Importance of NMDA Receptor Activation During Initial Exposure to a Stressor for Stress Response Habituation

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Importance of NMDA Receptor Activation During Initial Exposure to a Stressor for Stress Response Habituation

Nathan Riechers

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Abstract

Psychological stress contributes to the etiology of a wide variety of mood and anxiety disorders. We know that how an individual reacts to stress is based on prior stress experience. Our goal is to better understand the factors that modulate the development of stress response habituation. In order for an individual to habituate to stress, the brain must be able to form stress-associated memories. Formation of these memories requires a cellular consolidation process that proceeds for several hours. NMDA receptors in the brain are known to be important for many forms of learning and memory, although it is unknown whether they are necessary for stress response habituation. Specifically, our main study aimed to determine whether activation of NMDA receptors after initial exposure to a stressor is necessary in order for stress response habituation to occur in rats. In order to adequately interpret the results of this study, it was also necessary to perform preliminary studies to look at the acute effects of an NMDA receptor antagonist drug (MK-801) on the stress response. Our results show that blocking NMDA receptors immediately after initial stress experience prevented subsequent expression of stress response habituation. This indicates that NMDA receptor activation during initial exposure to a stressor is indeed an important factor contributing to stress response habituation. However, these results cannot be taken as definitive evidence owing to an unexpected long-lasting excitatory effect of MK-801 on the stress response in our preliminary studies. Intriguing explanations exist for why this drug effect may have occurred, and future studies should test for these hypotheses in an attempt to solidify the conclusions we have made here.
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Experiment 2: Acute microinfusion of MK-801 into the medial prefrontal cortex resulted in a slight but not statistically significant increase in the stress response.

Experiment 3: NMDA receptor inactivation by MK-801 during initial exposure to a stressor blocked the expression of habituation of the CORT and ACTH responses, but not of cFos mRNA expression. Also, MK-801 had an unanticipated, long lasting excitatory effect.

Discussion

- Study Goals and Expectations
- NMDA Receptor Activation Appears to be Important for the Expression of Habituation
- MK-801 had a Long-Lasting Excitatory Effect on the Stress Response
- The Ketamine Studies: A Possible Explanation for our Excitatory Drug Effect
- Future Directions
- Sources of Error
- Conclusion
**Introduction and Background**

**Introduction**

Stress is a subject with which most of us are all too familiar. Whether it is having to cram for multiple exams, dealing with relationship issues, or simply feeling overwhelmed by responsibility, humans encounter psychological stressors quite frequently. Psychological stress occurs when higher brain centers interpret a situation as a threat to the well being of the organism (39). Psychological stress can be differentiated from physical stress, in which injury or illness causes damage to the body. In our studies we were primarily concerned with psychological stress, as this is the type of stress that humans most often experience on a day-to-day basis. How an individual copes with recurring psychological stress is an important factor in determining the overall impact of the stress, including the possible development of physical and/or mental illness (14,40). For this reason it is important for us to learn about the mechanisms of stress response habituation, which is a healthy response to chronic stress. The more we learn about these mechanisms, the more informed we become regarding the diagnosis and treatment of disorders resulting from chronic psychological stress such as depression and post-traumatic stress disorder (13,29).

**Stress Response and the HPA Axis**

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In our experiments we analyzed the stress response in rats. This stress response is the product of a complex and well-regulated set of processes in the body that assist in coping with the stress in an adaptive manner. Two endocrine systems in the body react to a stress stimulus. The sympathetic nervous system (SNS) stimulates release of epinephrine from the adrenal medulla, which results in increased heart rate and increased blood glucose levels. The SNS also directly innervates the heart, pancreas, liver, and other organs. All of this SNS activity contributes to immediate arousal, which is adaptive in that it allows the organism to make a fast behavioral response to the stressor. The other important endocrine system that responds to stressful stimuli is the hypothalamic-pituitary-adrenal axis (HPA axis). Although this system is also activated during stress, it has less of an immediate impact and is more involved with sustaining and regulating a stress response in the organism as well as with helping it prepare for future stress. Because of this, the HPA axis is more involved with the response to chronic psychological stress than the SNS is. Our studies focused on various aspects of the HPA axis stress response.

In a normal HPA axis response to a stressor, many neural pathways converging on the paraventricular nucleus (PVN) of the hypothalamus are activated. Specialized cells in the PVN then secrete a neurohormone called corticotropin-releasing hormone (CRH), which acts in the anterior pituitary gland causing it to secrete adrenocorticotropic hormone (ACTH), a protein hormone. ACTH enters the bloodstream and acts at the adrenal cortex, causing it to secrete either cortisol in humans or corticosterone (CORT) in rats (11,23). CORT provides negative feedback
on the PVN and pituitary gland, decreasing CRH and ACTH release and thus helping the organism return to its resting state after the stressor is no longer present (19). We are interested in measuring levels of CORT and ACTH, as well as activity level in the PVN, in order to assess different aspects of HPA axis function. Dysregulation of the HPA axis response is strongly correlated with many psychiatric disorders (35,43). Figure 1 is a diagram of the HPA axis.

*Effects of Corticosterone*

Corticosterone is a type of glucocorticoid (a steroid); thus it binds to the glucocorticoid receptor (GR) in target cells. It is the primary effector hormone of the HPA axis in rats and is one of the most important players in the stress response. CORT is secreted by the adrenal cortex in response to input from the ACTH secreted by the anterior pituitary gland. It is found in the blood either as free CORT or bound to its carrier protein, corticosteroid-binding globulin. Bound GRs on the inside (usually) of target cells are then transported to the nucleus where they affect gene transcription in these cells. This results in various physiological effects including an increase in blood glucose and increased cognitive arousal (32). These effects are adaptive because they increase the organism's ability to properly cope with the stress and to prepare for
future stress. Because it is lipophilic and diffuses out of its secreting cell immediately following synthesis, CORT must be synthesized by the adrenal cortex on demand rather than stored in advance. Because of this, we do not begin to see an increase in CORT levels until about 30 minutes after stress onset. In contrast, ACTH (a protein hormone) levels peak 5-10 minutes after stress onset. This is because ACTH is lipophobic and will not immediately diffuse out of the secreting cell after synthesis, so it can be synthesized and stored in advance. After the conclusion of a stressor the PVN will no longer be stimulated to release CRH; thus ACTH and CORT will begin to fall back to basal levels. The CORT half-life in plasma is 25 minutes, so we would begin to see a decrease in CORT around that time. CORT is under tonic control, meaning some of it always being made by the adrenal cortex and secreted into the blood. Levels of tonic CORT secretion exhibit a circadian rhythm and are highest in the period just before the organism wakes up and lowest when it goes to sleep. The functional purpose of this is to prepare the body for activity before waking. As rats are nocturnal, we conducted our experiments in the morning when basal CORT levels were lowest. Basal levels at their lowest points during the day are around 4 µg cortisol/100 mL plasma in humans and 1 µg corticosterone/100 mL plasma in rats.

