The Relationship Between Whole Brain Catecholamine Depletion in Carassius auratus and the Exposure to Inescapable Shock in a Learned Helplessness Paradigm

Roderick J. Misunis

Eastern Illinois University

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THE RELATIONSHIP BETWEEN WHOLE BRAIN CATECHOLAMINE DEPLETION IN CARASSIUS AURATUS AND THE EXPOSURE TO INESCAPABLE SHOCK IN A LEARNED HELPLESSNESS PARADIGM

(TITLE)

BY

Roderick J. Misunis

B.Sc., University of Alberta, 1976

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Arts in Psychology

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

1983

YEAR

I HEREBY RECOMMEND THIS THESIS BE ACCEPTED AS FULFILLING THIS PART OF THE GRADUATE DEGREE CITED ABOVE

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COMMITTEE MEMBER

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COMMITTEE MEMBER

3/16/83
DATE

DEPARTMENT CHAIRPERSON
THE RELATIONSHIP BETWEEN WHOLE BRAIN CATECHOLAMINE DEPLETION IN CARASSIUS AURATUS AND THE EXPOSURE TO INESCAPABLE SHOCK IN A LEARNED HELPLESSNESS PARADIGM

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ABSTRACT OF A THESIS

Submitted in partial fulfillment of the requirements for the degree of Master of Arts in Psychology at the Graduate School of Eastern Illinois University.

CHARLESTON, ILLINOIS
1983
Abstract

Learned helplessness is a psychological concept that describes the subsequent escape-avoidance behavior of experimental subjects who are exposed to uncontrollable stressors. Subjects after treatment are unable to respond in situations where escape is possible. Two major theorists, Seligman and Weiss, have proposed explanations concerning the phenomena. Seligman feels that the subject is unable to respond due to the fact that the exposure to an uncontrollable stressor has caused him to learn to be "helpless". Weiss feels that the inability to respond can best be described by alterations in the subject's brain neurochemistry, specifically the neurotransmitter norepinephrine. This paper is an attempt to verify the J. M. Weiss theory of learned helplessness with goldfish.

Seventy-two experimentally naive goldfish were randomly assigned to the experimental conditions: A no shock control, conditioning, inescapable shock, and a group receiving inescapable shock and conditioning. These experimental conditions were tested at six different levels of increasing shock duration. The first three shock durations were at 6 volts and the longer three durations were at 10 volts. Twenty fish that received conditioning as part of their treatment had the number of escapes and avoidances tabulated for each of their conditioning trials. After each subject received its experimental condition it was immediately sacrificed and had its brain extracted. The brain was then assayed for three neurochemicals: norepinephrine, dopamine and epinephrine using High Pressure Liquid Chromatography with electrochemical detection.

The experimenter found for 72 subjects an overall mean of 460 ng/g and
standard error of 18.8 ng/g for norepinephrine and a mean of 180 ng/g and 
a standard error of 8.3 ng/g for dopamine. In only 44 percent of the 
144 samples was it possible to detect at least trace amounts of epinephrine.

The four by six factorial failed to detect an overall significant dif-
ference among the four learned helplessness treatment groups for both 
norepinephrine and Dopamine. Orthogonal contrasts comparing the groups 
also found nonsignificant differences. The six shock duration levels did 
produce significant main effect differences for both norepinephrine and 
dopamine. But since the apparent inverted quadratic function applied to 
the no shock controls, this finding is probably an artifact of different 
sized fish used at different levels of shock duration.

The partial correlation between amount of norepinephrine and dopamine 
found in fish brains adjusted for brain weight was $r (69) = .279$, 
$p = .009$.

Nonsignificant results were found when correlating the two neurochem-
icals to trials, escapes and avoidances. Comparisons of the two groups 
that received conditioning on avoidances, escapes and the number of fish 
achieving the criteria of two avoidances were nonsignificant. Proposals 
for future neurotransmitter depletion research using goldfish were made.
Acknowledgements

I would like to express my sincerest thanks and deepest gratitude to my advisor, Dr. John Rearden, without whose constant and untiring guidance this thesis would not have been possible. Further, I would like to thank Dr. Frank E. Hustmyer for being on my thesis committee and considering my manuscript. In addition I would like to thank Dr. David H. Buchanan whose analytical ability to investigate difficulties and willingness to afford time and guidance to this project were invaluable and are greatly appreciated. I would also like to thank Dr. Russell W. Carlson for the use of laboratory equipment and expert consultation at various stages of this project. I would finally like to thank the many Chemistry graduate students who offered help and assistance to a naive psychologist awkwardly making his way through the world of HPLC.
Dedication

I am dedicating this thesis to my mother and to my fiancée, Sharon Boyco, without whose love and support I would not have been able to complete this project.
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Statement of Problem

Over the course of the past sixteen years, a sizable body of research has accumulated around the concept of "learned helplessness." The learned helplessness phenomenon can be traced to an article by Overmier and Seligman (1967) in which a relationship between inescapable shock and subsequent escape/avoidance learning was hypothesized. In this investigation three experiments using dogs as subjects were conducted. These studies began with one group of dogs receiving a single treatment session of 3 hours duration in which 60 severe inescapable shocks of 5 seconds duration and of 6mA intensity were administered. A second group of dogs did not receive inescapable shock but later were tested in a shuttlebox. Afterwards, the dogs that were given inescapable shock were placed in a shuttlebox in which escape was possible. The dogs not given inescapable shock learned the shuttlebox task without difficulty. The dogs receiving inescapable shock prior to the shuttlebox task demonstrated significant decreases in their ability to escape in the shuttlebox. This interference of subsequent escape/avoidance learning was termed "learned helplessness" (Overmier, 1968; Overmier & Seligman, 1967; Seligman & Maier, 1967).

Seligman and Maier (1967) had assumed that the restrained dogs which received uncontrollable shock learned that their behavior was ineffective, and as a result learned to be "helpless" (Weinraub, 1979).

A different opinion of the learned helplessness phenomenon was advanced by Weiss (1970, 1974, 1981) who theorized on the basis of his investigations that maladaptive responses to situational stress were evidenced by an inability to respond to uncontrollable stressors. One of
these maladaptive responses was identified as "stress-induced depression," a condition synonymous with learned helplessness.

