THE EFFECTS OF AGGRESSION, DEFEAT, AND HORMONE ADMINISTRATION ON REGIONAL BRAIN LEVELS OF TOTAL RIBONUCLEIC ACID AND RIBONUCLEASE ACTIVITY IN C57BL/6J MICE

by

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TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

The recent emphasis on the involvement of ribonucleic acid (RNA) in the learning or memory process has been based on findings in which various forms of environmental stimuli were used for conditioning. In many cases, a stressful stimulus such as mild electric shock, combined with visual or auditory signals, provided the tool with which to study conditioned learning (Gaito et al., 1966; Agranoff et al., 1967; Shashoua, 1968; and Gaito et al., 1968). However, no one has attempted to use an environmental stimulus which is known to be both stressful and natural to most animal populations, namely, aggression. While aggression has not been linked directly with learning, Scott (1958) has stated with regard to his experiments with fighting mice, that aggression or the motivation to fight, has to be learned while the experience of winning reinforces aggressive behavior. In addition, Bronson and Eleftheriou (1965b) demonstrated a psychological component underlying defeat by aggression.

There are many ways in which fighting or aggressive behavior can be induced, although the physiological mechanisms are not well understood. Pain has been used extensively to elicit aggression (Ulrich et al., 1965), and mice will react to pain at an early age (Scott and Fredericson, 1951). Painful electric foot-shock will lead to fighting among paired animals both within species (Ulrich and Azrin, 1962; Ulrich et al., 1964; Miller, 1948; and Tedeschi et al., 1959) and between species (Ulrich et al., 1964).

Shock-induced aggression has been classified as reflexive or unconditioned fighting (Ulrich and Azrin, 1962) as well as conditioned fighting (Miller, 1948). However, the work by Ulrich and Craine (1964) would seem to support the view that shock-induced aggression results in a reflexive

or unconditioned response. They attempted to recondition shock-induced aggression by shocking rats after aggressive responses had begun and terminated the shock when fighting had ceased. However, they found that shock-induced aggression persisted despite this reverse conditioning. It is well established that electric intracranial stimulation can be used to produce aggression and/or rage responses of a reflexive nature (Stachnik et al., 1966; Wasman and Flynn, 1962; Delgado, 1966; Egger and Flynn, 1961; and de Molina and Hunsperger, 1959). The areas governing such responses also have been defined (Hunsperger et al., 1964; Brown and Hunsperger, 1963; Karli, 1956; and Ursin, 1960). In addition, frontal lobectomy (Karli, 1957) and lesions in the septal forebrain region (Brady and Nauta, 1953) also have been reported to result in hyperirritability, hyperexcitability, and aggressive behavior.

Conditioned aggression, however, has been produced through reinforcement. Scott (1958) has shown that mice can be conditioned to fight and win, and each victory reinforces the aggressive behavior. Ulrich et al. (1963) were able to condition rats to fight by water deprivation; the rats attained water, contigent upon their aggressive responses toward another rat. Reynolds et al. (1963) were able to elicit the same conditioned response with pigeons deprived of food and reinforced with food. A superior method of reinforcement was reported by Stachnik et al. (1966) in which rats were conditioned towards intra- and interspecies aggression by reinforcement with intracranial stimulation in the "pleasure centers"; the advantage here being that the animal can receive reinforcement without disrupting its aggressive behavior.

Among other painful stimuli which will initiate aggression are heat and physical blows (Ulrich, 1966). Many factors, however, are involved

in pain-elicited aggression such as the duration, intensity, and frequency of presentation of the stimuli, the sex, age, sensory impairment, and prior social experience of the animal (Ulrich, 1966 and Ulrich et al., (1965). Social experience before fighting has been found by Levine et al., (1956) to have different effects on two types of male mice; reducing aggressiveness in albino males, and increasing the latency of attack in black-agouti males. In addition, earlier studies by Levine (1959) on the effects of handling in infancy, showed that non-handled mice were far more susceptible to react to novel stimuli with greater emotional arousal than were handled mice. In turn, the lack of social experience, by rearing mice in isolation and without handling, will increase aggressiveness (Marsh et al., 1959; Scott and Fredericson, 1951; Bourgault et al., 1963; and Coleman, 1967).

The use of drugs also has played an important role in suppressing (Sturtevant and Drill, 1957 and Tedeschi et al., 1959) and inducing aggression (Everett, 1965; Plotnikoff and Everett, 1965; Everett et al., 1964; and Reinhard et al., 1960).

Two more recent studies have shown that there still are other ways in which aggression can be initiated. Southwick (1967) found that fostering male mice of a non-aggressive strain, at birth, to females of an aggressive strain brought about a significant elevation in aggression when the mice mature. Archer (1968), in turn, has found that mice, housed together from 4-5 weeks of age with little aggressive interaction, will exhibit heightened aggression when paired in a cage containing the odor of a strange male of another strain.

With the preceding discussion of the ways in which aggression may be initiated, it now becomes important to consider the physiological effects

of aggression. The subordinate animal, who is the continual receptor of aggression, obviously is existing under a high stimulus level and therefore should be physiologically different from the aggressor. Numerous experiments have, indeed, confirmed this observation. Defeated or subordinate mice tend to lose weight (Davis and Christian, 1957), have larger adrenal weights than aggressors or dominants (Davis and Christian, 1957; Bronson and Eleftheriou, 1963; Southwick, 1964; and Bronson and Eleftheriou, 1964), have reduced adrenal levels of ascorbic acid (Bronson and Eleftheriou, 1963 and 1964), and elevated levels of circulating corticosterone relative to increases in unbound hormone (Bronson and Eleftheriou, 1965a). In addition, Eleftheriou and Church (1967) found elevated plasma levels of luteinizing hormone (LH) in defeated mice while fighters showed no increase in plasma LH but significantly higher levels of pituitary LH than controls. This is somewhat contradictory to the report by Mathewson (1961) that dominance relationships and aggression in starlings could be changed by injecting pituitary LH. However, the difference may be related to species variance.