*Stress Response Habituation and Learning*

In our studies, we were interested in the mechanisms underlying habituation of the stress response upon repeated exposure to a stressor. Habituation is one of
multiple types of learning that are seen in animals. Learning can be divided into two general categories: associative learning and non-associative learning (8).

Associative learning includes operant and Pavlovian conditioning, which both involve forming an association either between two stimuli or between a behavior and a stimulus. In this way, a stimulus or behavior is eventually associated with an outcome. In contrast, non-associative learning involves changing of the response to a stimulus without any association with a positive or negative outcome. One example of this is sensitization, where a subject will show an increased response to a stimulus after first being presented with a strong or novel stimulus. Once sensitized to the stimulus, the subject will show a stronger response when presented with that stimulus in the future. The other type of non-associative learning is habituation, where a subject frequently subjected to a stimulus will eventually show a reduction or total elimination of the response to that stimulus without positive or negative reinforcement (8). In our studies, we were interested in the mechanisms underlying this type of non-associative learning.

When humans experience stress, that stress response is put into the context of a background of previous stress experience. In order for a person to make an adaptive response to repeated stress, the ability to habituate to this stress is essential. Habituation to repeated stress experience, then, is a critical factor in determining the impact of the stress on an individual. Impaired ability to habituate to stress may exacerbate the negative consequences of chronic stress. Many studies have demonstrated the phenomenon of stress response habituation in rats (3,7,15,21,44). In these studies repeated exposure of a subject to a particular
stressor resulted in decreased SNS and HPA axis activity when the subject was later presented with the same stressor.

**Role of the Medial Prefrontal Cortex in the Stress Response**

The medial prefrontal cortex (mPFC) is one of the most evolutionarily advanced brain areas, and is known to be important for emotional control, goal-oriented planning, expression of personality, and complex behaviors (46). It also plays an important role in comparing current experiences with past experiences and in regulating other brain areas that mediate behavior (30). The mPFC indirectly modulates both HPA axis and SNS responses (2,37,38). Disrupted mPFC activity has been shown to be associated with stress-related mental conditions such as depression and post-traumatic stress disorder (20,27).

Recent findings in our lab suggest that the mPFC plays a role in modulating stress response habituation (45). Our lab found that when the mPFC in rats

*Figure 2*  
Inactivation of the mPFC prior to restraint on Days 1 and 2 completely blocked the expression of stress response habituation on Day 3

*Figure 3*  
The only condition in which mPFC inactivation interfered with the expression of stress response habituation was the condition in which the mPFC was active for the first time during the Day 3 restraint challenge.
was inactivated during two days of restraint stress, stress response habituation was able to occur. However, when the mPFC was allowed to become active again for the first time during day 3, habituation was not seen. This was evidenced both by a lack of habituation in the mPFC and PVN on day 3 and by corticosterone levels (Figures 2,3). So, activation of the mPFC on day 3 caused dishabituation to occur. This means that neural activity in the mPFC is not necessary for a normal response to acute stress, but that the mPFC has the capacity to modulate that response. In other words, the mPFC appears to modulate habituation of HPA axis activity. However, we still are not sure as to the exact mechanisms of this modulation. Thus, we are interested in measuring levels of activity in the mPFC during the stress response in order to observe any effects it might be having on stress response habituation and HPA axis function.

The NMDA receptor

N-methyl-D-aspartate (NMDA) receptors are best known for their control of learning and memory (42). NMDA receptors are ion channels (ionotropic) and are located on the phospholipid bilayer of cells in many different brain regions. When its cell is at rest, the inside of the NMDA receptor is blocked by Mg$^{2+}$ (See Figure 4). When the cell membrane depolarizes and both glutamate and glycine bind to the NMDA receptor, the Mg$^{2+}$ is expelled from the inner portion of the receptor (28). At this point the active channel is permeable to Ca$^{2+}$, Na$^{+}$, and K$^{+}$. Ca$^{2+}$ flows into the cell through the NMDA receptors and activates signaling molecules, which then
regulate the number and activity level of $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors on the cell membrane. Changes in the amount of active AMPA receptors alter the effectiveness of these synapses, and thus alter the post-synaptic current that results from a given input. It is this regulation of AMPA receptors and synaptic efficacy by NMDA receptors that is thought to be the catalyst of memory formation in the brain's cells.

NMDA receptors are unique in that they are able to form associations between separate neural events, and are able to cause increased strength between related neural inputs. This happens by a process called coincidence detection, which is when a cell can detect the occurrence of two or more simultaneous but spatially separate signals (36). For example, NMDA receptors can be activated by multiple separate inputs that are close together in time, but not by one input alone. When the NMDA receptor receives these simultaneous signals repeatedly over time, it is able to strengthen the connections between those inputs and thus cause a much larger response. It does this by upregulating the number of nearby AMPA receptors so that the cell will have a greater response to these inputs in the future (24). It is in this way that NMDA receptors contribute to associative learning, where an organism is able to form an association between two previously unrelated stimuli. In our research, however,
we studied habituation, which is a form of non-associative learning. If we find that NMDA receptor activation is also important to this type of learning, then this information could reshape the way we think about the underlying processes of habituation.

NMDA receptors relate to our research on stress habituation because adaptation to recurring stressful events requires that the brain be able to form memories of those events. Several studies have found that there appears to be a critical period of ongoing NMDA receptor activity after the end of an experience that is necessary for memory formation (1,41). The brain undergoes NMDA dependent changes that appear to be critical for the formation of these new memories (10,31). It has also been shown that inhibiting NMDA receptors in the mPFC after an event interferes with the formation of long-term memories of that event (1). If NMDA receptors in this region and others are important for the consolidation of these memories after initial stress exposure then we would expect to see a lack of habituation to the stressor when this consolidation is blocked immediately after the initial stressful experience. Thus for our studies it was important that we were able to inactivate these receptors during the initial stress exposure. We did this using Dizocilpine (MK-801), one of many known NMDA receptor antagonists. MK-801 is a non-competitive NMDA receptor antagonist and has been shown to effectively block glutamate from having an effect on target cells via the NMDA receptors (9). Another NMDA receptor antagonist, ketamine, has also been extensively studied and will be important in helping us to interpret the effects of MK-801 that we saw in our studies.
Study Goals