Weiss, investigating the relationship between stress-induced depression (learned helplessness) and uncontrollable stressors (inescapable shock), found that rats, when exposed to shock over which they have no control, exhibit a subsequent disruption in the function of brain catecholamines, specifically in the neurotransmitter norepinephrine. Weiss felt that a better explanation of the learned helplessness phenomenon was that a physiological change takes place in the brain after inescapable shock occurs, the subjects' subsequent decrement in performance on the shuttle box task being explained as due to a depletion in the neurotransmitter norepinephrine. This depletion is viewed as an interference of the acquisition of avoidance behavior to electrical shock in the shuttle box. These differing explanations of the learned helplessness phenomenon have lead to vigorous polemics between the two principle authors, Seligman and Weiss (Weinraub, 1979).

Learned helplessness has been demonstrated to occur in a number of mammalian species such as rats, dogs, and cats (Seligman, 1976). However, only a few studies have substantiated its occurrence in goldfish (Padilla, Padilla, Ketterer & Giacolone, 1970). The goldfish (*Carassius auratus*) has been the subject of extensive neurochemical research (Olson, Kastin, Montalbano-Smith, Olson, Coy & Michell, 1978). The neurochemical norepinephrine and its relationship to learned helplessness has not been studied in goldfish.

This thesis is an attempt to verify the norepinephrine depletion explanation of learned helplessness in a species other than mammals by an examination of whole brain catecholamines in goldfish that have gone through the learned helplessness paradigm. The learned helplessness
treatment was the presentation of inescapable shock prior to conditioning. This experiment will add to the large body of knowledge already accumulating under the topic of "Learned Helplessness."
The following topics will be reviewed in this chapter: Norepinephrine and catecholaminergic function, synthesis, and metabolism; theories of norepinephrine depletion; learned helplessness in goldfish; and the use of goldfish in neurochemical studies.

Norepinephrine and Catecholamine Metabolism and Function

In this section the physiological basis of norepinephrine and the related catecholamines is discussed, highlighting its metabolism and action at the receptor site.

Norepinephrine is one of the monoamines. It is a catecholamine (as opposed to an indoleamine). Catecholamines are a class of molecules that contain a catechol nucleus (a benzene ring with two adjacent hydroxyl substituents) and one amine group. The neurotransmitters norepinephrine, dopamine, and epinephrine are collectively known as the catecholamines. Figure 2 represents the chemical structures of the three catecholamines. In this thesis the abbreviations for norepinephrine will be N.E., for dopamine D.A., and for epinephrine E.

The synthesis of the catecholamines involves four enzymes. The precursor for N.E. and the other catecholamines is the amino acid tyrosine. Phenylalanine is converted to tyrosine in the liver by the action of Phenylalaninehydroxylase. Phenylalanine does not cross the blood-brain barrier and therefore its action in the synthesis of the catecholamines can only be termed as an "indirect precursor" (Green & Costain, 1981). The amino acid tyrosine is hydroxylated (addition of a hydroxyl group) to dopa via tyrosine hydroxylase. Tyrosinehydroxylase requires molecular
oxygen, a pteridine cofactor and iron \( \text{Fe}^{2+} \) for its activity. This is the rate-limiting step for the synthesis of the catecholamines. Rate-limiting is the slowest step in the reaction which determines the overall rate of synthesis of the end product of the pathway, thus the level of tyrosine hydroxylase available ultimately determines the rate of catecholamine production. Dopa is then decarboxylated (removal of a carboxyl group) to D.A. by dopa decarboxylase. The dopa and D.A. steps in the catecholamine synthesis occur in the cytoplasm of neurons. D.A. is a neurotransmitter in some neurons. D.A. is converted to N.E. by another hydroxylation enzyme, dopamine-beta-hydroxylase. D.A. is selectively taken up by storage vesicles where it can be \( \beta \)-hydroxylated and synthesized to N.E. Dopaminergic neurons do not have dopamine-beta-hydroxylase so that no N.E. is synthesized in these cells. In the adrenal gland a methyl group is added via phenylethanolamine-N-methyltransferase to produce E.

Tyrosine hydroxylase is sensitive to the concentrations of its end products, D.A. and N.E. High levels of D.A. and N.E. regulate the system by inhibiting the activity of the enzyme so that less D.A. and N.E. are then synthesized. When N.E. and D.A. are at low levels the enzyme causes the production of more D.A. and N.E. Tyrosine hydroxylase is also activated by \( \text{Ca}^{2+} \) and AMP which stimulate enzyme activity by increasing the affinity constants for the substrate so that the enzyme binds more tyrosine and synthesizes more dopa.

"Once N.E. re-enters the synaptic cleft after release it is finally degraded by two enzymes that exist there: Monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). MAO metabolizes D.A. to 3,4-dihydroxyphenylacetaldehyde and N.E. to 3,4-dihydroxyphenylglycolaldehyde. These aldehydes are then dehydrogenated to the corresponding acids dihydroxyphenylacetic acid (DOPAC) and dihydroxymandelic acid (DOMA) and these are methylated by COMT. If first acted on by COMT they are methylated to 3-methoxytyramine or normetanephrine involving the methyl donor system: \( S \)-adenosyl methionine. The end product for D.A. is homovanillic acid (HVA) and for
N.E. it is vanillylmandelic acid (3-methoxy-4-hydroxymandelic acid, VMA).

In the case of N.E. metabolism, 3,4-dihydroxyphenylglycolaldehyde can be reduced by aldehyde reductase to 3,4-dihydroxyphenylglycol (MOPEG or MHPG). This compound exists in the urine both in the free state and as a sulfate ester conjugate. There have been several studies suggesting that in some animal species including man MOPEG or its sulfate ester are the main N.E. metabolites in the brain, and it has further been suggested that most urinary MOPEG is formed from the brain with VMA being the peripheral N.E. metabolite."

(Green & Costain, 1981, p. 23)

**Postsynaptic Function**

The neurotransmitter, N.E. or D.A., is synthesized within the neuron, stored in vesicles of the nerve terminals and released from there by nerve impulse.