Defeated mice also exhibit greater plasma and pituitary levels of thyrotropin (TSH) than unfought controls (Eleftheriou et al., 1968) and lower eosinophil levels (Vandenbergh, 1960).

Welch (1967) has found that fighting leads to an increase in brain norepinephrine (NE), serotonin, and dopamine which is accompanied by an enhanced release of epinephrine, but not NE, from the adrenal. Eleftheriou and Church (1968a) found a similar increase in brain serotonin in defeated mice but a general decline in brain NE, with the exception of the frontal cortex, which exhibited a progressive elevation in NE with days of defeat. The same reciprocal relationship between serotonin and

NE was reported by Freedman et al. (1962) and Barchas and Freedman (1963) who subjected rats to physiological stress. Studies with the enzymes involved in the biosynthesis and inactivation of these amines, 5-hydroxytrytophan decarboxylase and monoamine oxidase, have shown that behavioral manipulation in the form of aggression and defeat can alter the enzyme activity in the brain (Eleftheriou and Church, 1968b and Eleftheriou and Boehlke, 1967).

Other investigations have shown that aggression or exposure to stress retards leukemia (Lemonde, 1959) and the growth of Ehrlich carcinoma in mice (Marsh et al., 1959) and furthermore, differentially affects the mating response of male mice (Kahn, 1961).

Thus many physiological effects of aggression or defeat by aggression have been studied in great detail, while many other doors remain to be opened. One possibility certainly would be the changes that might occur in the nucleic acid content of such animals. A number of individuals have attempted to relate one or both of the nucleic acids (i.e., RNA and DNA) to learning (Hyden, 1959; Gaito, 1961; Dingman and Sporn, 1961; McConnell, 1962; and Landauer, 1964). Hyden and his co-workers have used a number of elegant experiments concerned with RNA changes (e.g., in amounts of RNA and base-ratios in RNA) during behavioral events to suggest the involvement of RNA in memory (Hyden, 1961, 1962; Hyden and Egyhazi, 1962, 1963; and Hyden and Lange, 1965); others, too, support this hypothesis (Shashoua, 1968; Flexner and Flexner, 1966; and Gaito et al., 1968). If then the principles of complex learning apply to agonistic behavior, as Scott (1958) tentatively has concluded, the use of aggression as the tool with which to manipulate an animal's environment and study molecular events has unlimited possibilities. At present, however, the more simple

aspects of behavior, such as stimulation and reduction of stimulation, although not as exciting to many as have been learning aspects, are basic to the understanding of more complex behavior. In general, it has been found that moderate stimuli tend to increase the content of RNA in the brain (Vladimirov et al., 1961; Baranov and Pevzner, 1963; Talwar et al., 1961; and Attardi, 1957). Furthermore, overstimulation or overexcitation of the nervous system, as a rule, brings about exhaustion and fatigue and a general decline in RNA; presumably, because its synthesis is unable to keep pace with its utilization (Mihailovic et al., 1958 and Talwar et al., 1961). Such stimulation has varied from electroshock (Mihailovic et al., 1958 and Noach et al., 1962) to drug-induced convulsions (Talwar et al., 1961).

It is well established that aggression, when used against another animal, is a stressful stimulus to that animal. Thus, the overall objective of the present study was to make use of aggression as the tool of stimulation and to determine the effects that it might have on the total RNA content and ribonuclease activity within certain brain areas. This type of stimulation, in which mice were subjected to short, daily attack by trained fighters, is also known to be normal for many rodents in field conditions.

MATERIALS AND METHODS

Experimental Animals

Animals used in this study were male mice of the C57BL/6J strain, Mus musculus. All mice were isolated at 30 ± 4 days of age in cages of 5.5 x 7.5 inches. Food and water were provided ad libitum.

In order for the mice to establish and maintain home territories, the cages were not cleaned during the course of the entire experiment, with the exception of those mice trained to fight (see below).

All mice remained undisturbed for 30 days.

Group Separation

From the total population of 467 mice, six major groups were used for the present study. Group I consisted of 50 mice which were trained to become fighters. Group II was comprised of 145 mice which were subjected to attack by the trained fighters and was designated "naive" mice. Group III contained 40 mice which were trained to fight, but for a shorter period of time than those of Group I. These mice were designated as "early fighters". In Group IV, 200 mice were administered with various hormones. Group V consisted of 26 mice which were not exposed to fighters or any other specific environmental stimuli and served as the intact control group. Group VI, composed of a small group of 6 mice, were used solely for the training of fighters and designated as "bait" mice.

Hormone Administration

The 200 mice of Group IV were subdivided into four groups of 50 mice each. When these animals were 60 ± 4 days old, one group received 200 micrograms per 0.1 ml of dopamine in saline per day; a second group received 200 ug/0.1 ml of corticosterone in sesame oil per day; a third group received 100 ug/0.1 ml of testosterone in oil per day; and the final group received 50 ug/0.1 ml of 5-hydroxytryptamine (serotonin) in oil per day. All hormones were administered via cervical subcutaneous injection and administration was carried out for five days. On the sixth day, the 200 mice were sacrificed by cervical dislocation and brain parts removed.

Training of Fighters

The training of mice to fight was by a modification of the method of Scott (1946).

At the age of 57 ± 4 days, 50 mice from Group I were removed from their cages and placed (one mouse per cage) in clean 11 x 7.5 inch cages. In order to maintain as stable a home territory as possible, the shavings from each initial cage were transferred to the clean cage. This cage change was necessary so that the mice would have ample space in which to maneuver during the training period and, more important, during the actual contest with the "naive" mice.