Our overall goal is to understand the factors that modulate the expression of stress response habituation. More specifically, our main study aimed to determine whether activation of NMDA receptors after initial exposure to a stressor is necessary in order for stress response habituation to occur in rats. We inactivated NMDA receptors with MK-801 immediately after the end of restraint on days 1 and 2, then measured the response to the same stressor on day 3 (in the absence of drug). To measure the stress response in these rats on day 3 we looked at struggling behaviors, CORT levels, ACTH levels, and neural activity in the PVN and mPFC. We expected that restricting the brain’s NMDA receptors on days 1 and 2 would disrupt the rats’ expression of stress response habituation on day 3 due to an inability of the rats to form memories of the stress. This would indicate that the changes elicited by these receptors after a stress challenge are indeed necessary for the encoding of information that leads to habituation, and that certain illnesses in humans resulting from a maladaptive response to chronic stress may be due at least in part to NMDA receptor dysfunction.

Prior to performing our main study, it was important to perform preliminary studies to look at the acute effects of MK-801 on the stress response. This would tell us about the effects MK-801 has on the stress response unrelated to habituation. It was necessary for us to lay this groundwork about the acute effects of our drug in order to later make accurate conclusions about our main study. Because blocking
NMDA receptors inhibits the activity of the affected neurons, we expected that administration of MK-801 prior to a stressor would have a dampening effect on the subsequent stress response.

**Methods and Experimental Procedures**

**Subjects**

Subjects were young adult male Sprague-Dawley rats from Harlan Labs (Indianapolis, IN). They weighed approximately 300 grams at the time of experimentation. Rats were allowed a 2-week acclimation period before use in order to minimize their stress resulting from human handling on test day. Animals were housed in polycarbonate tubs with wood shavings and were allowed food and tap water ad libitum. Rats were housed in pairs, except for when experimentation required guide cannulae in the brains, in which case rats were housed individually to prevent them from chewing on each other’s guide cannulae. The housing rooms were maintained on a 12 hour light/dark cycle with lights on at 0700 hr and room temperature held between 20°C and 24°C. Testing began after 0800 hr. and was finished before 1300 hr. Animals were each given three minutes of human handling on each of the three days leading up to the start of the experiment. All care, handling, and use of animals followed ethical guidelines and were approved by the University of Colorado Institutional Animal Care and Use Committee and adhered to the National Institutes of Health Guide for Care and Use of Laboratory Animals.
**Surgeries**

For our main experiment surgical placement of indwelling guide cannulae into the medial prefrontal cortex was required. To accomplish this, deeply anesthetized rats (Halothane) were placed in a stereotaxic apparatus and implanted with 26-gauge stainless steel bilateral guide cannulae (Plastics One, Roanoke, VA, USA) into the medial prefrontal cortex: +3.2 mm anterior to bregma, ±.5 mm relative to midline, and -1.8 mm ventral to dura. Cannulae were fixed to the skull with dental cement and three stainless-steel screws. After surgery a dummy cannula that extended 1 mm beyond the end of the implanted guide cannula was inserted. Rats recovered for approximately 1 week after surgery prior to experimentation.

**Intraperitoneal (IP) Drug Injections**

25 and 5/8 gauge needles connected to syringes were used for IP injections. These injections resulted in diffusion of the drug throughout the body. Doses of 0.15 mg/kg body weight MK-801 (Sigma) in 0.3 mL saline solution were based on previous studies concerning the drug’s effect on NMDA receptor function in the brain (17,18). Rats were removed from their home cage and grabbed over the shoulder to prevent movement and biting. The abdomen was exposed and the needle was
inserted in the lower right or left quadrant of abdomen in order to avoid hitting the bladder, liver, or other internal organs. The drug was then slowly squeezed through the syringe into the IP cavity. Figure 5 shows an IP injection.

**Drug Microinfusion**

Rats were first wrapped in a soft towel. Dummy cannulae were removed and replaced with a 33-gauge microinjector (Plastics One) attached to polyethylene 50 (PE-50) tubing through the indwelling guide cannula. The distal end of the PE-50 tubing was attached to a 10 µL syringe that was mounted on an automated infusion pump. Microinjectors extended 1 mm into the brain beyond the tip of the guide cannulae. MK-801, an NMDA receptor antagonist, was dissolved in 0.9% saline solution using sonication at a ratio of 6.25 µg MK-801 per 0.3 µL saline solution. Vehicle treatment was 0.9% saline solution. The MK-801 solution or vehicle was infused bilaterally (0.3 µL/hemisphere over 3 minutes), then microinjectors were left in place for an additional minute after infusion. Drug doses were based on previous studies involving microinfusions of MK-801 into localized brain regions (5,48). Rats were replaced into their home cage after microinfusion for 30 minutes before further manipulation.

**Restraint Stress Challenge**
For our main, experiment, in which restraint was chosen as the stress challenge, plexiglass cylindrical tubes (15±.5 cm long, with a diameter of 6.3 cm and several air holes) were used. The length of the restrainers was adjustable. Rats in the stress challenge groups were placed in these restrainers for 30 minutes. The restrainers prevented major movement but allowed for normal breathing. Restraint took place in a room next to, but separate from, the home cage sites. Figure 6 shows a rat in restraint.

Platform Stress Challenge

For experiments in which platform stress was chosen as the stress challenge, rats were placed on a platform (29 X 29 cm) that was 64 cm off the ground. The rats were on the platform for 30 minutes each and were free to roam the platform. Rats were challenged with platform stress in groups of two, with each rat placed on a separate platform about two feet apart from its neighbor. Platform stress took place in a room next to, but separate from, the home cage sites. Figure 7 showcases platform stress.
Videos and Scoring of Behavior

Video was taken of each rat on test day during their stress challenge. Struggling behavior of each rat was scored manually from the video using event-recording software (courtesy J. Christianson). Heavy mobility, defined as active struggling, rapid running, or escape behaviors, was scored. These behaviors were divided into one-minute bins and were reported in seconds.