"The released neurotransmitter (N.T.) activates a N.T.-sensitive adenylate cyclase present in the membrane of the postsynaptic cell, leading to the production of cyclic AMP in the immediate vicinity of the postsynaptic membrane. The newly formed cyclic AMP activates a cyclic AMP-dependent protein, kinase, present in the post-synaptic membrane. This activated protein-kinase catalyzes the phosphorylation of a substrate protein, also present in the post-synaptic membrane, converting it from the nonphosphorylated to the phosphorylated state. A key element of this model is that this substrate protein controls the permeability of the post-synaptic membrane. Phosphorylation of the substrate protein leads, through a change in either ion conductances or in the rate of an electronic pump, to a change in the membrane potential. The terminating steps in this sequence are the enzymes phosphodiesterase that hydrolyzes the cyclic AMP to 5' AMP and a phosphoprotein phosphotase that converts the substrate protein back to the non-phosphorylated form, leading to the termination of the post-synaptic potential." (Greengard, P., 1976, p. 104.)

**Catecholamines in the Brain**

In the human brain, D.A. is located in three major systems: First in the nigrostriatal pathway with cell bodies in the substantia nigra, zona compacta and projecting into the striatum containing the nucleus caudatus (caudate nucleus) and putamen. Second, a system which has cell bodies in the interpeduncular nucleus and project on a tract lying close
to the nigrostriated tract to the mesolimbic forebrain (nucleus Accumbens) and olfactory tubercles. The third and final system is that of the tuberoinfundibular system with the cells mainly in the arcuate nucleus and projecting to the median eminence.

Cell bodies containing norepinephrine are present in the pons medullary region. Noradrenergic projections to the forebrain pass along the ventral and dorsal bundle and N.E. has a wide distribution including the forebrain, hypothalamus and cerebellum.

Epinephrine has two major cell body groups in the lateral segmental system and dorsal medulla, with projections to the hypothalamus, locus coeruleus and spinal cord.

No mapping of the N.E. or associated catecholamines in the neuroanatomy of the goldfish brain has been done.

**Norepinephrine Depletion Studies**

Although there are many ways to experimentally deplete N.E. in an animal's brain, by far the most popular technique has been the use of a neurotransmitter toxin called 6-Hydroxydopamine (6-OHDA). 6-OHDA is closely related to N.E. and D.A. and will be taken up selectively by catecholaminergic-containing neurons.

When the drug has reached a sufficient cytoplasmic concentration, it kills the catecholamine-containing neuron. It can be injected directly into specific regions of the brain or injected intraperitoneally where it will also destroy the peripheral autonomic nervous system noradrenergic neurons.

In a review of the literature on N.E., Mason (1981) reviewed several theories of N.E. action. He presented supporting evidence and negative evidence for each theory.
The most promising theory is Mason's theory of attentional dysfunction. This theory states that the basic function of noradrenergic neurons in the dorsal bundle is to screen out incoming sensory stimuli by classifying them as being relevant or irrelevant to the task at hand.

A consequence of the lesion-induced loss of this filtering mechanism is that animal subjects treated with 6-OHDA are forced to sample more stimuli in the environment than are controls. The preponderance of stimuli cause a greater number to be associated with reinforcement during acquisition. During extinction there are more Stimulus-Response links to maintain, owing to increased stimulus sampling during acquisition which causes the animal to continue to respond much longer than a control. The basic problem for the lesioned animal is that it does not know which stimuli are relevant and which are irrelevant, thus he is unable to ignore irrelevant stimuli and responds to all stimuli. An important consideration for this theory is that innate salience of the stimuli must be taken into account. If a stimulus to which the animal responds innately is used, no effect will be noticed. Also, motivational states can have an effect on attention and could easily "swamp" the effects of a brain lesion by focusing attention to the relevant stimuli. By this means, motivational states may act to overcome the effects of the brain lesion (Mason, 1981).

The syndrome of forebrain N.E. depletion as seen in experimental rats elicits the following behaviors: Increased distractibility, a perseveration of inappropriate behavior, and a failure to filter out irrelevant stimuli. Mason implicates the role of N.E. depletion in schizophrenia, Korsakoff's psychosis and the attentional deficit disorder. He proposes that in schizophrenia there may exist an antagonistic D.A.-N.E. interaction. D.A. may work in opposition to the attentional role of N.E. It has been observed that D.A.-depleted rats show sensory neglect (Marshall,
1976; Ungerstedt, 1971; and Marshall, 1971) compared to the N.E.-depleted rat which has been observed to sample too many stimuli in its environment. Neuroleptics may be therapeutic in the treatment of schizophrenics by helping to partially restore a D.A.-N.E. balance (Antelman & Caggiula, 1977). It is suggested that a deficit in dorsal bundle N.E. might give rise to some of the attentional alterations seen in human schizophrenics.

Cassens et. al. (1980) have reported that changes in N.E. can be elicited through classical conditioning implicating environmental manipulation in the etiology of neurotransmitter dysfunction.

In a study using rats exposed to uncontrollable stress (shock), Weiss (1976) found depressive behavior as outlined in the DSM III classification of symptoms of depression. From this he theorized that such stress-induced depression in rats represents a good model of clinical depression in humans.

In 1981, Weiss, Goodman, Giardina, Corrigan, Cherry, & Bailey reported an experiment in which the effect of uncontrollable shock on behavioral responses as well as catecholamine levels in many regions of the brain was measured. They found that this response decrement "depression" correlated more with depletion of N.E. within the brain stem than with any other neurochemical charge. Weiss determined that the area of maximal N.E. depletion is the Locus Coeruleus region of the brainstem. Weiss did not find that changes in other neurotransmitters (D.A. and serotonin) were related to stress-induced depression.

Weiss feels that stress, usually operationalized as uncontrollable shock, causes a dramatic rise in the release of N.E. Because postsynaptic N.E. receptors (alpha-2) can't utilize all of the N.E., it is released back into the synaptic cleft, reabsorbed by the neuron and destroyed, causing a lowering of N.E. levels.
Weiss (Note 1) correlated the ability of animal subjects to recover from helplessness and the time required for induction of tyrosine hydroxylase in the locus coeruleus region of the brain, which causes the synthesis of more N.E. in that area. Weiss claims that this finding coincided with recent observations that electro-convulsive shock treatment has a therapeutic effect.

Weiss feels that stress-induced depression can be overcome by the direct injection into the brain of drugs that stimulate the alpha-2 receptors. Other studies have implicated the neurotransmitters serotonin and acetylcholine along with N.E. in the process of depression (Anisman & Remington, 1979; McRae-Deguearce & Pujol, 1979; Vizi, 1980).

