexual behavior is inhibited in the golden hamster [17], the rat [9], and the mouse [20]. We have found that bulbectomizing the female mouse results in a high incidence of pup killing [7], while bulbectomizing the male virtually eliminates intermale aggression [3,19].

As yet neural mediators of these behavioral changes remain obscure. However, some investigations have indicated that alterations in the 5-hydroxytryptamine transmitter system can have effects on some of the same behaviors which are affected by olfactory removal. Sheard [22] has reported an increase in aggressive behavior of male rats when treated with parachlorophenylalanine (PCPA), a drug known to inhibit 5-HT synthesis at the hydroxylation of tryptophan [13]. More recently, DiChiara [4] has shown an increase in the incidence of mouse killing by rats treated with PCPA. In the same study, DiChiara showed that mouse killing could also be induced by performing bilateral bulbectomies and, further, that this surgically induced killing could be inhibited by raising levels of brain 5-HT with 5-hydroxytryptophan its immediate precursor.

Although these pharmacologically induced reversals of behavior were in the rat, they led us to carry out a series of experiments with bulbectomized mice in order (1) to investigate the possibility of biochemical alterations in the 5-HT system, and (2) to see whether chemical intervention can reverse the abnormal behavior in the bulbectomized mouse.

EXPERIMENT 1: EFFECT OF BULBECTOMY ON SEVERAL BIOCHEMICAL PARAMETERS

In the first series of experiments, both male and female mice were bulbectomized or sham-operated. Possible changes in various biochemical parameters were examined 6 days following surgery. In all cases, whole brain except for the olfactory bulbs was the tissue investigated. In the case

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of the sham operates the olfactory bulbs were removed before homogenization.

Method

Tryptophan hydroxylase. Using a modification of the method of Ichiyama [11], tryptophan hydroxylase was assayed in a whole brain homogenate fraction by measurement of $^{14}CO_2$ formed from L-tryptophan-1-C^{14}. The reaction mixture (1 ml) contained 100 μmoles of Tris-acetate, pH 8.1, 10 mM iproniazid phosphate, 0.033 μc of L-tryptophan-1-C^{14} and 0.5 ml of tissue preparation. Tissue was homogenized in 4 volumes of 0.32 M sucrose. L-tryptophan-1-C^{14} was stored in 0.01 N HCl to retard decomposition. The reaction was carried out in 10 ml Erlenmeyer flasks with a central well holding a glass insert containing 0.2 ml hydroxide of hyamine. After 1 hr of incubation at 37°C with constant shaking, 0.3 ml of 10% trichloroacetic acid was added and the mixture allowed to shake another hour. During this period, the $^{14}CO_2$ released from the acidified medium is absorbed by the hydroxide of hyamine. The insert is then removed, placed in a counting vial along with 5 ml of toluene base scintillation fluid (4.9 g PPO and 0.1 g POPOP/liter) and the radioactivity determined in a Packard Tri-Carb Liquid Scintillation Spectrometer. Enzyme activity is expressed as DPM/mg protein.

5-Hydroxytryptophan decarboxylase. 5-HTP decarboxylase was assayed in whole brain using a modification of the procedure described for tryptophan hydroxylase above. The substrate mixture contained 25 nc of carboxyl-labelled 5-hydroxytryptophan in 0.5 ml of 2 M Tris-HCl, pH 8.5, also containing 1 μmole cold 5-HTP and 250 μg of pyridoxal phosphate, and 0.5 ml of tissue homogenized in 4 volumes of 0.32 M sucrose. The assay procedure follows that for tryptophan hydroxylase outlined above.

Tyrosine hydroxylase. The method of Nagatsu [18] was modified as follows: 100 mmoles of L-tyrosine containing 2 $\times 10^5$ cpm of tyrosine tritiated on the 3 and 5 positions of the aromatic ring, 5 μmoles of tetrahydrofolate, 0.5 μmole $FeSO_4$ $(NH_4)_2SO_4$, 200 μmole 1 M acetate buffer, pH 6.0, 200 μl of tissue (whole brain) homogenized in 4 volumes of 0.01 M mercaptoethanol composed the reaction mixture. The samples were incubated at 37°C with shaking for 30 min. 50 μl of glacial acetic acid were then added and the mixture transferred with a Pasteur pipette to a small column of Dowex 50 W^+ (0.5 × 3 cm). The test tube in which the reaction was carried out was washed with 1 ml of water and this was also added to the column after the first effluent had run through. The combined effluents were collected in counting vials, 5 ml of Bray's solution [1] was added and enzyme activity expressed as dpm/mg protein.