Tissue and Blood Collection

Rats were taken either directly from their home cages or from the restrainers/platforms and immediately decapitated with a guillotine. Brains were obtained and flash frozen in isopentane (dry ice chilled between -30 and -20°C). Brains were stored frozen at -80°C. Using a cryostat 12-μm coronal sections were collected from the mPFC, and PVN. These frozen sections were placed on glass slides (poly-L-lysine-coated) in the order in which they were sectioned and stored at -80°C. Trunk blood was collected in ethylenediaminetetraacetic acid (EDTA)-coated tubes and placed on ice. EDTA stops the blood from coagulating. The tubes were then centrifuged for 15 minutes at 4°C to separate the plasma from the blood cells. The plasma was then aliquoted and stored at -80°C.

cFos mRNA and In Situ Hybridization
In order to measure neural activity in brain regions of interest, we measured expression of cFos mRNA in the brain tissue. cFos is an immediate early gene that codes for the FOS protein. cFos mRNA is an important measure of neural activity because it is expressed in the cell at very low levels under basal conditions until stimulated above threshold, when it is expressed in much higher levels (22). cFos is induced within a couple of minutes following stimulation, peaking in levels 30 to 60 minutes later (22). Thus, presence of this mRNA in a brain region would indicate neural activity in that region. Drawbacks of using cFos to measure neural activity are that both excitatory and inhibitory neurons express FOS, and it is not expressed in every cell type. However, the great cellular resolution and sensitivity that cFos provides make it a useful marker for our purposes, and it is expressed in the CRH containing neurons of the PVN that are of primary interest to us.

The technique of in situ hybridization was used to measure levels of immediate early gene expression, specifically levels of cFos mRNA, in brain slices from the regions of interest. Slides with tissue sections were fixed in buffered 4% paraformaldehyde for 45 minutes at room temperature, washed in 2x standard saline citrate solution (SSC, 1x.15M NaCl, .015 M sodium citrate), acetylated in .1M triethanolamine containing .25 acetic anhydride, pH 8 for 10 min, rinsed in double distilled water, dehydrated through graded ethanol baths and air dried. For the generation of the probes, plasmids containing a fragment of cFos cDNA were used. 35S-labeled complementary RNA probes were generated using standard in vitro transcription reagents. Briefly, .5-1 µg of linearized plasmid were incubated at 37°C for 2 hours in the presence of 7.5 µL UTP, 40 µM each of CTP, ATP and GTP, 10 mM...
DTT, 40 units of RNAse inhibitor, and 20 units of T7 RNA polymerase. Following purification through a G50/50 Sephadex column, the probe was applied to slides at 750,000-1,500,000 counts-per-million per slide in 65 µL hybridization buffer. Hybridization was performed in a 50% formamide humidified atmosphere at 55°C overnight. The following day, sections were treated with RNase A, 200 µg/mL at 37°C (1 hr), washed in decreasing concentrations of SSC at room temperature and finally in 0.1x SC at 70°C for 1 hr. Dehydrated sections were exposed to X-ray film for 2-4 weeks before the film was developed.

*Image Analysis*

After the X-ray film from our in-situs were developed, photographs of individual brain slices were obtained from the resulting autoradiographs. At this point the slices would appear darker where the mRNA was present. The regions of interest (PVN and mPFC) were determined on the images using the Paxinos and Watson (1998) rat brain atlas for guidance. Background areas were chosen in the white matter close to the regions of interest and were compared to signal areas using an optical density quantifying program called ImageJ (NIH shareware). The uncalibrated optical density values from ImageJ for each brain slice served as a quantification of the amount of cFos mRNA present in a given brain region.

*Measuring Corticosterone Levels*
Levels of corticosterone (CORT), the main stress hormone in rats, were measured in duplicate using 20 µL plasma from the trunk blood of the sacrificed rats. For this, an enzyme immunoassay kit (Assay Design, Ann Arbor, MI) was used. The sensitivity of this assay is 130 ng/100 mL.

Measuring ACTH Levels

Adrenocorticotropic hormone (ACTH) was measured using radioimmunoassay. ACTH was measured in duplicate from plasma collected on test day. This single-staged RIA procedure is adapted from a previously established protocol (33,34). $^{125}$I radiolabeled ACTH was obtained from Diasorin (Minneapolis, MN) and rabbit antiserum (Rb7, final dilution 1:30,000) was donated by Dr. Bill Engeland (University of Minnesota). The assay was sensitive to ACTH concentrations of approximately 15 pg/mL.

Statistical Analysis

All statistical tests were performed using the SPSS statistical analysis program 10.5 for Macintosh operating system. The data were analyzed using 2-way ANOVAs to determine whether there was a main effect of either stress or of drug treatment, and whether there was an interaction between the stress and drug treatments. To analyze behavioral scoring over 30 minutes, repeated measures ANOVAs were used to determine whether there was a main effect of drug treatment.
or time on the amount of struggling. Fisher’s least significant difference post-hoc test (FLSD) was then conducted on all measures to determine if there was a significant pair-wise difference between the treatment groups. α-levels are reported as either p<.05, p<.01, or p<.001.

Experiment 1: Effect of acute, peripheral MK-801 treatment on HPA axis activity.

Before we could study the effects of NMDA receptor inactivation on habituation of the stress response, it was necessary to perform an experiment looking at the acute effects of the NMDA receptor antagonist MK-801 on the stress response. This would allow us to get an understanding of what, if any effect MK-801 had on the stress response without the added question of stress habituation. We expected that by inhibiting NMDA receptors, treatment with MK-801 in the rats would inhibit the stress response and cause lower neural activity (as measured by cFos mRNA) than would be seen in the corresponding rats that received vehicle. If this hypothesis were correct then we would know that the drug could block NMDA receptor activity effectively without producing confounding or unexpected effects.

After a two-week acclimation period, rats were either challenged with 30 minutes of platform stress or were left in their home cage. The rats were given an intraperitoneal (IP) injection (0.3 mL total) of either MK-801 (.15 mg/kg body weight in saline solution) or vehicle 30 minutes before stress onset. After the conclusion of either a 30 minute stress challenge or 30 minutes in the home cage, rats were immediately sacrificed. The brains were then extracted, frozen in
isopentane, and stored at -80°C. Trunk blood was also collected for subsequent analysis of corticosterone and ACTH levels in the plasma. Videos were recorded of each rat on the test day for behavioral scoring. Lastly, we measured cFos mRNA levels in the PVN as well as the mPFC to observe the acute effects of this drug treatment on neural activity. The treatment groups for experiment 1 are listed below.

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<td>MK-801 30 min before stress onset</td>
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Experiment 2: The effect of acute microinfusion of MK-801 into the medial prefrontal cortex on HPA axis activity.