Weiss proposes two models which explain the effects of learned helplessness. The first is called the N.E. depletion hypothesis in which a brief, very strong uncontrollable stressor can disrupt normal catecholamine functioning and temporarily impair the mediation of motor behavior. The second model describes the effects of a long-term stressor (long-duration shock). Weiss states that exposure to shock of long duration results in inactivity in the rat because the rat, through an associational process, pairs termination of shock with his inactivity. Weiss feels that brain N.E. plays an important role in mediating the ability of an animal to perform actively when presented with an appropriate stimulus. The long shock will disrupt this mediator and cause an inability to perform active motor tasks.

Interesting questions have arisen concerning the threshold needed to produce a N.E. effect in the brain. Weiss found that some shock durations do not produce the learned helplessness effect in studies where the shock only lasted two, three, and four seconds, but with shocks up to five and six seconds the effect became evident (Weinraub, 1980). Seligman has
reported learning deficits in dogs exposed to half a second or five seconds of inescapable shock. Kelsey (1977) and Altenor, Key & Richter (1977) have reported deficits with shock exposures of less than three seconds' duration. Anisman, Decatanzaro & Remington (1978) have found that the long-term deficit does not occur when one uses inescapable shocks of short duration (two seconds), but that it is critical to use a shock of six seconds to obtain an effect in a one-hour shock session. From the previous discussion we can see the exact amount of shock needed to obtain an effect is still in question.

Some investigators have implicated N.E. in a number of human mental and physical states; for example, the amount of N.E. turnover and the ability of a person to operate in a state of aroused power motivation (McClelland, 1980). Ward (1982) has implicated N.E. in pain and depression. Finally, increases in N.E. have been correlated with anxiety and panic disorders (DeVito, Note 2).

**Learned Helplessness in Goldfish (Carassius auratus)**

Maier & Seligman in a 1976 review of the literature on the topic of "learned helplessness" found that the learned helplessness effect had been demonstrated in a number of mammals and in goldfish. Only two published studies and one unpublished master's thesis have been done using goldfish as subjects for a learned helplessness paradigm of inescapable shock. Padilla, Padilla, Ketterer & Giacolone (1970), using 28v of inescapable shock in two experiments, found that the interference with subsequent escape/avoidance conditioning was still present 48 hours after treatment presentation. The effect did dissipate after 72 hours which agrees with the Weiss findings (1981) that the learned helplessness effect will disappear after 48 hours. Padilla (1973), using a higher shock level of 45v,
again found that a learned helplessness effect could be produced with prior or interpolated exposures to inescapable shock. He found that a learned helplessness effect could be produced in goldfish when unsignaled inescapable shock is interpolated between escape/avoidance conditioning. He also found that the learned helplessness effect could also be produced with prior inescapable shock. In a recent paper, Kimbara (Note 3) tested the hypothesis that there may be a difference between prior and interpolated shock exposure and found that prior inescapable shock did affect subsequent escapes and avoidances. The use of interpolated shock, however, produced a significant decrement in the performance of fifty-three of the ninety-eight fish in this study.

There is a problem of determining the level of shock intensity necessary to produce learned helplessness effects. In the Padilla studies very high shock intensities (28v and 45v) were used whereas Bintz (1971) found that using a shock level of 18v in avoidance conditioning in goldfish was lethal for some of the fish tested in his experiment.

The optimal level of shock intensity to be used in avoidance conditioning of goldfish was found to be between 6v and 7v (Behrend & Bitterman, 1963; Gallon, 1972; and Scobie & Herman, 1972). Bintz (1971) and Zerbolio & Wickstra (1975) proposed a higher level (9v to 15v) as the optimal escape avoidance conditioning in goldfish. Kimbara's unpublished thesis (Note 3) failed to find significant differences between shock levels of 6v and 10v.

**Neurochemical Studies with Goldfish as Subjects**

Although goldfish have not been used as subjects in a neurochemical learned helplessness experiment specifically, goldfish have been used to assess whole brain N.E. Bogdanski, L. et. al. (1963) assessed the levels
of two neurotransmitters, N.E. and 5HT. He found a level of 490 ng/g of N.E. in the brain of the goldfish (C. auratus). In a Russian study on oil pollution, Vosilene, N. E. (1978) found values of 234 ng/g-176 ng/g of N.E. in the whole brain of goldfish controls over a three-month period. Goldfish have been used in pharmacological experiments dealing with learning. Satake (1978) studied the effect of puromycin on the retention of specific learning tasks which included avoidance conditioning. Tate, D. S., Galoan, Louis, Urgar & Georges (1976) used goldfish to isolate and identify two learning-induced brain peptides. Kaplan (1975) used goldfish in his studies on the biochemical correlates of behavior. Kaplan found that neurochemical lesion effects the subsequent performance of goldfish on learning tasks. Richard D. Olson et. al. (1978) used goldfish to test the effect of 21 neuropeptides injected intracranial or intraperitoneally on general behavior as measured by a swimming test. Finally Dan C. McIntyre et. al. (1979) measured the whole brain monoamine levels of trout fingerlings. McIntyre's results are incongruent with Weiss' (1981) observations that there is a decrease of N.E. in rats which show signs of depressive behavior. McIntyre's study is of interest in that he was able to measure catecholamines in a fish. He found that when the dominance hierarchy was determined in six groups of rainbow trout fingerlings, the dominant fish exhibited significantly lower levels of N.E. and higher levels of D.A. than the submissive members.

Although learned helplessness has been shown to exist in goldfish, no studies have been done to investigate the neurochemical connection in the phenomena of learned helplessness. This experiment is designed to assess the behavioral and neurotransmitter effects of inescapable and escapable shock on goldfish. As a test of the Weiss depletion theory, the following hypotheses are proposed:
1. There will be a significant difference in the level of N.E. in the treatment groups.
   
a. There will be a significant difference in N.E. between the control group which receives no shock and the groups that receive conditioning and inescapable shock.
   
b. There will be a significant difference in N.E. between the group that receives conditioning and the group that receives inescapable shock.
   
c. There will be a significant difference between the groups that receive inescapable shock or conditioning and the group that receives both.