Surgical technique. Bilateral olfactory bulb removal was performed under chloral-hydrate anesthesia (0.4mg/g body weight). A 2 mm hole was placed in the skull directly over the bulbs using a dental drill, and the bulbs were removed by aspiration. No aspiration was used in the sham operations.

Animals

Rockland Swiss Albino mice, randomly bred within our closed colony, were used in these experiments. All animals were approximately 60 days of age, and all females were nulliparous.

After recovery from surgery (1–2 hr), they were housed individually in translucent cages (11 × 7 × 5 in.) containing shavings. Food and water were given ad lib. The animals were kept on a cycle of 13 hr of light and 11 hr dark.

Results

The results are summarized in Table 1. Tryptophan hydroxylase, the rate-limiting enzyme in serotonin synthesis, was found to be approximately 30% lower in activity in males and females six days following surgery (male t = 2.92, female t = 3.07; df = 18, $p<0.01$, in both instances). In contrast, 5-hydroxytryptophan decarboxylase was not affected by bulbectomy.

In order to determine whether we were observing a biochemical phenomenon unique to the 5-HT system, we measured the activity of tyrosine hydroxylase, the rate-limiting enzyme in norepinephrine synthesis. Tyrosine hydroxylase activity was not altered by bilateral olfactory bulb removal.

EXPERIMENT 2: THE EFFECTS OF BULBECTOMY ON 5-HT SYNTHESIS

The lowered activity of tryptophan hydroxylase in bulbectomized animals suggested a possible alteration in the synthesis rate of 5-HT. In the next study we measured the rate of synthesis of 5-HT in bulbectomized males and females.

Method

Animals were bilaterally bulbectomized as described above and were housed individually for 6 days following surgery. Again, whole brains, except for the olfactory bulbs, were removed after decapitation and serotonin was determined by a modification of the method of Bogdanski [2].

Serotonin determination. Whole mouse brains were homogenized in 4 volumes of 0.1 N HCl (containing ascorbate). After centrifuging at 27,000 × g for 20 min, 1.6 ml of supernatant is transferred to a tube containing 3.2 ml n-butanol, 0.4 ml Na_2CO_3, 1ml borate buffer (pH 10.4) and 1 g NaCl. The tube is shaken for 15 min, centrifuged to separate the phases, and 2.7 ml of the butanol phase is removed and added to a tube containing 2 ml borate buffer. This tube is shaken for 15 min, centrifuged, and 2.5 ml of the butanol phase is removed and added to a tube containing 4 ml n-heptane and 0.15 ml 0.1 N HCl (with ascorbate). The tube is shaken for 30 min, centrifuged, and the upper phase is removed by aspiration. 100 μl of the acid phase is added to 50 μl of concentrated HCl to develop the fluorescence. Samples were read at an excitation wavelength of 295 nm and an emission wavelength of 540 nm.

The rate of 5-HT synthesis was determined by injecting paragyline hydrochloride (80 mg/kg) IP, an MAO inhibitor. At T = 0, 30, 45, 90 min after injection the amount of serotonin was measured. Serotonin accumulation was linear for 90 min. The rate of synthesis was expressed as ng 5-HT formed/g tissue/hour.