Another important step in developing an understanding of what effects the drug MK-801 has in rats was to inject it directly into a brain area of interest. We chose to inject the drug into the mPFC due to our interest in this area potentially modulating HPA axis activity (45). It was important to do this experiment as well as experiment 1 because the IP injections resulted in the diffusion of MK-801 throughout the whole body, thus inhibiting NMDA receptors everywhere. Because of this, it was impossible to know from experiment 1 data if the drug effects observed were due to a direct drug action in a brain region of interest or if the
results were due to MK-801 activity elsewhere. Experiment 2 would provide more specific information on what effects the drug had locally on the tissue surrounding the infusion site as well as other effects due to possible alteration of the function in the mPFC.

After a two-week acclimation period, rats were either challenged with 30 minutes of platform stress or were left in their home cage. The rats were given a bilateral microinfusion of either MK-801 (6.25 µg MK-801 in .3 µL saline per side) or vehicle 30 minutes before stress onset. The rats were sacrificed immediately after the stress period ended. The brains were then extracted, frozen in isopentane, and stored at -80°C. Trunk blood was also collected for corticosterone analysis. Videos were recorded of each rat on the test day for behavioral scoring. We will measure cFos mRNA levels in the PVN as well as the mPFC to observe the acute effects of this drug treatment on neural activity, although these data are not yet available at the time of writing. The treatment groups for experiment 2 are listed below.

<table>
<thead>
<tr>
<th>Group</th>
<th>Test Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Microinfusion into mPFC</td>
</tr>
<tr>
<td>1</td>
<td>Vehicle</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle 30 min before stress onset</td>
</tr>
<tr>
<td>3</td>
<td>MK-801</td>
</tr>
<tr>
<td>4</td>
<td>MK-801 30 min before stress onset</td>
</tr>
</tbody>
</table>

Experiments 3: Importance of NMDA receptor activation during initial exposure to a stressor for subsequent stress response habituation

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This experiment was designed to impair the NMDA receptor mediated aspect of the brain’s post-experience neuroplasticity process on days 1 and 2, and to see if this had an effect on the expression of habituation to a stressor on day 3. The deactivation of these NDMA receptors impairs the affected cells’ ability to adapt. In this case we expected this to cause the affected cells to be unable to consolidate new memories and thus for stress response habituation not to occur. The drug MK-801 was administered to rats peripherally, after exposure to a stressor, on days 1 and 2 and its effect on the stress response on day 3 was examined. If the drug prevented rats from habituating to stress, then we would know that activation of NDMA receptors after exposure to a stressor is indeed necessary for habituation to occur. This would mean that the brain’s NMDA receptor mediated new memory consolidation is important to habituation.

During days 1 and 2, rats were either put through 30 minutes of stress in a restrainer or were left in their home cage. Then the rats were given an intraperitoneal (IP) injection (.3 mL total in saline) of either MK-801 (.15 mg/Kg body weight) or vehicle. For rats restrained on days 1 and 2, drug was either administered immediately after the end of restraint, or was given 3 hours after the end of restraint. The 3-hour delay group was included so we could observe how close in time the injection had to be to the restraint experience in order to prevent new memory consolidation. Studies indicated that injection with the 3-hour delay would not disrupt memory formation (16). On day 3, none of the rats received an infusion and all rats were challenged with 30 minutes of restraint. The rats were sacrificed 30 minutes after the onset of restraint on day 3. The brains were
extracted, frozen in isopentane, and stored at -80°C. Trunk blood was also collected for corticosterone and ACTH analysis. Videos were recorded of each rat on the test day for behavioral scoring. We measured cFos mRNA levels in the PVN as well as the mPFC to assess neural activity in these areas. The treatment groups and predicted results are listed below.

<table>
<thead>
<tr>
<th>Group</th>
<th>Days 1 and 2</th>
<th>Day 3</th>
<th>Predicted response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infusion</td>
<td>Stressor</td>
<td>Stressor</td>
</tr>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>Home cage</td>
<td>Restraint</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle</td>
<td>Restraint</td>
<td>Restraint</td>
</tr>
<tr>
<td>3</td>
<td>MK-801</td>
<td>Home cage</td>
<td>Restraint</td>
</tr>
<tr>
<td>4</td>
<td>MK-801 immediately following restraint</td>
<td>Restraint</td>
<td>Restraint</td>
</tr>
<tr>
<td>5</td>
<td>MK-801 3 hrs after restraint</td>
<td>Restraint</td>
<td>Restraint</td>
</tr>
</tbody>
</table>

n=6 rats per treatment group  N=30 total rats

**Results**

*Experiment 1: Acute peripheral MK-801 injections resulted in an increase in HPA axis activity*

Intraperitoneal injections of 0.15 mg/kg body weight MK-801 resulted in increased HPA axis activity in rats that were challenged with stress as well as in rats that were not challenged with stress. The drug resulted in increased activity in the medial prefrontal cortex only in rats that were not challenged with stress. The rats were either given MK-801 or vehicle 30 minutes before either being challenged with 30 minutes of platform stress or left in their home cage.
There was a strong main effect of MK-801 treatment on CORT levels in rats (F\textsubscript{1,20} = 185.80, p<.001). There was not a main effect of stress on CORT levels. However, there was a stress-by-drug interaction (F\textsubscript{1,20} = 36.38, p<.001). Rats that received drug had significantly higher levels of corticosterone than their vehicle counterparts in both the stress (p<.001, FLSD) and no-stress (p<.001, FLSD) groups. Curiously, the drug/no stress group had higher CORT levels than the drug/stress group (p<.01). These results were unexpected and indicate a strong drug effect on CORT independent of stress state. This is unexpected because we had no reason to predict that MK-801 would cause any increase in HPA axis activity, let alone a large increase like this. Also, the vehicle/stress group had significantly higher corticosterone than the vehicle/no stress group (p<.001, FLSD), which is to be expected since stress results in increased CORT levels under normal conditions. See Figure 8 for specific CORT data.
**ACTH**

ACTH levels in this experiment closely mirrored CORT levels. There was a main effect of MK-801 treatment on ACTH levels in rats ($F_{1,20} = 34.03, p<.001$). There was not a main effect of stress on ACTH levels. Rats that received MK-801 had significantly higher levels of ACTH than the vehicle rats in both the stress (p<.01, FLSD) and no-stress (p<.001, FLSD) groups. The drug/no stress group again had higher ACTH levels than the drug/stress group, though for ACTH this difference was not significant. This result confirms that the HPA axis was stimulated in an unexpected manner by MK-801. There was slightly more ACTH expressed in the vehicle/stress group than the vehicle/no stress group, but the difference was not significant. See Figure 9 for specific ACTH data.