2. There will be a significant reduction in the following measures: trials, avoidances and escapes and trials to criteria between the group that receives conditioning and the group that receives both inescapable shock and conditioning.

3. There will be a significant decrease in N.E. as the shock duration is increased.
Methods

Subjects

The 72 goldfish (Carassius auratus) used in the study were acquired in three different purchases that were made approximately two weeks apart from a local dealer. The first lot consisted of 36 large fish (wt. $\bar{M} = 9.67g$, S.D. = 2.37). The second lot consisted of 27 small fish (wt. $\bar{M} = 2.30g$, S.D. = .50). The final lot consisted of 9 medium size fish (wt. $\bar{M} = 5.00g$, S.D. = 1.28). The fish were housed in 10-gallon aquaria maintained at 76°F.

Table 1. The Mean, Standard Deviation, and Range of the Body Weight and Brain Weight for the 72 Goldfish.

<table>
<thead>
<tr>
<th></th>
<th>M</th>
<th>S.D.</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Wt. (g)</td>
<td>6.366</td>
<td>3.857</td>
<td>1.460 - 15.470</td>
</tr>
<tr>
<td>Brain Wt. (g)</td>
<td>.077</td>
<td>.034</td>
<td>.031 - .162</td>
</tr>
</tbody>
</table>

The first six fish had the two brain samples analyzed 24 hours apart. For all other fish the two samples were analyzed consecutively. Due to errors in the work-up procedure, five fish had to be replaced. These replacement fish were run on the following day in addition to the scheduled fish.
Apparatus

All treatment fish were tested in the Lafayette Aquatic A-660 type shuttle tank. Lafayette apparatus consisted of a plexiglass container which held approximately one gallon of water. A plastic barrier with an opening in it separated the two sides of the tank. Shock could be given selectively to either side as electrode plates are positioned horizontally on both sides. For fish which received inescapable shock a glass barrier was inserted between the halves of the tank. The shuttle tank was connected to two Hunter timers which pulsed the shock with a 1.5-second interpulse interval. The amount of shock was cumulative for each fish. The shuttle tank was surrounded with cardboard with a small aperture for observation. Shock intensities of 6 volts A.C. and 10 volts A.C. were used. The water in the apparatus was maintained at a constant pH of 7.6.

The bioassays of the goldfish brain were done by the method outlined by Keller, Oke, Mefford and Adams (1976) and an LCEC Application Note.

Calibration of the HPLC. The data were analyzed by the method of internal standard. The synthetic chemical 3,4-dihydroxybenzylamine (DHBA) was chosen as an internal standard because it was related structurally to norepinephrine, epinephrine and dopamine. DHBA separates in the column in the same general region but does not overlap with any of the catecholamines. DHBA did not react with any of the components in solution. DHBA's solubility, volatility and detector response are very close to that of the catecholamines.

After the choice of DHBA as the internal standard had been made, two solutions of differing known concentrations were prepared from solutions 3 and 4 (see Reagents section). The values are presented in Table 2.
Table 2. The Concentration, Retention Time, Average Area, Slope and Intercept for Norepinephrine, Dopamine, Epinephrine, and the Internal Standard.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Sample</th>
<th>RT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>k</th>
<th>b</th>
<th>Area</th>
<th>Concentration</th>
<th>Area</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.E. (ng/mL)</td>
<td>7</td>
<td>2.13</td>
<td>1.198</td>
<td>.04261</td>
<td>2054571.5</td>
<td>40.92</td>
<td>1364302.5</td>
<td>27.28</td>
</tr>
<tr>
<td>D.A. (ng/mL)</td>
<td>8</td>
<td>6.86</td>
<td>.7265&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-.022&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4723396</td>
<td>59.59</td>
<td>3194406</td>
<td>39.73</td>
</tr>
<tr>
<td>E. (ng/mL)</td>
<td>7</td>
<td>3.36</td>
<td>.00053</td>
<td>.4471</td>
<td>1089530.5</td>
<td>23.62</td>
<td>1371031</td>
<td>31.49</td>
</tr>
<tr>
<td>I.S. (ng/mL)</td>
<td>8</td>
<td>4.17</td>
<td>1</td>
<td>0</td>
<td>3361319.5</td>
<td>52.81</td>
<td>4636445.5</td>
<td>70.41</td>
</tr>
</tbody>
</table>

<sup>a</sup>RT = Retention Time  
<sup>b</sup>These figures were calculated manually.

These standard solutions were injected into the HPLC and from the relative areas of the peaks produced, values for the slope of the line and intercept were programmed into the Shimadzu CR-1B Integrator. The following equation was used to calculate the weights of the catecholamines in each sample:

\[ W_x = W_s (b + k \frac{A_x}{A_s}) \]

where \( W_x \) = weight of the unknown in the sample solution, \( W_s \) = weight of the internal standard (DHBA) known, \( b \) = intercept on y axis, \( k \) = slope of the line determined through calibration run, \( A_x \) = area of sample, \( A_s \) = area of the internal standard.

Injections of brain solution were then made. Each injection produced a trace on which each catecholamine was represented as a spike on a trace base-line. The areas of these catecholamine spikes were always compared to the area of the internal standard.

The weights and retention times for N.E., D.A. and E. were programmed into the recorder after the calibration runs. Added to each brain sample was 300 µL of internal standard. The recorder measured the areas of
catecholamine and internal standard, completed the above equation and made automatic calculations for the amount of N.E., D.A. and E. in each sample solution.

Reagents

Dilute perchloric acid was made up by diluting 8.55 mL of 70% perchloric acid to 1.00 L; this produced the required 0.1 M HClO₄.

The internal standard—3,4-dihydroxybenzylamine (DHBA)—was made up by dissolving 0.0165 g of DHBA in 100 mL of 0.1 M HClO₄. The solution was then mixed well for approximately 10 minutes. A 100 µL sample was diluted to 100 mL with 0.1 M HClO₄ producing the required 100 mg/mL DHBA solution. The final DHBA solution was kept refrigerated.