Training began when the mice were 60 ± 4 days of age. For two 5-minute periods each day a "bait" mouse was dangled by the tail, before the head of the trainee. These two 5-minute training periods were conducted not less than 4-5 hours apart. The response of the trainee initially was that of escape-avoidance or assuming the defense position (i.e., a corner crouch with the forelegs raised). However, after one week, the

trainees began to show aggressive behavior towards the "bait" mice; frequently biting the ears and/or tail of the "bait" mice. The training was then altered by holding the "bait" in the trainee's cage and withdrawing the "bait" several inches each time the trainee approached within contact range. This was alternated with the former procedure and as soon as the antagonized trainee made any attempt to attack, the "bait" was removed rapidly.

At the end of two weeks, the trainees exhibited good aggressive responses towards the "bait" mice. The "bait" mice then were dropped in the cages of the fighters and withdrawn at the trainee's attack. By the third week, the latent period (i.e., the time lapse between the entry of the "bait" and the attack) had decreased to within ten seconds. At this time, the trainee was considered a good fighter.

Training of Early Fighters

The procedure for training early fighters essentially was the same as that described previously. The exception was that these mice were trained for only five days. Consequently, few, if any, became good fighters. They were sacrificed not more than 5 minutes after the final training period and brain parts removed.

"Naive" Mice

The 145 mice of Group II were subdivided into five groups. In the first group, composed of 50 mice, each was exposed to a trained fighter for two 5-minute periods (separated by not less than 4-5 hours) for one day. The second, third, and fourth groups consisted of 24 mice each. These groups were exposed to a trained fighter for two 5-minute periods per day, but for two, four, and eight days, respectively.

The fifth group of 23 mice was exposed to the trained fighters for sixteen days.

Removal of Brain Areas

All mice were sacrificed by cervical dislocation. The amygdala, hypothalamus, frontal cortex, and cerebellum were dissected out as rapidly as possible (in the case of fighters, "naive" mice, and early fighters, within five minutes after the final fight, exposure, or training period, respectively) and frozen in ether-dry ice. These tissues than were stored at -30°C for later analysis.

Isolation of Total Ribonucleic Acid

The particular brain areas (amygdala, hypothalamus, frontal cortex, and cerebellum) were pooled so as to have not less than 100 mg of tissue from each area for subsequent analysis. Total ribonucleic acid (RNA) was extracted by a modification of the method of Santen and Agranoff (1963). Tissues were homogenized in distilled water, 100 mg of tissue per ml of water, by means of a conical glass homogenizer. One ml of homogenate then was shaken with 8 volumes of 10% trichloroacetic acid (TCA). The resultant precipitate was collected on filters cut from Whatman chromatography paper No. 3MM, using a filtering apparatus which enabled ten precipitates to be collected simultaneously. Each precipitate was washed once with 10% TCA, twice with chloroform-methanol (2:1, v/v), once with chloroform-methanol-water (38:19:3, v/v), once with 95% ethanol, twice with 5% TCA, followed by another 95% ethanol wash. After a final wash with absolute ethanol, followed by an ether rinse, the filter paper discs were removed carefully and stored in vials at -30°C for later quantitation of total RNA. All washing solutions used in this procedure were kept at 4°C.

Determination of Total RNA

A modification of the Schmidt and Thannhauser (1945) chemical fractionation procedure was used to separate the deoxyribonucleic acid (DNA), RNA, and protein fractions. Two ml of 1N KOH was added to each filtrate and the filtrates were hydrolyzed for 3 hours at 37°C, and neutralized with 0.4 ml of 6N HCL. The contents of each vial were checked for precipitate formation, and 2 ml of cold 5% TCA was added to each filtrate. This mixture was centrifuged at 1500 rpm for 10 minutes. One ml of supernatant from each vial was removed and transferred to separate colorimeter tubes. Five standards (ranging from 5 to 100 ug), taken from a stock solution of yeast RNA, 100 ug/ml, were run for each analysis. The samples and standards were brought to a volume of 1.5 ml with distilled water. An orcinol test was used to determine total RNA content (Schneider, 1957). After the addition of 3 ml of acid reagent (0.5 ml of 10% hydrous ferric chloride - $FeCl_3 \cdot 6H_2O$ — in 100 ml of concentrated HCl), the tubes were shaken and 0.2 ml of 6% orcinol in 95% distilled ethanol (made fresh daily) was added. Again the tubes were shaken and placed in a boiling water bath for 20 minutes. After removal and subsequent cooling, the tubes were read at 660 mu in a Spectronic 20 spectrophotometer.

An average factor was used to calculate the concentration of total RNA (in ug/100 mg) in the experimental samples by the equation:

$$\frac{\text{ug of total RNA}}{\text{100 mg tissue}} = \frac{\text{O.D. of sample}}{\text{average factor}} \times \text{dilution (4.4 ml)}$$

This average factor was obtained by dividing the optical density (O.D.) reading of each standard sample by its respective known concentration of RNA and obtaining an average from the total standards which were determined.

Preparation and Isolation of Labelled RNA

Pregnant mice in the 16-18th day of gestation period were asphyxiated with CO_2 fumes using a mixture of dry ice and water. Embryos were removed aseptically; head and the limbs of embryos were severed; the remaining tissue minsed, washed three times in phosphate buffered saline (PBS) solution and trypsinized for 10 minutes with 0.25% trypsin solution at room temperature. The supernatant was decanted, centrifuged at 600 x G for 5 minutes and the cell pellet resuspended in 5 to 10 ml of Eagle's medium containing 10% fetal calf serum. The process of trypsinization was repeated 5-6 times. The resulting cell suspension was counted in a hemocytometer and diluted in Eagle's medium with 10 fetal calf serum. Glass or Falcon plastic petri dishes (60 x 15 min) were seeded, with 5 ml of the cell suspension containing 1.2 million cells/ml. The cultures were incubated at 37°C in a humidified CO_2 incubator.