**cFos mRNA**

cFos mRNA expression in the paraventricular nucleus (PVN) of the hypothalamus mirrored ACTH and CORT levels, which comes as no surprise since these three measures are all direct results of HPA axis activity. There was a main
effect of MK-801 treatment on cFos mRNA expression in the PVN in rats \((F_{1,20} = 22.84, p<.001)\). There was not a main effect of stress on cFos mRNA expression. In both the stress \((p<.05, \text{FLSD})\) and no-stress \((p<.001, \text{FLSD})\) groups, rats that received the drug had significantly more cFos mRNA expression than the corresponding rats that received vehicle, meaning there was more neural activity in the drug groups. Since neurons expressing cFos in the PVN are responsible for the secretion of corticotropin releasing hormone (CRH), this most likely indicates that MK-801 caused an increase in CRH secretion. Once again, there was more expression in the vehicle/stress group than the vehicle/no stress group, but Fisher’s LSD post-hoc test did not reveal this difference to be significant.

cFos expression in the medial prefrontal cortex differed from the pattern seen in our other measures of the stress response. As in the other measures, we saw
a main effect of MK-801 treatment on cFos mRNA levels \( (F_{1,20} = 5.10, p<.05) \).

We also saw a stress-by-drug interaction \( (F_{1,20} = 5.00, p<.05) \). There were higher levels of the mRNA in the drug/no stress group than the vehicle/no stress group \( (p<.01, \text{FLSD}) \).

However, in the mPFC the drug/no stress was group significantly lower than both of the groups that were challenged with stress; the drug/stress group \( (p<.01, \text{FLSD}) \) and vehicle/stress \( (p<.01, \text{FLSD}) \) group. The two stress groups were not different. It appears that in the mPFC our drug injection had an excitatory effect on neural activity only in the rats that were not challenged with stress. It is possible that the lack of an effect in the rats that received stress was due to a ceiling effect of cFos expression. This means that there is a maximum amount of cFos expression that is possible within the tissue, and these levels may have been reached in both our vehicle/stress and drug/stress groups. See Figures 10-13 for specific cFos data.
Behavioral analysis

There was a significant main effect of MK-801 treatment on heavy movement behaviors in rats ($F_{1,280} = 112.10, p<.001$), accounted for by higher activity level in the drug group. This effect did not change significantly across the 30 minutes of stress. From observing the rats that received drug during the platform stress, the rats appeared fearless and unorganized as they darted in an apparently random manner around the platform. Two rats fell off the platform to the floor. It is apparent at this point not only that our peripheral MK-801 treatment had a very strong excitatory effect on the stress response, but also that it caused disorganized behavior in the rats. See Figure 14 for behavioral data from experiment 1.
Experiment 2: Acute microinfusion of MK-801 into the medial prefrontal cortex resulted in a slight but not statistically significant increase in the stress response

Bilateral microinfusions of MK-801 (6.25 µg MK-801 in .3 µL saline per side) into the prelimbic medial prefrontal cortex resulted in an increase in the stress response in rats as well as an increase in heavy movement behaviors during the stress challenge. The effect on behavior was significant but the effect on CORT was nonsignificant. Rats that were challenged with stress had a significantly greater response than those that were not challenged. The rats were either given microinfusions MK-801 or vehicle into their prefrontal cortex 30 minutes before either being challenged with 30 minutes of platform stress or being left in their home cage. We have not yet collected ACTH or cFos mRNA data from experiment 2.

Corticosterone

There was a strong main effect of stress on CORT levels in rats ($F_{1,20} = 30.99$, p<.001). There was not a main effect of MK-801 treatment on CORT levels. In both the stress and no-stress groups, rats receiving MK-801 did not have significantly different levels of CORT than their vehicle-receiving counterparts. However, there was a statistically nonsignificant trend toward MK-801’s resulting in increased CORT levels. This information indicates that MK-801 may have had a slight, nonsignificant excitatory effect on HPA axis activity. This trend is consistent with
the results from experiment 1, although the effect here was much less pronounced. See Figure 15 for specific corticosterone data.

*Behavioral analysis*

There was a significant main effect of MK-801 treatment on heavy movement behaviors in rats ($F_{1,300} = 18.82, p<.001$) accounted for by higher activity level in the drug group (see Figure 16). The movement behaviors

![Figure 15](image15.png)

**Figure 15**

Corticosterone levels were not significantly affected by microinfusion of MK-801 into the mPFC in rats that were challenged with stress nor in those that were not challenged with stress.

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* indicates significantly higher value in drug group compared to corresponding vehicle group as revealed by Fisher’s LSD post-hoc test

![Figure 16](image16.png)

**Figure 16**

Struggling behavior (as indicated by time spent in heavy movement) was significantly affected by microinfusion of MK-801 into the mPFC in rats that were challenged with stress, and struggling decreased significantly over the 30 minutes
decreased over the course of the 30 minutes, as there was a main effect of time
\((F_{1,300} = 10.77, p<.001)\). This effect appears to be less pronounced than in
experiment 1. This information confirms that the infusion of MK-801 into the mPFC
had some excitatory effect on the stress response as well as a stimulatory effect on
movement behaviors.

*Experiment 3: NMDA receptor inactivation by MK-801 during initial exposure to
a stressor blocked the expression of habituation of the CORT and ACTH
responses, but not of cFos mRNA expression. Also, MK-801 had an
unanticipated, long lasting excitatory effect.*

After intraperitoneal injections of MK-801 (.15 mg/Kg body weight) or
vehicle immediately after exposure to a stressor on days 1 and 2, the stress
response after a stress challenge on day 3 was observed. Rats were challenged with
30 minutes of restraint on day 3 then immediately killed. Levels of the hormones
CORT and ACTH indicate that inactivation of the NMDA receptors with MK-801
prevented habituation from occurring, but cFos mRNA levels in the PVN and mPFC
indicated that habituation did occur. Also the drug had an unexpected, long lasting
excitatory effect on all measures of the stress response. Due to camera malfunction
behavioral data was not collected for experiment 3.
Corticosterone