The standard solutions for E., D.A. and N.E. were made in two separate solutions: Standard 3 was made by adding 0.0155 g of N.E. bitartrate and 0.0147 g of 3,4-dihydroxyphenylethylamine hydrochloride salt to 100 mL of 0.1 M HClO₄. The second standard, standard 4, was made by adding 0.0087 g of E. bitartrate and 0.0165 g of 3,4-dihydroxybenzylamine hydrobromide to 100 mL of 0.1 M HClO₄. The contents were mixed until all the components were dissolved. Both standards were then refrigerated in the Chemistry Department cold room.

Calculations for each of the components were done to obtain the molecular weight as a free base: 3,4-dihydroxybenzylamine hydrobromide — mol. wt. = 220.1, as a free base 139.1; E. bitartrate — mol. wt. = 333.3, as a free base 183.21; 3,4-dihydroxyphenylethylamine hydrochloride salt — mol. wt. = 189.6, as a free base 153.1; and N.E. bitartrate — mol. wt. = 319.3, as a free base 169.21.

The tris buffer/EDTA was made up by the combination of 45.0 g of tris base (Sigma T-1503), 5.00 g Na₂EDTA and approx. 200 mL deionized water. The mixture was stirred until all the solids were dissolved. A constant pH
was obtained by the addition of concentrated HCl dropwise, until a pH of 8.6 was registered on a pH meter. The solution was then diluted to 250mL and refrigerated in the cold room.

The alumina oxide AAO(Rainin) used had not been washed with acid as required by the bioanalytic procedure. Therefore 20.00g of the alumina was washed twice with 0.1M HClO₄ and once with distilled water. The washed alumina was dried in an oven at 105°C for 24 hours.

A stock mobil phase for the HPLC was prepared as follows:
28.3g of monochloroacetic acid (MCAA), 9.35g NaOH and 1.5g Na₂EDTA were dissolved in 2.00L of distilled, deionized water (HPLC grade). The pH of the solution was then adjusted to a pH of 3.00-3.05 by the addition of solid NaOH until the required value appeared on the pH meter.

To adjust the reverse phase separation to the desired selectivity a working mobile phase was prepared by adding .2678g of sodium octyl sulfate to 1.00L of stock mobile phase. The resulting solution was filtered through a 0.45µM-Millipore filter.

The liquid chromatograph used was a Beckman-Altex Model 112 pump with a Model 210 injector system. The system used a recycling 1.50L mobile phase (described above) which was circulated at a flow rate of 2.4mL/min. The column used to separate the catecholamines in solution was a Biophase ODS 5µ column (250 x 4mm). The detector consisted of a Bioanalytic systems LC-3 electronic controller, LC-17 oxidative flow cell and TL-3/CP-O working electrode which operated at an applied potential of +640mV vs. Ag/AgCl. The actual amount injected into the column via the model 210 injector was 20µL. All instruments operated at room temperature.
Procedure

The design of the experiment was a 4 x 6 factorial. There were four conditions related to learned helplessness:

<table>
<thead>
<tr>
<th>Inescapable Shock</th>
<th>Conditioning (Escapable Shock)</th>
<th>Shock Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (control)</td>
<td>No shock</td>
<td>No conditioning</td>
</tr>
<tr>
<td>Group 2</td>
<td>No shock</td>
<td>Conditioning</td>
</tr>
<tr>
<td>Group 3</td>
<td>Shock</td>
<td>No conditioning</td>
</tr>
<tr>
<td>Group 4</td>
<td>Shock</td>
<td>Conditioning</td>
</tr>
</tbody>
</table>

The six levels of shock were confounded with voltage; each level was an increment of 8.75 sec. of shock with the first three durations at 6v. After preliminary analysis of 36 fish showed no group differences, it was decided that the last three increments of shock duration would be run at 10v. Eighteen goldfish were randomly assigned to each of the LH treatment combinations.

The procedure for giving inescapable shock consisted of a series of .5-second pulses with an interstimulus interval of 1.5 seconds. The Lafayette timer was recycled after each 35-second interval until the fish received its allotted shock time.

There were six durations of shock for each group:

<table>
<thead>
<tr>
<th>Shock Intensity</th>
<th>Shock Duration</th>
<th>Group 1</th>
<th>Groups 2 &amp; 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 volts</td>
<td>duration 1</td>
<td>0</td>
<td>8.75 sec.</td>
<td>17.5 sec.</td>
</tr>
<tr>
<td></td>
<td>duration 2</td>
<td>0</td>
<td>17.5 sec.</td>
<td>35.0 sec.</td>
</tr>
<tr>
<td></td>
<td>duration 3</td>
<td>0</td>
<td>26.25 sec.</td>
<td>52.50 sec.</td>
</tr>
<tr>
<td>10 volts</td>
<td>duration 4</td>
<td>0</td>
<td>35 sec.</td>
<td>70 sec.</td>
</tr>
<tr>
<td></td>
<td>duration 5</td>
<td>0</td>
<td>43.75 sec.</td>
<td>87.50 sec.</td>
</tr>
<tr>
<td></td>
<td>duration 6</td>
<td>0</td>
<td>52.50 sec.</td>
<td>105.00 sec.</td>
</tr>
</tbody>
</table>
Six fish were randomly chosen for each day with at least one fish in each of the four treatment groups. In a two-day period three fish were run from each group. Each fish in the conditioning group was run until they had received approximately as much shock as fish got during the inescapable shock treatment. A trial consisted of a 15-second period of light without shock followed by a 20-second period of light and pulsed shock. The timer was manually recycled at the end of a trial when light and shock were turned off. When a fish avoided by swimming to the other side in the first 15 seconds of a trial or escaped by crossing to the other side in the interval from 15 to 35 seconds, the trial was aborted which stopped the accumulation of shock time. The timer was manually recycled and a new trial was begun immediately.

For fish receiving both inescapable shock and conditioning, inescapable shock was given first, then the shuttle barrier was removed. The timer was reset for conditioning and the fish received conditioning trials until total time was registered on the cumulative times.

For conditioning trials shock pulse time was reduced to .25 from .5 sec. but the interpulse interval remained the same. Another trial was presented unless the fish had accumulated the allotted shock time.

In the last 36 goldfish a record of escapes and avoidances per trial was tabulated for each treatment group of fish.