The cells were prelabeled 24 hours after plating with 2 microcuries of ^{3}H-uridine per ml of medium and allowed to grow for an additional 48 hours. Whem a complete monolayer had formed, the labeled media was decanted and the cells washed once with PBS. Ten ml of phenol in Tris buffer was added to one culture dish and the monolayer was freed by scraping. The contents of this dish were transferred to another culture dish with subsequent scraping. The process was repeated until 10 culture dishes had been scraped. Never more than 10 ml of phenol was used to scrape more than 10 culture dishes (i.e., another 10 ml must be used for the next set of 10 dishes). The resultant mixture was transferred to a centrifuge tube, shaken vigorously for 5 minutes and centrifuged at 2500 rpm for 20-30 minutes. After centrifugation, the aqueous phase (top) was removed and the phenol layer was re-extracted with 3-5 ml of PBS. The aqueous layers were pooled,

layered with 3 volumes of cold absolute ethanol, and the tube then inverted gently several times. The mixture was placed at 4°C for 3-4 hours to allow the RNA to precipitate. The precipitate was collected by centrifugation for 10 minutes and redissolved in 4 ml of PBS. After the addition of 4 ml of chloroform, the mixture was shaken vigorously for 2 minutes and after a 10 minute centrifugation, the aqueous layer again was precipitated with 3 volumes of cold absolute ethanol for 1 hour at 4°C. The precipitate again was collected by centrifugation, redissolved in 4 ml of PBS, shaken with 4 ml of chloroform, and the aqueous phase again precipitated with absolute ethanol for 1 hour at 4°C. This precipitate was collected by centrifugation, redissolved in 4 ml of PBS and treated with 10 ug/ml of electrophoretically pure deoxyribonuclease (DNase) for 1 hour at 37°C. Following the incubation period, 4 ml of chloroform was added, the mixture shaken and after centrifugation the RNA again was precipitated as described previously. The precipitate was collected by centrifugation and finally redissolved in 5 ml of 0.85% saline.

Isolation of Unlabeled RNA

Since the radioactivity of the tritium-labeled RNA was extremely high, it was necessary to reduce its specific activity by the addition of unlabeled ("cold") mouse RNA. The source of this "cold" RNA was the livers and kidneys of the mice from which the embryos had been removed (see previous section). These organs were minced and washed repeatedly in PBS until free of blood. The tissue then was homogenized and added rapidly to an equal volume of phenol in Tris buffer. The remaining isolation procedure was the same as that described for ^{3}H-RNA with the exception that the purification steps were doubled.

Determination of the Concentration of ^{3}H-RNA and "cold" RNA

The orcinol test was used for the determination of both ^{3}H-RNA and "cold" RNA concentration. Both were expressed as ugm/0.1 ml.

Determination of the Radioactivity of ^{3}H-RNA

Radioactivity of the ^{3}H-RNA was determined by adding 0.1 ml of ^{3}H-RNA to 10 ml of aqueous scintillation fluid and counting on a Packard Tricarb Liquid Scintillation Spectrometer. The radioactivity was expressed as counts per minute (CPM) per 0.1 per 0.1 ml of ^{3}H-RNA. Dilutions were made with the "cold" RNA so that the average CPM and concentration was 28,500 CPM/100 ugms/0.1 ml of ^{3}H-RNA.

Determination of Ribonuclease Activity

Each brain area was analyzed separately for ribonuclease (RNase) activity by a modification of the method of MacLeon et al. (1963). All samples and solutions were kept at 4°C unless stated otherwise. Each tissue was homogenized in 0.5 ml of 0.05 M sodium acetate in 0.05 M ethylenediaminetetraacetate tri-sodium salt (EDTA) buffer, pH 5.7, and centrifuged at 2500 rpm for 30 minutes. After centrifugation, 0.1 ml of each supernatant was placed in separate tubes containing 0.05 ml of 0.05 M EDTA, and 0.45 ml of 0.05 M acetate buffer, pH 5.7. These were the experimental tubes. Another 0.1 ml of each supernatant was placed in separate tubes containing the same solutions but in addition, 0.2 ml of a 25% perchloric acid solution containing 0.75% uranyl acetate. These were the control tubes. An additional 0.05 ml of each supernatant was removed for protein determination.

The experimental tubes were incubated for 30 minutes at 37°C with 0.1 ml of ^{3}H-RNA (28,500 CPM/100 ug/0.1 ml); while the control tubes were

incubated with 0.1 ml of ^{3}H-RNA and 0.1 ml of yeast RNA (100 ug/0.1 ml). Following incubation, the contents of each tube were thoroughly mixed. Next, 0.1 ml of yeast RNA was added to the experimental tubes, followed by the addition of 0.2 ml of the perchloric acid-uranyl acetate solution. Both sets of tubes then were placed in an ice bath for 40-60 minutes. Following centrifugation at 2500 rpm for 30 minutes, 0.1 ml of the supernatant from each tube was added to 10 ml of aqueous scintillation fluid and the radioactivity was measured by scintillation spectrometry.

The control counts were subtracted from the experimental counts to give the net counts per minute for each sample. This value then was divided by the concentration of protein (ugms/0.1 ml) in 0.1 ml of the supernatant (from the original homogenate) to obtain the specific activity of the ribonuclease.

Determination of Protein

The method of Lowry et al. (1951) was used for the determination of protein in the supernatant fraction of each brain homogenate (see previous section). An aliquot of each supernatant (0.05 ml) was added to tubes containing 0.5 ml of 1 N NaOH and the tubes were heated for 30 minutes at 100°C. Following the addition of 0.5 ml of doubly distilled water, the tubes were allowed to cool. Four standards (20, 40, 60, and 80 ugms) of bovine serum albumin (50 ug/0.1 ml) were run with each analysis. The standards were brought to a volume of 1 ml with distilled water. To each of the tubes was added 5 ml of a solution consisting of the following mixture: 0.5 ml of 1% $CuSO_4 \cdot 5H_2O$ and 0.5 ml of 2% NaK tartrate with 50 ml of 2% Na_2CO_3 in 1 N NaOH. The reaction mixture was incubated at room temperature for 10 minutes. Then 0.5 ml of diluted Folin reagent

was added and the contents of the tubes mixed rapidly. After incubating 30 minutes at room temperature, the optical density was read at 600 mu on a Spectronic 20 spectrophotometer.