In rats that received drug on days 1 and 2, habituation of CORT levels was not seen on day 3, as CORT levels in the naïve drug group and repeated restraint drug group were not significantly different. There was clear habituation in the rats that received vehicle on days 1 and 2, as the repeated restraint vehicle group had significantly lower CORT levels than the naïve vehicle group (p<.05, FLSD). In addition, the group that received MK-801 three hours after the end of restraint on days 1 and 2 had higher CORT levels than the repeated restraint vehicle group, though this difference was not significant. That effect is important because it means the drug had some unexpected excitatory effect well after the consolidation of memory of the stressor was complete. This also means that this drug effect persisted in the rats’ systems through to day 3, when no injections were given. So although giving the drug on days 1 and 2 appeared to block habituation of the stress response as expected, it obviously did something else to the rats that we did not anticipate. See figure 17 for specific CORT data.
In rats that received drug on days 1 and 2, habituation of ACTH levels was not seen on day 3, as ACTH levels in the naïve drug group and repeated restraint drug group were not significantly different. Unlike with CORT, we did not see clear habituation as indicated by ACTH on day 3 in the rats that received vehicle on days 1 and 2, as the repeated restraint vehicle group did not have significantly lower ACTH levels than the naïve vehicle group. However, it appears there was at least some habituation because the repeated restraint vehicle group was slightly lower than the naïve vehicle group. Again the group that received MK-801 three hours after the end of restraint on days 1 and 2 had higher ACTH levels than the repeated restraint vehicle group, though this difference was not significant. This furthers our suspicion that the drug might indeed have had some unexpected effect well after consolidation of the memory of the stressor was complete. See figure 18 for specific ACTH data.
In rats that received drug on days 1 and 2, habituation of cFos mRNA expression was seen on day 3, as expression in the repeated restraint drug group was significantly lower than in the naïve drug group. This effect was seen in both the PVN (p<.05, FLSD) and the mPFC (p<.001, FLSD). This indicates that neural activity on day 3 was lower in rats that had been exposed to stress than in rats that were naïve to the stress. In other words, treatment with MK-801 on days 1 and 2 did not block habituation. This is different from what we observed in the CORT and ACTH data, where there was no habituation in the drug group. The result in the PVN is especially surprising since this area is closely linked to CORT and ACTH secretion as part of the HPA axis. We saw nonsignificant habituation in the PVN in the rats that received vehicle on days 1 and 2, as the repeated restraint vehicle group had nonsignificantly lower cFos levels than the naïve vehicle group. In the mPFC, there was significant habituation of cFos mRNA expression in the rats that

![Experiment 3 PVN cFos mRNA](image)

On test day, PVN neural activity (as indicated by cFos mRNA levels) in the naïve drug group and the repeated restraint drug group were significantly different, meaning there was habituation of the this response in rats that had received MK-801 on days 1 and 2. Also there was a non-significant long lasting drug effect in the 3 hr delay group.

-- * indicates significant habituation relative to the corresponding naïve group as revealed by Fisher’s LSD post-hoc test

RR: received restraint on days 1 and 2
Naïve: Did not receive restraint on days 1 and 2
received vehicle on days 1 and 2, as the repeated restraint vehicle group had significantly lower cFos levels than the naïve vehicle group (p<.05, FLSD). As mirrored in the CORT and ACTH data, high levels of cFos mRNA in the 3 hour delay drug group in the PVN once again indicated a long lasting excitatory effect of MK-801 beyond the time necessary for memory consolidation. It should also be noted that the levels of neural activity in the PFC in the naïve drug group were higher than in any other group. We will discuss possible reasons for all of these surprising results. See figures 19 and 20 for specific cFos mRNA data.

Discussion

Study Goals and Expectations

In order to determine whether NMDA receptor activation immediately after initial exposure to a stressor is important for stress response habituation in rats, we inactivated NMDA receptors with MK-801 after the end of 30 minutes of restraint on
days 1 and 2 and then measured the response to the same stressor on day 3 (in the absence of drug). To lay some groundwork for the interpretation of the results of this study, we also performed two preliminary studies to look at the acute effects of MK-801 on the stress response. We hypothesized that NMDA receptor activation would indeed be necessary after initial exposure to a stressor in order for stress response habituation to occur. We also expected, in our preliminary studies, that acute administration of MK-801 30 minutes before stress onset would have a dampening effect on the stress response.

**NMDA Receptor Activation Appears to be Important for the Expression of Habituation**

The experiment 3 CORT and ACTH data indicate that the inactivation of NMDA receptors after exposure to a stressor on days 1 and 2 prevented the expression of stress response habituation on day 3. The rats that received MK-801 after stress challenge on days 1 and 2 had similar levels of CORT or ACTH as rats that were exposed to stress for the first time on day 3. This result supports our hypothesis.

However, NMDA receptor inactivation on days 1 and 2 did not block the habituation of neural activity (as indicated by levels of cFos mRNA expression) in the PVN or the mPFC. The results in the PVN were unexpected because we would expect the pattern of activity in the PVN, which can be largely attributed to active CRH-secreting neurons, to match the patterns of CORT and ACTH levels. Still, CORT and ACTH levels are strong indicators of HPA axis activity, and the fact that...
habituation of these responses was not seen on day 3 tells us that NMDA receptor activation during initial exposure to a stressor is at the very least an important factor contributing to stress response habituation.

*MK-801 had a Long-Lasting Excitatory Effect on the Stress Response*

Experiment 3 data revealed surprisingly high levels of stress response measures in the rats that received MK-801 three hours after the conclusion of stress challenge on days 1 and 2. We did not expect this group to have a greater response than the rats that received vehicle after the conclusion of the stressor on days 1 and 2, because the critical period for initial memory consolidation, as described in previous studies (1,41), had passed before these rats received MK-801. In other words, we did not expect the drug to block habituation in this group. However, the CORT and ACTH data both indicate that habituation did not occur in the 3-hour delay group (it had roughly equivalent stress response measures as the corresponding vehicle group). This not only means that drug administration resulted in an excitatory effect in this group, but also that whatever caused this effect persisted in the rats through to day 3 (when no drug treatment was given).

Experiment 1 data show that MK-801 had a strong excitatory effect on HPA axis activity independent of the presence of a stressor. The trend in experiment 2 corroborates the effects seen in experiment 1. We would have expected MK-801 either to have no effect on the stress response or to result in a decrease. Because NMDA receptor activity in a cell is associated with excitation of that cell, we
predicted the drug would cause a decrease in activity of affected cells in the brain due to the blockage of NMDA receptor activity in these cells. In experiment 3, the rats that received drug and were not stressed on days 1 and 2 had very high expression of cFos in the PVN and mPFC. This group had the highest level of activity on day 3 in the PVN, and even higher levels relative to the other groups in the mPFC. Because no rats received drug on day 3, this means that the excitatory effect of MK-801 was long-lasting and persisted in the rat at least 24 hours after administration.