Brain tissue was extracted by first making a deep incision posterior to the basioccipital portion of the skull cap. Using spring loaded scissors lateral cuts were made on either side of the skull cap. A lateral cranial cut was made which allowed the skull cap to be removed. The optic nerves and posterior cranial nerves were severed, allowing the removal of the entire brain from the brain case. The brain weight and remaining carcus weight were recorded.
The following is a synopsis of the pre-injection work-up that each brain underwent:

1. Brain tissue (.080 grams) was added to 2mL of .1M HClO₄ and 300µL of internal standard. 70% of tissue is water.

2. The tissue was then homogenized by grinding it in a special test tube.

3. The sample was centrifuged at 9,000g for 15 minutes; its supernatant was transferred with a clean pasteur pipette each time into a test tube.

4. The supernatant had added to it 50mg (.05g) of AAO + 1mL of tris buffer (which increases the pH to 8.6). It was then shaken on vortex for 5 minutes. The AAO was then allowed to settle.

5. The supernatant was then removed from each test tube with the same pasteur pipette.

6. The remaining alumina was washed twice with deionized water (3mL). The same pipette was used to remove water.

7. The alumina then had 0.5mL H₂O (deionized) added to it. It was then transferred to a Beckman microfuge tube using a separate pipette each time. It was allowed to spin for 5 minutes. The supernatant was then removed.

8. The alumina then had added to it 200µL (.200mL) of 0.1M HClO₄. It was then vortexed, left to stand for 5 minutes, and finally vortexed again.

9. The final solution was placed in a Beckman microfuge Model 11 and centrifuged for 10 minutes at 11,000 RPM.

10. From this supernatant 20µL was injected into HPLC.

Each subject brain solution was injected into the HPLC twice, therefore each subject produced two data points. All experimental subjects
had their brains assayed. A Shimadzu CR-1B Integrator Recorder was used
to record the chromatogram and calculate the concentration of the cate-
cholamines for each subject. For subject samples where areas were too
small for automatic calculations, the calculations were done by hand
using triangulation to approximate peak areas.

For each subject sample injection, E. was classified by group and
duration. Due to extremely small concentrations of E. present in the fish
brain, no actual concentrations were calculated. A line was drawn through
the HPLC trace baseline. If a white space appeared above the baseline at
the correct retention time for E., it was tallied as an indicator of
presence of E.

All data points were then collected and stored as a data file in the
MICC computer system.
Results

The means and standard deviations for concentrations (ng/g) of N.E. and D.A. by treatment group and shock level are presented in Table 3.

The differences in amount of N.E. and D.A. among the treatment groups was not significant (see Table 4). There was a significant difference between shock level and amount of N.E. and D.A. (see Table 4).

The Chi square comparisons of E. presence for the different treatment groups was 1.56 (3), p > .05 and 0.486 (5), p > .05 for the comparison of the different levels of shock.

The linear contrasts comparing the first three shock levels at 6 volts was not significant for N.E. (t(66) = -1.705, p ≤ .093) or for D.A. (t(66) = 1.109, p = .271). A second contrast comparing the last three shock levels at 10 volts was significant for N.E. (t(66) = 4.882, p < .000) and for D.A. (t(66) = 3.943, p < .0001).

Three orthogonal contrasts comparing the differences in amounts of catecholamine among the groups were not significant.

For the contrast comparing the control group with the groups that received shock the values of t were t(68) = .419, p = .677 for N.E. and t(68) = .691, p = .492 for D.A. For the contrast comparing the conditioning and inescapable groups with the group that received both, the values of t were t(68) = -1.075, p = .286 for N.E. and t(68) = -.964, p = .338 for D.A. For the contrast comparing the group that received conditioning with the group that received inescapable shock the values of t were t(68) = -.939, p = .351 for N.E. and t(68) = .167, p = .868 for D.A.
Table 3. The means and standard deviations of Norepinephrine (ng/g) and Dopamine (ng/g) of fish brains within the treatment group and shock level for 72 fish.

<table>
<thead>
<tr>
<th>Shock Duration</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>SD</td>
<td>M</td>
<td>SD</td>
<td></td>
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<td>50</td>
<td>150</td>
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<tr>
<td>4</td>
<td>290</td>
<td>20</td>
<td>260</td>
<td>60</td>
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</tr>
<tr>
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<td>Dopamine</td>
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<td>SD</td>
<td>120</td>
<td>80</td>
<td>80</td>
<td>110</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Analysis of variance of Norepinephrine and Dopamine adjusted for brain weight by treatment groups and shock level.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Degrees of Freedom</th>
<th>Norepinephrine</th>
<th></th>
<th>Dopamine</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean Square</td>
<td>F</td>
<td>Mean Square</td>
<td>F</td>
</tr>
<tr>
<td>Groups</td>
<td>3</td>
<td>17589.152</td>
<td>1.256</td>
<td>2591.292</td>
<td>1.883</td>
</tr>
<tr>
<td>Shock</td>
<td>5</td>
<td>155474.627</td>
<td>11.098*</td>
<td>33629.359</td>
<td>11.457*</td>
</tr>
<tr>
<td>Groups &amp; Shock</td>
<td>15</td>
<td>11496.784</td>
<td>.821</td>
<td>3960.737</td>
<td>1.349</td>
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<tr>
<td>Residual</td>
<td>48</td>
<td>14008.619</td>
<td></td>
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<tr>
<td>Total</td>
<td>71</td>
<td>23591.635</td>
<td></td>
<td>5298.910</td>
<td></td>
</tr>
</tbody>
</table>

*p < .001

A correlation comparing ng/g of N.E. to ng/g of D.A. adjusted for brain weight (r(69) = .28, p = .009) was significant. The partial correlations of ng/g of N.E. and total number of trials/sec. of shock for each fish (r(35) = .1925, p = .130) and ng/g of D.A. with total trials (r(35) = .1284, p = .228) proved to be nonsignificant.

Multiple regressions using N.E. and D.A. as dependent variables and trials, total trials, escapes plus avoidances, and trials to criteria of two avoidances was not significant (F(4,13) = 1.703, p = .209) for N.E. or D.A. (F(5,12) = 1.76, p = .195).

The 20 fish, where avoidances were tallied, were classified by group and whether they achieved the criteria of two avoidances. Although seven of the conditioning fish achieved criteria and eight of the fish which received inescapable shocks before conditioning failed to achieve criteria, the 2 x 2 chi square was not significant (chi sq.(1) = 3.71, p > .05).