Results

In males, 6 days following surgery, the 5-HT synthesis rate decreased from 240.0 ng/g/hr in sham animals to 72.0 ng/g/hr. In females, under the same conditions the synthesis rate decreased from 300 ng/h/hr to 114 ng/g/hr. An analysis of variance of each sex separately found significant

TABLE 1

EFFECT OF BULBECTOMY ON BRAIN MONOAMINE ENZYMES (MEAN ± SE) IN EXPERIMENT 1, N = 10 PER GROUP

	Male		Female	
Enzyme	Sham	Bulbx	Sham	Bulbx
Tryptophan Hydroxylase	40.6 ± 2.3*	29.0 ± 1.6	42.0 ± 1.8	28.5 ± 0.3
5-Hydroxy-Tryptophan Decarboxylase	1021 ± 57	974 ± 83	931 ± 42	943 ± 35
Tyrosine Hydroxylase	1150 ± 61	1142 ± 94	1109 ± 44	1073 ± 72

*Activity is expressed as DPM/mg Protein

interactions between the bulbectomized and sham curves over the 90 minutes, $F(3,24) = 5.20$ (male); 7.24 (female); $p<0.01$ in both instances. These data are shown in Fig. 1. An inspection of the figure reveals that the reason for the significant interactions was due to the greater rate of synthesis of the shams relative to the bulbectomized mice.

EXPERIMENT 3: THE EFFECT OF 5-HTP TREATMENT ON PUP KILLING AND BRAIN SEROTONIN

The data from Experiments 1 and 2 suggest that, in both male and female mice, bilateral removal of the olfactory bulbs leads to an abnormal synthesis of serotonin, presumably resulting from decreased tryptophan hydroxylase