RESULTS

The results of this study are presented in Tables I and II. Table I shows the mean levels of total ribonucleic acid (RNA) from the four brain areas studied, while Table II shows the mean values for ribonuclease (RNase) activity in these same areas.

Total RNA in Unfought Controls

The total amount of RNA in the normal, unfought control differed from area to area, with the highest values found in the cerebellum and amygdala. The lowest value was found in the frontal cortex while the hypothalamus showed an intermediate value.

Total RNA in Defeated Mice

Exposure to daily attack and defeat by trained fighters caused an initial decline (after 1 day) of total RNA in all four brain areas of the defeated mice.

In the amygdala, there was a progressive decline up to 8 days of defeat, at which time, it was 25% below the control value. From the 8 to the 16 day of exposure, the total RNA rose by only 5%, and thus, it still remained 20% below the control level at 16 days of defeat.

The total cerebellar RNA also declined progressively, but only to 2 days of defeat. After 4 days of defeat, a 12% increase was found over the 2 day exposure. This increase reached the control level after 8 days of defeat and then immediately declined again at day 16.

The frontal cortex exhibited a slight decline in total RNA after 1 day of defeat, but this was followed at 2 days by an 11% increase over the

control value. This increase was followed by a slight decline below the control level (5%) at day 4, and declined further after 8 days of defeat. A second peak, 6% above control, again occurred after the 16-day exposure.

In the hypothalamus, the total RNA values showed a variable decline from 1 to 4 days of defeat. However, a 19% increase over the control value occurred on day 8, but was followed at 16 days by a 13% decline. This value at day 16 still remained above the control value by 6%.

Thus, the exposure to trained fighters brought about an initial decline in total RNA in all four brain areas. With the exception of the frontal cortex, the RNA values remained well below control after 4 days of defeat. By day 8, the RNA levels had increased to control value in the cerebellum and exceeded control values in the hypothalamus. On day 16, these values declined, but the hypothalamic RNA content was still greater than control at this time. The RNA content of the frontal cortex exceeded the control value at 2 and 16 days of defeat, with near control levels between these days. The RNA content of the amygdala remained well below the control value throughout the period of 16 days.

Total RNA in Early and Late Fighters

The experienced fighters (late fighters) showed a decline in total RNA in all brain areas except the frontal cortex at the end of 16 days of daily fighting. The RNA content of the frontal cortex was only 2% above the control level, and that of the cerebellum was only 3% below the control level. The hypothalamic and amygdaloid RNA values were 9 and 14% lower, respectively, than control values.

In contrast, the RNA values were greater than controls in all areas except the amygdala after 5 days of aggressive training (early fighters).

The total RNA of the amygdala declined by 10%. The cerebellar RNA content was only very slightly elevated over the control level, while the hypothalamus and frontal cortex showed 7 and 13% increases, respectively.

Thus, the trained fighters exhibited a general decline in the total RNA content of all brain areas except the frontal cortex, while the inexperienced fighters showed a general increase in RNA in all brain areas with the exception of the amygdala.

Total RNA in Hormone-Treated Mice

Dopamine increased the total RNA in all brain areas except the amygdala (3% lower than control). The cerebellar RNA increase was only slightly above the control value, while the hypothalamus and frontal cortex values were 6 and 20% greater, respectively.

Corticosterone administration caused an increase in the total RNA content of all four brain areas. The cerebellum and frontal cortex showed the greatest percentage increases (17 and 20%, respectively).

Serotonin and testosterone brought about an increase in the total RNA in all areas except the cerebellum, which dropped 9% below the control value with serotonin administration and 4% with the administration of testosterone.

Thus, the administration of dopamine, serotonin, and testosterone, in general, increased the RNA content in most brain areas (the exceptions were the amygdala with dopamine and the cerebellum with serotonin and testosterone) while corticosterone elevated the total RNA in all four brain areas.

RNase Activity in Unfought Controls

The greatest RNase activity in the normal unfought control was exhibited by the hypothalamus, while the amygdala displayed the least enzyme activity. Intermediate values were shown by both the cerebellum and frontal cortex.

RNase Activity in Defeated Mice

The RNase activity showed a progressive decline in both the frontal cortex and the cerebellum after 1 and 2 days of defeat by a trained fighter. However, on the 4th day following defeat, the activity in both areas rose well above the control levels (21% increase in the frontal cortex and 23% increase in the cerebellum). By the 8th and 16th day of defeat, the cerebellar RNase activity had dropped progressively, but still remained above the control value. The enzyme activity in the frontal cortex, however, continued to rise and was 26% greater than control by the 8th day of defeat. However, by day 16, this increase in enzyme activity had dropped 5% below the control value.

On the other hand, both the hypothalamus and amygdala showed an increase in RNase activity after 1 day of fighter exposure, 41 and 12%, respectively. By the 2nd day of defeat, both areas exhibited a decline over the 1-day exposure, 32 and 14% lower for the hypothalamus and amygdala, respectively. The enzyme activity in both areas then began to rise progressively, until at 4 and 8 days of defeat the values were 13 and 28% greater, respectively, than control in the hypothalamus and 28 and 41% greater in the amygdala. By the 16th day of defeat, the enzyme activity had declined from the 8-day exposure by 21% for the hypothalamus and 28% for the amygdala. However, these values were still 8 and 15% greater than control levels for the hypothalamus and amygdala, respectively.

RNase Activity in Early and Late Fighters

After 16 days of two fights per day, the RNase activity of experienced (late) fighters exhibited a decline in all four brain areas with the hypothalamus and amygdala showing the greatest percentage decrease (38 and 39%, respectively).