Reliability of HPLC electro-chemical detection was calculated by correlating the readouts of the two injections made from the same brain sample for the 72 fish. The correlations were .97 for N.E. (df = 71, p = .001) and .58 for D.A. (df = 71, p = .001). Correlated t Tests com-
paring the two injections were not significant: $t(71) = 1.95, p = .055$
for N.E. and $t(71) = 1.62, p = .109$ for D.A.

The relationship between the group receiving conditioning and the group
receiving both conditioning and inescapable shock and trials divided by
shock time was not significant. The value of $t$ was $t(16) = 1.25, p < .229$. 
Discussion

It was hypothesized that given longer durations of inescapable shock, a depletion in whole brain N.E. should result. The failure to detect significant differences by main effect analysis and orthogonal comparison affords little support for the N.E. depletion hypothesis.

The major contribution of this experiment is the reliable quantification of the amounts of N.E. and D.A. in the brain of the goldfish. This reliability is shown by the high correlation of the amounts of neurotransmitter for the two injections from the brain of a single fish.

The large variance among the individual fish poses analytical problems for future research. Using goldfish of the same size and breeding stock should reduce within group variance.

The amounts of N.E. and D.A. in fish brains did not differ by treatment groups while a significant effect was noted in the effect of duration for both catecholamines. A curvilinear relation was found when contrasts were done comparing the first set of durations with the last set. These results are flawed by the fact that the control group, which received no shock, varied in the same pattern as the treatment group, thus making this shock duration effect largely uninterpretable.

A partial correlation between N.E. and D.A. adjusted for brain weight was found to be significant. These results do not support the theory proposed by Marshall (1976) which would expect a selective decrease in D.A. compared to N.E. in experiments requiring the animal to attend to a particular stimulus.
There was no significant correlation between N.E. and trials. This nonsignificant correlation is compatible with the observation that there is no evidence to support the relationship of N.E. and acquisition learning (Mason, 1981). Finally, the data concerning escapes, avoidances, and criteria fails to find a learned helplessness effect, in that the group that received both inescapable shock and conditioning should have had a significant decrease in the number of escapes and avoidances when compared to the group that receives conditioning alone.

It was noted that smaller fish (shock levels 4 and 5) had more N.E. and D.A./ng/g. This leads one to believe that the brain size plays an important part in the amount of the final catecholamine detection.

Parametric studies of shock intensity, pulse length and interstimulus interval are needed to increase the precision of future learned helplessness research.

A more fruitful avenue of future research than assaying whole brain monamines after treatment may be the selective chemical lesioning of fish brain tissue. Lesioning with the neurotoxin 6-Hydroxodopamine (6-OHDA) will result in the selective destruction of noradrenergic neurons in the brain. The amount of depletion could be checked through a random sampling of lesioned fish brains. An experiment of this nature could allow the observer to record the behavior of a subject that is known to be depleted in N.E. Since goldfish have been studied using intracranial injection (Olson, R. D. et. al., 1978) the probability of success of such an investigation is high.

Another suggestion for future research would be the use of an extreme group design, where monamine content of goldfish that have done well on
the escape-avoidance task are compared to fish with poorer performance. This comparison would involve a large sample of fish because in this study, of the 20 fish studied, only three fish made over 30 avoidances. This disparity highlights the large individual differences found in the ability of goldfish to perform learning tasks.

The inexpensive and robust goldfish with an easily accessible central nervous system and history of use in learning experiments could become the research animal of choice when studying the role of neurochemical dysfunction in mental and physical disorders provided refinement in methods and greater control of subject variable are utilized.
References


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Reference Notes


Footnotes

Bioanalytical Systems Incorporated LCEC Application Note No. 14,
Plasma Catecholamines. West Lafayette, Indiana.
Figure 1. Catecholamine Pathways.

The Biochemistry of the Central Nervous System

A. Catecholamine Pathways

1. phenylalanine
   \[ \text{phenylalanine} \xrightarrow{\text{phenylalanine hydroxylase (PH)}} \text{tyrosine (liver)} \]
   \[ \text{tyrosine} \xrightarrow{\text{tyrosine hydroxylase (TH)}} \text{dopa (pathways)} \]

2. tyrosine
   \[ \text{tyrosine} \xrightarrow{\text{tyrosine hydroxylase (TH)}} \text{dopa (pathways)} \]

3. dopa
   \[ \text{dopa} \xrightarrow{\text{dopamine \& \: noradrenaline (DA \& NE)}} \text{dopamine} \]

4. dopamine
   \[ \text{dopamine} \xrightarrow{\beta\text{-hydroxylase (DBH)}} \text{norepinephrine (NE)} \]

5. norepinephrine
   \[ \text{norleucine (PNMT)} \]
   \[ \xrightarrow{\text{norleucine methyltransferase}} \text{epinephrine (EP)} \]
   \[ \xrightarrow{\text{catechol O-methyltransferase (COMT)}} \text{noradrenaline (NM)} \]
   \[ \xrightarrow{\text{monoamine oxidase (MAO)}} \text{NE aldehyde derivative} \]
   \[ \xrightarrow{\text{NE alcohol oxidase}} \text{3,4-dihydroxyphenylglycol (NE alcohol derivative)} \]

6. 3,4-dihydroxyphenylglycol
   \[ \text{3,4-dihydroxyphenylglycol} \xrightarrow{\text{COMT}} \text{3-methoxy-4-hydroxyphenylglycol (MHPG)} \]

7. 3,4-dihydroxymandelic acid
   \[ \text{3,4-dihydroxymandelic acid} \xrightarrow{\text{COMT}} \text{3-methoxy-4-hydroxymandelic acid (VMA)} \]

*TH = Rate-limiting enzyme in catecholamine synthesis

**DD = Aromatic amino acid decarboxylase (non-specific enzyme; e.g., acts on any aromatic amino acid)

*** DA, NE + EP = brain neurotransmitters

**** MHPG + VMA = major NE metabolites.

Figure 2. Chemical Structures

Dopamine
4-(2-Aminoethyl)-1,2-benzenediol

Norepinephrine
4-(2-Amino-1-hydroxyethyl)-1,2-benzenediol

Epinephrine
4-[1-Hydroxy-2-(methylamino)ethyl]-1,2-benzenediol

Internal Standard
3,4-dihydroxybenzylamine (DHBA)