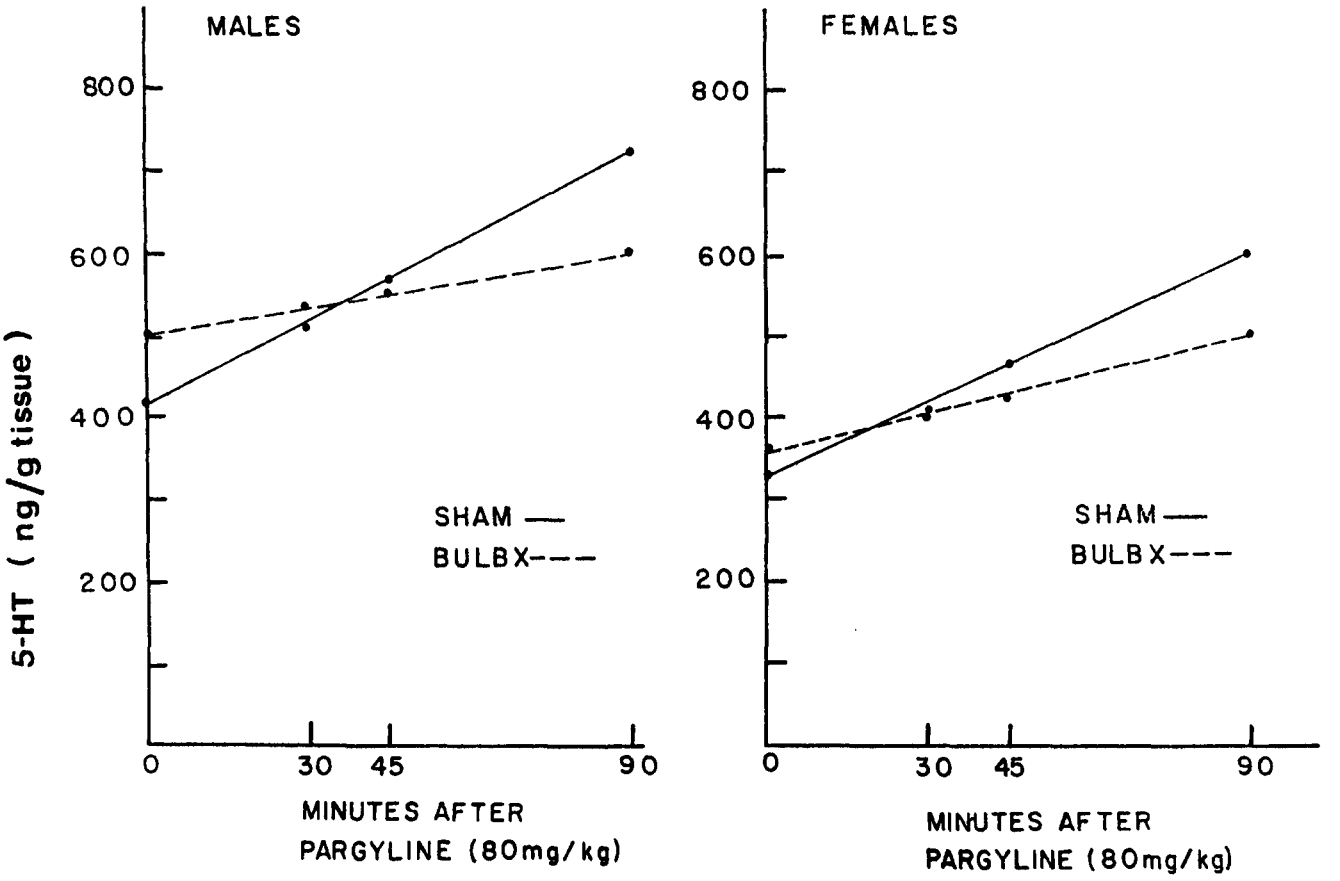

FIG. 1. 5-HT Synthesis: Sham vs Bulbectomized Mice (6 days following surgery).

activity. In the following studies the effects of 5-HTP injection on the synthesis of 5-HT and on the pupkilling behavior of the bulbectomized females were investigated.

Procedure

Twenty-eight virgin female Swiss Albino mice approximately 70 days of age, were bilaterally bulbectomized as described previously and housed individually for 7 days. On the eighth day 14 randomly selected animals were given an IP injection of 5-HTP (200 mg/kg body weight in saline). The other 14 mice received a vehicle injection only. The animals were then left undisturbed for 60 min. At the end of this period, one mouse pup (1–5 days old) was placed in each cage and observations were taken every minute for 2 hr. The occurrence of pup killing and the latency in minutes to kill the pup (with 120 min being the maximum latency) were recorded.

In addition, twelve other virgin females were bulbectomized and housed individually for 7 days. On the seventh day, half of these animals were injected with 5-HTP (200 mg/kg) and the other half with saline. After 2 hr, these animals were killed and their brains were analysed for 5-HT content.

Results

The 5-HT determination showed that the injection of 5-HTP was sufficient to significantly increase levels of brain 5-HT from 1.51 to 2.43 μg/g brain ($t = 2.76$; $df = 10$; $p<0.02$). This 60% increase verified the effectiveness of this dosage.

In the group of animals tested for pup killing, two animals in the saline control group proved to have incomplete olfactory bulb removal and were not used in the analysis. The results are recorded in Table 2. These data show that even though all animals in both groups killed the pup in the allotted two hours, the latency in the 5-HTP group was over three times that of the saline injected group. A t test comparing the means found a difference which approached significance at the 0.06 level ($t = 1.91$; $df = 24$).

TABLE 2

MEAN LATENCY (± SE) TO KILL PUPS AS A FUNCTION OF 5-HTP TREATMENT IN EXPERIMENT 3

Treatment	N	Percent Killing	Mean Latency to Kill (min)
5-HTP in saline	14	100	27.6 ± 8.8
Saline	12	100	8.8 ± 2.5

EXPERIMENT 4: REPLICATION OF EXPERIMENT 3

The failure to find a significant effect in Experiment 3 could have been due to our way of storing the 5-HTP. Although the 5-HTP seems to be reasonable soluable in saline, some visible precipitation occurs if the solution is not kept above room temperature. In order to eliminate a heterogenous injection solution as a source of error, the amine was dissolved in a tris buffer (pH 6.8) in which it was totally soluable at room temperature. The procedure for testing remained the same. The 26 animals used in Experiment 3 were randomly assigned to one of two treatment groups: 5-HTP in tris buffer or a control injection of tris buffer alone. A period of three days had elapsed between the completion of Experiment 3 and the beginning of Experiment 4, making a total of an 11-day interval between surgery and this testing time.

Results

The results are shown in Table 3. In this experiment all the control animals and 85% of the 5-HTP treated animals killed the pup in under 120 min. However, the 5-HTP treated animals had a significantly longer latency than did the control animals ($t = 2.85$; $df = 24$; $p<0.01$). This difference is still significant if the two non-killers are eliminated from the analysis ($t = 2.58$; $df = 22$; $p<0.02$).