The enzyme activity of inexperienced (early) fighters was slightly elevated over controls in all areas except the frontal cortex.

Thus, while the trained fighters exhibited a decline in the activity of RNase in all four brain areas, the early fighters exhibited a general increase in the enzyme activity in all areas except the frontal cortex.

RNase Activity in Hormone-Treated Mice

The administration of dopamine elevated the RNase activity in all four brain areas with the frontal cortex and amygdala showing the greatest percentage increases of 22 and 17%, respectively.

Corticosterone increased the enzyme activity of the hypothalamus by 12% and decreased the activity in the cerebellum and amygdala only slightly. The frontal cortex remained essentially unchanged.

Serotonin administration brought about an increase of enzyme activity in all four brain areas. The hypothalamic RNase activity was elevated by 20%, the cerebellar and amygdaloid by 17 and 15%, respectively, and finally, the frontal cortical enzyme activity rose by 5%.

Testosterone caused a decline of RNase activity in all areas except the amygdala, which rose only 4% over the control level. The greatest decrease occurred in the cerebellum (23%), and the hypothalamus showed an 11% decline.

Thus, dopamine and serotonin increased the RNase activity in all four brain areas, whereas testosterone decreased the enzyme activity in all areas except the amygdala. Corticosterone caused a decline in two areas, the cerebellum and amygdala, and an increase in the hypothalamus, with the frontal cortex remaining unchanged.

TABLE I

The mean levels of ribonucleic acid (RNA in micrograms/100 milligrams of brain tissue) in different regions of the mouse brain in relation to trained aggression (early and late fighters), defeat (exposure to trained fighters for two 5-minute periods for 0, 1, 2, 4, 8, and 16 days), and 5 days of hormone treatment (with dopamine, corticosterone, serotonin, and testosterone). The standard deviations are also given (± SD).

No. of days of 2 fights/day	Frontal Cortex	Amygdala	Hypothalamus	Cerebellum
Unfought control (0)	128.09 ± 26.99	160.18 ± 30.95	144.45 ± 65.91	160.37 ± 25.47
1	120.61 ± 29.16	125.12 ± 26.30	100.35 ± 23.22	130.84 ± 29.10
2	160.18 ± 19.60	106.38 ± 38.79	128.82 ± 30.70	104.40 ± 20.59
4	117.86 ± 47.05	101.34 ± 10.03	114.93 ± 19.24	134.12 ± 20.11
8	104.21 ± 34.96	96.10 ± 29.03	211.32 ± 53.22	167.32 ± 44.11
16	143.58 ± 33.92	105.66 ± 34.21	164.04 ± 55.85	109.65 ± 54.93
Fighters				
Early fighters	165.72 ± 37.09	131.68 ± 27.75	165.00 ± 26.10	161.14 ± 20.60
Late fighters	133.16 ± 19.20	120.66 ± 22.50	120.61 ± 34.54	151.45 ± 23.27
Hormone Treatment				
Dopamine (200 ug)	191.99 ± 25.62	151.77 ± 24.78	161.91 ± 5.79	167.06 ± 44.50
Corticosterone (200 ug)	191.05 ± 56.93	176.11 ± 32.49	179.47 ± 7.66	226.18 ± 10.96
Serotonin (50 ug)	162.52 ± 35.15	171.80 ± 46.45	166.04 ± 58.87	133.99 ± 23.78
Testosterone (100 ug)	183.33 ± 42.69	182.45 ± 18.97	154.39 ± 55.03	148.46 ± 30.62

TABLE II

The mean levels of ribonuclease (RNase) activity (in counts per minute/microgram of protein) in different regions of the mouse brain in relation to trained aggression (early and late fighters), defeat (exposure to trained fighters for two 5-minute periods for 0, 1, 2, 4, 8, and 16 days), and 5 days of hormone treatment (with dopamine, corticosterone, serotonin, and testosterone). The standard deviations are also given (± SD).

No. of days of 2 fights/day	Frontal Cortex	Amygdala	Hypothalamus	Cerebellum
Unfought control (0)	39.68 ± 5.64	43.25 ± 4.08	54.69 ± 8.55	32.19 ± 7.67
1	38.69 ± 6.74	55.29 ± 13.21	129.82 ± 14.33	31.25 ± 6.98
2	33.68 ± 6.33	41.65 ± 10.00	66.30 ± 16.98	31.02 ± 3.46
4	60.89 ± 9.74	76.46 ± 5.75	70.32 ± 11.75	51.75 ± 3.54
8	66.84 ± 10.98	104.39 ± 15.49	97.97 ± 10.92	46.11 ± 7.51
16	35.88 ± 7.39	59.07 ± 9.22	63.82 ± 9.98	32.39 ± 2.66
Fighters				
Early Fighters	38.88 ± 3.53	50.14 ± 9.84	60.59 ± 12.96	33.57 ± 6.43
Late fighters	38:62 ± 3.38	19.04 ± 10.94	24.57 ± 8.12	28.83 ± 3.19
Hormone Treatment				
Dopamine (200 ug)	61.83 ± 12.67	60.69 ± 10.39	63.21 ± 14.49	36.45 ± 7.43
Corticosterone (200 ug)	39.59 ± 10.61	42.87 ± 5.62	70.22 ± 11.80	30.08 ± 4.27
Serotonin (50 ug)	44.07 ± 4.46	58.50 ± 12.54	82.86 ± 4.90	45.01 ± 3.79
Testosterone (100 ug)	39.07 ± 9.53	47.28 ± 14.66	44.27 ± 14.34	20.08 ± 5.31