EXPERIMENT 5: EFFECT OF 5-HTP ON AGGRESSIVE BEHAVIOR IN MALE MICE

Since treatment with 5-HTP in the female mouse increased the latency for pup killing, the next question we asked was whether the same treatment could restore normal levels of aggression in the male mouse.

Procedure

Animals. Twenty-four adult male mice 70 days of age were bilaterally bulbectomized as described previously. After the operation these mice were housed individually in opaque mouse cages measuring 11 X 7 X 5 in. Approximately 24 hr before the first test, each of these animals was placed into one half of an aggression testing box. At this time the animals were randomly assigned either to a group which was to receive 5-HTP injections or to the control group receiving vehicle injections. These injections were given as in Experiment 4 except in this instance injections were given twice a day, one at 6:00 p.m. and another one hour before testing. The bulbectomized males were paired against normal males of the same strain as described below.

Aggression testing. The wooden fighting boxes measured 12-1/2 X 15-1/2 X 6-1/2 in. and were divided into 2 equal size compartments by means of a removable metal partition. The floors of both compartments were covered with pine shavings and standard mouse cage lids, fitting into the top of each side, supplied food and water. Immediately prior to testing, the two cage tops were replaced with a flat metal grid in order to facilitate observation. All testing occurred at approximately the same time each morning. The test began by removing the partition and was continued for five minutes or until an attack was initiated. Upon the occurrence of an attack the animals were separated and returned to their respective compartments. Testing followed a quasi-round robin schedule in which the normal males were rotated so that they faced a new bulbectomized opponent each day. Each pair was given the opportunity to fight once a day with the following data recorded: presence or absence of an attack, the latency of attack, and a notation indicating which of the pair had initiated the attack. In those instances where no fight occurred in the five-minute period, a latency of 300 seconds was assigned for that test.

TABLE 3

MEAN LATENCY (± SE) TO KILL PUPS AS A FUNCTION OF 5-HTP IN TRIS BUFFER TREATMENT IN EXPERIMENT 4

Treatment	N	Percent Killing	Mean Latency to Kill (min)
5-HTP in Tris Buffer	13	85	40.0 ± 12.1
Tris Buffer	13	100	5.1 ± 1.7

TABLE 4

TOTAL NUMBER OF FIGHTS OCCURRING, MEAN NUMBER OF TIMES ATTACKED AND THE MEAN LATENCY TO BE ATTACKED (SEC) AS A FUNCTION OF 5-HTP TREATMENT IN EXPERIMENT 5

Treatment	N	Number of Attacks Initiated	Mean Number of Times Attacked	Mean Latency to be Attacked (sec)
Bulbectomy + 5-HTP	11	0	2.27 ± 0.19	245 ± 45
Bulbectomy + Tris Buffer	12	0	2.08 ± 0.31	165 ± 41

Results

In no case did a bulbectomized male of either treatment group initiate a fight and in no case did a bulbectomized animal fight back when attacked. There was also no difference in the number of fights elicited or the latency to attack between the treatment groups. These results are shown in Table 4. The results of this experiment indicated that, unlike pup killing in the female, 5-HTP treatment in the male does not result in a return to more species typical behavior.

DISCUSSION

Surgical removal of both olfactory bulbs in mice results in a lack of aggression in the male and a lack of maternal behavior in the female [3,7]. The studies presented in this paper show that substantial changes in brain 5-HT synthesis are a further result of this treatment. On the basis of the biochemical data, we postulated that augmentation of 5-HT synthesis might reestablish more normative behavior patterns.

Using peripheral injections of 5-hydroxytryptophan, an intermediate in the 5-HT pathway which bypasses tryptophan hydroxylase and penetrates the brain readily, we were able to delay atypical behavior in the female, but were unable to alter atypical male behavior.