DISCUSSION

The effect of a totally novel and stressful situation upon brain RNA clearly can be seen by the decline in total RNA in all four brain areas of mice subjected to their first day of attack by trained fighters. Similar decreases were found by Mihailovic *et al.* (1958) in various parts of the cat brain following electroshock stress. The accompanying increase in RNase activity, found only in the hypothalamus and amygdala at this time, would tend to suggest that some factor, either neurochemical or hormonal, caused an increased activation or production of RNase in these areas. The general elevation of RNase in the hypothalamus and amygdala of defeated mice throughout the 16-day period would tend to indicate that this increase in RNase activity was a result of daily *de novo* enzyme activation or synthesis. However, high levels of RNA would be needed for such enzyme synthesis: therefore, without additional data (e.g., amino acid accumulation and incorporation rates, RNA turnover rates and RNA polymerase activity) one can only speculate at best as to the exact nature of the increase in enzyme activity. Nonetheless, the high level of enzyme activity in the hypothalamus and amygdala would explain the decline of total RNA which occurred in both areas as a result of repeated exposure to aggression and defeat. However, the increased levels of hypothalamic RNA after 8 and 16 days of defeat are inconsistent with the high hypothalamic RNase activity at these same days. A possible explanation for this is suggested later in discussion of RNA levels in early and late fighters.

The RNase activity and the total RNA content in the frontal cortex exhibited a reciprocal relationship throughout the entire 16 days of fighter exposure. The greatest decline in enzyme activity, at 2 days of

defeat, was accompanied by the greatest elevation of total RNA content and the same was true for the reversal at 8 days of defeat.

The RNA of the cerebellum generally remained below the control level from 1 to 16 days of defeat despite the level of enzyme activity. It is interesting to note that whatever the cause in the immediate (after 1 day of defeat) increase in the activity of RNase in the hypothalamus and amygdala, it does not change as rapidly in the frontal cortex or cerebellum until later (i.e., after 4-8 days of defeat). This may implicate the importance of the two former brain regions as areas where integration of incoming stimuli and subsequent processes of adaptation occur initially. Such an adaptation response is seen with fighter training. The early trainees exhibited the same high levels of RNase activity in the hypothalamus and amygdala as did the defeated mice. However, by the time aggressive behavior had become firmly established (i.e., in the late fighters), the RNase activity had declined below the control level, thus implicating the occurrence of some type of adaptation by the time fighters had completed their training.

The general increase in total RNA in the brain areas of early fighters and the general RNA decline in late fighters is inconsistent with data one would expect in view of the RNase activity in early and late fighters. However, it is in agreement with the findings which have shown that neural RNA increases during moderate stimulation (Vladimirov et al., 1961; Baranov and Pevzner, 1963; Talwar et al., 1961; and Attardi, 1957) and declines during decreased stimulation or overstimulation (Mihailovic et al., 1958; Noach et al., 1962; and Talwar et al., 1961). The early fighters are not subjected to the same type of daily attack as are the defeated mice and thus, one could suspect that the early fighters receive a moderate degree

of stimulation while the defeated mice are certainly overstimulated. Nonetheless, the psychological component underlying the degree of stress suffered by these two groups of mice may be very similar, indeed. Certainly, the similarity of the hypothalamic and amygdaloid RNase levels of early fighters and defeated mice would suggest this, especially since both areas, being components of the limbic system, react in similar fashion. However, the situation with late fighters is not as easy to explain and interpret. When training mice to fight by Scott's method (1946), the first stimulus toward aggression appears to be pain and much later the fighter is stimulated by winning every aggressive encounter. Obviously, some type of adaptation has occurred in the process of learning to win every encounter, but because the beaten mouse always runs and rarely challenges the fighter, the fighter's attacks gradually become less and less severe. An aggressive encounter during the later stages of fighting (i.e., between 8 and 16 days of defeat) may be reduced to a simple threat by the fighter. Since all late fighters in this experiment were sacrificed after 16 days of aggressive encounters with "naive" mice, it is easy to observe that they might differ in their degree of stimulation as compared to fighters sacrificed at an earlier time. This decreased stimulation would tend to explain the decline of total RNA which occurred in the amygdala, hypothalamus, and cerebellum of late fighters. By the same token, the defeated mice may adjust physiologically to such a decline of direct daily attacks (i.e., overstimulation) by the fighters. If this were the case, then one or two mild threats by the fighter during such encounters would be enough to keep the defeated mice under a moderate degree of stimulation rather than under severe stimulation. Such an hypothesis would tend to explain the total RNA increase which occurred within the hypothalamus of the

defeated mice on the 8th and 16th days of defeat as well as in the cerebellum on the 8th day.

In studying the data on hormone administration, one's attention has to be drawn to the increases which occur in RNase activity in all four brain areas with dopamine and serotonin administration. This becomes even more significant when one notices that aside from the hypothalamus, the RNase activity in all four brain areas is near or below the control level with corticosterone and testosterone administration. Serotonin is known to be a neurotransmitter while dopamine is a precursor of the neurotransmitter, norepinephrine, and both serotonin and norepinephrine are known to be high in situations of environmental stress. Perhaps, it is these and other neurohumoral agents, released as a result of the stimulus of aggression, which cause the increase in hypothalamic and amygdaloid RNase activity in defeated mice *in vivo* rather than the stimulus of aggression *per se*. However, as one who likes to think that the release of these neurohumors during stress aids the animal in making stress adjustments, it becomes hard to understand how the subsequent increase in RNase activity would be beneficial to such stress adjustments. Such a neurohumoral-induced increase in RNase activity becomes even more difficult to understand in view of the increase which occurred in the total RNA of most brain areas with the same neurohumors, as well as with corticosterone and testosterone.

The administration of the two neurohumoral agents, to some degree, simulates the actual physiological response to aggression since these neurohumors are found in high levels in defeated mice. However, their administration cannot account for the psychological component of the actual aggressive encounter. This can be likened to an *in vitro* versus an *in vivo* situation, where *in vitro* (neurohumoral administration), the total RNA

increased despite the elevated activity of RNase. In vivo (actual exposure to aggression), only the RNase activity increased. This may implicate the existence of a psychological component which is capable of inhibiting the neurohumoral-induced increase in total RNA in specific brain areas. However, the factual information is not adequate at present to justify fully the validity of this implication.

Certainly, further research into the changes which may occur in specific fractions of RNA during aggression and defeat, in addition to RNA polymerase activity and turnover rates, may be very illuminating to our understanding of the complexity of aggressive behavior.

SUMMARY

A study of the effects of aggressive behavior, exposure to aggression and defeat, and hormone and neurohumoral administration on brain levels of ribonuclease and total ribonucleic acid has revealed some interesting differences. The results would seem to agree with the findings that neural RNA increases under conditions of moderate stimulation. Such moderate stimulation is experienced by early fighter trainees or defeated mice during the later weeks of fighter exposure, where a dominant-subordinate relationship may develop between the aggressor and defeated mouse, in which aggression has become confined to one or two mild threats by the fighter. Such conditions of moderate stimulation are accompanied by increases in the activity of RNase in these same animals. Because the activity of RNase increases with dopamine and serotonin administration, these neurohumors possibly may be the cause of the increased enzyme activity in defeated mice, where they are known to be at elevated levels. It is difficult to understand, however, why these same neurohumors and the hormones, corticosterone and testosterone, elevate the total RNA in most of the brain areas studied.

Conditions of overstimulation or decreased stimulation such as in the early stages of defeat or in late fighters, respectively, resulted in a general decline of total RNA in most of the four brain areas. Furthermore, overstimulation produced a general elevation in the activity of RNase whereas a decline of stimulation resulted in the decline of total RNA in late fighters.

Generally, it was found that by using aggression as an environmental stimulus, profound effects were evident, particularly in RNase activity. Whether such changes can be attributed directly to the stimulus of

aggression or indirectly to the action of hormones or neurohumoral agents is not clear at the present time. The significant changes observed in RNase activity as a result of aggression, defeat, and hormonal activity may provide a possible avenue of research toward a better understanding of the neural RNA changes which so many have found with neural stimulation. Certainly, the further study of macromolecular changes which do or do not occur during aggression and defeat may help to elucidate the very nature of such complex behavior as aggression.

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APPENDIX

PHOSPHATE BUFFERED SALINE (PBS)

Basic Solutions:

Solution A

$Na_2HPO_4 \cdot 7H_2O$ . . . . . 268 gms in 2 liters of water

or

$Na_2HPO_4 \cdot 12H_2)$ . . . . . 358.22 gms in 2 liters of water

Solution B

$NaH_2PO_4 \cdot H_2O$ . . . . . 138.06 gms in 2 liters of water

Buffer Stock, pH 7.15

Solution A . . . . . 386 ml

Solution B . . . . . 114 ml

Deionized water . . . . . 500 ml

Buffered Saline, pH 7.2 (2 liters)

NaCl . . . . . 17 gm

Buffer stock . . . . . 80 ml

Deionized water . . . . . 1920 ml

Autoclave 10 lbs for 10 minutes.

AQUEOUS SCINTILLATION FLUID

2, 5-diphenyloxazole (PPO) . . . . . 5 gm

Napthalene . . . . . 80 gm

Toluene . . . . . 360 ml

Dioxane . . . . . 360 ml

Absolute Ethanol . . . . . 216 ml

THE EFFECTS OF AGGRESSION, DEFEAT, AND HORMONE ADMINISTRATION ON REGIONAL BRAIN LEVELS OF TOTAL RIBONUCLEIC ACID AND RIBONUCLEASE ACTIVITY IN C57BL/6J MICE

by

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ABSTRACT

As a result of the findings by others that neural ribonucleic acid increases with moderate stimulation and declines with decreased stimulation or overstimulation, it was thought that aggression might be a useful tool with which to study the effects of a stressful environmental stimulus on regional brain levels of ribonucleic acid (RNA) and ribonuclease (RNase) activity in C57BL/6J mice. An attempt to elucidate the possible role of hormones in this phenomenon was undertaken by the administration of dopamine, serotonin, corticosterone, and testosterone, nearly all of which are high in the stressed animal.

The significant increases in RNase activity that occurred in all four brain areas with dopamine and serotonin administration suggested that similar increases in the hypothalamus and amygdala of defeated mice may result from neurohumoral induction rather than the stimulus of aggression per se. However, the increases in total RNA, which occurred with all hormones administered, did not manifest themselves in defeated mice. This was to be expected in view of the elevated activity of RNase but, nonetheless, the increases in both RNA and RNase activity with hormone administration certainly obscure the role of hormones in this situation.

The possible role of a psychological component, capable of inhibiting the hormone-induced increase in RNA during the actual agressive encounter, was considered. However, the data is not adequate at present to justify such an assumption.

The results did show a definite effect of aggressive stimulation on regional brain levels of RNA and RNase activity. During the early

stages of fighter exposure, when the "naive" mice were under a severe degree of stress or overstimulation, the RNA levels of the hypothalamus and amygdala were elevated while RNase activity declined. During the later weeks of fighter exposure, a dominant-subordinate relationship may develop between the aggressor and the defeated mouse, where agression is confined to one or two mild threats by the fighter. Thus, the defeated mouse is receiving only a moderate degree of stimulation from the fighter, whereas the fighter is receiving a decreased amount of stimulation from the defeated mouse. It was under these conditions that the RNA increased in the 8-16 day defeated mice and declined in the late fighters. RNase activity also declined in late fighters, suggesting again the possibility of a hormonal relationship to the degree of stimulation.

The early trained fighters, existing under a more moderate degree of stimulation than the early defeated mice, also showed increases in total RNA. Furthermore, the increase in RNase activity which they exhibited, once again, leads one to speculate on a hormone influence upon RNase activity relating to the degree of stimulation.

Certainly, further research into the changes which may occur in specific fractions of RNA during aggression and defeat, in addition to RNA polymerase activity and turnover rates, may be very illuminating to our understanding of the complexity of aggressive behavior.