Since there are no ascending serotonergic pathways to the olfactory bulbs from other brain regions [15], the present data suggest that surgical removal of the bulbs leads to a fall in tryptophan hydroxylase activity accompanied by a decreased rate of 5-HT synthesis which is modulated transsynaptically. This hypothesis is given support by studies of Heller [10]. Destruction of the medial forebrain bundle produces marked reductions in brain serotonin and norepinephrine secondary to a loss of enzymatic activities essential for the biosynthesis of these monoamines. Comparisons between the distribution of medial forebrain bundle axons to various anatomic loci and areas of brain in which monoanimes decrease suggest that the lesion effect represents a disruption of neuronal control of monoanime biosynthesis mediated across a polyneuronal system in the brain. We proposed the following model to account for our data.

Upon removal of the olfactory bulbs, the primary afferent fibers are severed. These fibers synapse onto secondary neurons, some of which are serotonergic. Thus, upon removal of the bulbs, there is a decreased stimulation of the serotonergic fibers, resulting in a decreased rate of firing and lessened release of 5-HT from the presynaptic bouton.

Referring to Fig. 1, we see that the basal level of 5-HT is not statistically different in operated and sham-operated mice, suggesting that a steady-state level of the transmitter in the nerve ending is being maintained. To this end, the excess intraneuronal 5-HT feeds back on the rate limiting enzyme to decrease its activity. This, in turn, leads to a decreased rate of synthesis of the amine. Other investigators have attempted to implicate feedback regulation as a means of regulating 5-HT synthesis [15,16]. Much evidence has been adduced to date suggesting that feedback of norepinephrine onto tyrosine hydroxylase is an important regulatory mechanism in the synthesis of catecholamines [17,23].

Administration of 5-hydroxytryptophan bypasses the affected enzyme and increases the amount of 5-HT in the presynaptic bouton. Some of the excess amine is degraded by monoamine oxidase (in the bouton), but a portion of the amine, free in the axoplasm, may leak out of the bouton into the synaptic space, thus stimulating a postsynaptic site. Alternatively, the excess free amine in the nerve ending may cause the release of already packaged amine in greater quantities than usual to return the 5-HT concentration to the original steady state level. In either case, the postsynaptic site would be acted upon.

Karli and Vergnes [13] suggest that there are inhibitory serotonergic fibers in the limibc system. We propose that the serotonergic fibers we are discussing here are also inhibitory in nature and, when functioning properly, serve to prevent pup-killing behavior from occurring in the female mouse.

The female must make use of several cues in determining that her pups are not foreign objects. Sight and smell would seem to be most important. If a pup has body hair, a bulbectomized female is less likely to kill it. This can be related both to the presence of hair and to the size of the pup [8]. When the pups are too young to have body hair, it would seem that smell, a very highly developed sense in rodents, would be the major restraining cue. Cutting off this input, with the possible concomitant transsynaptically caused depletion of brain 5-HT, would remove inhibitory influences on this particular type of aggression, at a time when other cues are incapable of taking over from smell.

Recent findings substantiate this conclusion. Vandenberg [23] showed that anosmia induced by zinc sulfate treatment can simulate, to a partial extent, the effects of bulbectomy on pup-killing behavior in the female mouse. Data from our laboratory (Seegal, personal communication) suggest that under different conditions zinc sulfate treatment can give the same effect on pup-killing behavior as does bulbectomy and this effect is dependent on the parity of the female [21].

In contrast to this, Edwards [5] reports that zinc sulfate treatment in male mice will not necessarily have a debilitating effect on intermale aggression. He concludes that the reduction in aggression seen after bulbectomy is not due to anosmia alone. Perhaps the olfactory bulbs of mice made anosmic with zinc sulfate are still capable of spontaneous firing and thus can still send impulses into the brain proper. This tonic level of stimulation of certain brain regions may be enough to maintain intermale aggression at near normal levels. In this case no fiber tracts have been destroyed. In the bulbectomized male, however, we may assume some axonal degeneration six days following surgery. Although the 5-HT system is similarly affected in male and female following bulbectomy, the fact that 5-HTP replacement therapy has no effect in the male leads one to hypothesize that non-serotonergic, possibly degenerating, nerve tracts are causing the absence of intermale aggression. Thus, we are arguing for a dual mechanism to explain the behavioral abnormalities seen in both sexes following removal of the olfactory bulbs, that in the female being serotonergic while that in the male remaining as yet unknown.

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