This drug effect complicates our interpretation of the experiment 3 results. Although the results of the experiment agree with our hypothesis that NMDA receptor activation is necessary for habituation, it is difficult to make any definitive conclusions when our NMDA receptor antagonist (MK-801) was obviously having unintended effects on the rats. It is possible that the expression of an underlying neural habituation process was masked by this long-lasting neural excitatory effect of the drug. So although the CORT and ACTH data from experiment 3 serve as strong evidence in support of our hypothesis, it is important for us to better understand all of the effects of MK-801 administration before making definitive conclusions.

*The Ketamine Studies: A Possible Explanation for our Excitatory Drug Effect*

Because MK-801 was given to the rats peripherally in experiments 1 and 3 and therefore circulated throughout the entire body, there are a multitude of possible explanations for its excitatory effect on the stress response. However, since
the trend in experiment 2 (microinjections of MK-801 into the mPFC) is consistent with the results from experiments 1 and 3, we will focus our discussion of this drug effect on MK-801’s activity at the level of the brain.

We know that NMDA receptors are glutamatergic and are found throughout the brain (28). Since we administered the drug peripherally in experiments 1 and 3 we expect that it was able to inhibit all of the NMDA receptors in the brain, both in the cortex and in deeper brain areas. In the mPFC, for example, a subset of neurons called pyramidal cells are responsible for sending output from the cortex to other cortical and subcortical brain areas. We expected that the blockage of the glutamate binding sites on the NMDA receptors on these cells by MK-801 would inhibit these cells and thus decrease their neural output. So why did we see an increase in neural activity in the drug groups in experiments 1 and 3?

A possible explanation can be taken from a set of studies performed with the drug ketamine (another NMDA receptor antagonist) by Ron Duman and his colleagues. These studies are centered on the fact that ketamine has been found to be effective in treating people with major depression who are resistant to traditional anti-depressants, possibly by causing increased synaptic signaling and increased number and function of new spine synapses in the prefrontal cortex (4,6,12,25,26,47). These researchers have developed an alternative model for how this type of drug might be acting in the brain.

In this model it was proposed that there is another factor in NMDA receptor inactivation that could lead to an overall disinhibition of a cell’s response.
Specifically, it was proposed that there is a subset of NMDA receptors on GABAergic inhibitory neurons in the cortex that are responsible for this disinhibition. When these receptors are “turned on” by glutamate, the GABAergic neurons would increase their activity and thereby increase their release of GABA into the synapse. GABA is the major inhibitory neurotransmitter in the brain. It exerts its effects on target cells through both GABAa and GABAb receptors. These GABAergic neurons synapse onto the glutamatergic pyramidal cells, causing decreased glutamate release by the pyramidal cells and thus a decrease in neural output from the cortex. This would explain, then, why inhibition of the GABAergic cells via NMDA receptor inactivation could lead to increased activity and output by the many pyramidal cells in the cortex. This increased neural output could then cause increased stimulation of the anterior pituitary gland and the adrenal gland, resulting in greater ACTH and CORT secretion.

This model is limited by the fact that the output cells of the cortex receive input from thousands of other cells, making it difficult to know exactly what is having a significant influence on them. However, if this model is accurate and MK-801 inhibited a large number of NMDA receptors on GABAergic neurons in our studies, then that would explain the excitatory drug effects we saw in the rats in our experiments. If this were indeed the case, then the effect we saw in rats that received MK-801 on days 1 and 2 in experiment 3 could be due to this disinhibition of neural output in the cortex rather than an inability to habituate. In the future it will be important for us to conduct studies either to test or to control for this alternative hypothesis.

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The results of these ketamine studies appear on the surface to be at odds with the results from our studies. We found that blocking NMDA receptor activity blocks habituation. We would predict that the disruption of habituation in humans could exacerbate the development of mental illness resulting from chronic stress. Why then does blocking these receptors with ketamine appear to be beneficial in patients with depression? In truth, it is difficult to directly compare the ketamine studies to our studies, as many aspects of the studies differ greatly. These depressed patients were given ketamine continuously for a period of time, whereas our rats were given MK-801 either acutely or after the end of a stressor. So our studies didn’t look at how NMDA receptor antagonism affected any aspects of the stress experience during the stressor as they relate to the development of habituation. The ketamine given to depressed patients, however, could have had effects on aspects of stress experiences that are beyond the scope of our studies. So these ketamine studies can still be informative as to how this type of drug may be acting in the body, but it is not productive to compare the results of these studies to our MK-801 studies in great depth.

But setting aside this confounding issue of the unexpected excitatory effects on the stress response of MK-801, our results from experiment 3 have some important implications for our understanding of the mechanisms underlying the development of habituation. We already know that NMDA receptors are important for associative learning (24,28,42). The receptors are able to form associations between multiple inputs that are close together in time. Ca^{2+} flow through active NMDA receptors catalyzes responses within the cell that lead to a greater response
to these inputs in the future. However, it is unknown if NMDA receptors are important for forms of non-associative learning, such as habituation. Our results, if supported by future research, provide support for this. Our data indicate that NMDA receptor activation after exposure to a stressor is important for habituation of the HPA axis response. It could be that, by modifying synaptic efficacy between cells involved with the excitatory response to a stressor, NMDA receptors are involved with dampening those cells’ response to that same stressor in the future. In this way, memories would be formed in the brain of the stress experience, leading to a lower response when exposed to the same stressor later on (i.e. habituation).

Future Directions

Future studies should be performed both to clarify the results we found here and to test possible alternative hypotheses. The first step will be to determine the effect of microinfusion of MK-801 on neural activity, as well as to look at the local effects of the drug in the mPFC around the infusion site. We will accomplish this by performing in situ hybridizations to analyze levels of cFos mRNA in the PVN and mPFC in tissue from experiment 2. If we see a large amount of cFos around the infusion sites, then it would tell us that the drug directly caused disinhibition of neural activity in this region.

Next, we will want to determine whether there were different levels of cFos expression in GABAergic cells versus glutamatergic cells in rats that received MK-801 treatment in our experiments. To accomplish this, we will perform double-label
in situ hybridization on the tissue from these experiments. This will involve using 2 separate riboprobes with 2 different detectable tags (one tagged with a radioactive atom and the other with a fluorescent molecule) in order to localize the specific RNA sequences coding for FOS as well as for some sequence unique to GABAergic cells within one tissue sample. This unique sequence could be the GAD gene, which is concerned with the synthesis of GABA and is expressed in GABAergic cells. For example, a section of tissue with low expression of cFos but high amounts of GAD would indicate low activity of GABAergic cells in that region. If we saw this as well as higher activity in regions without GAD expression, then this would tell us that the GABAergic neurons may indeed be experiencing inhibition, leading to disinhibition of the pyramidal cells of the cortex. In other words, information from a double-label in situ would be useful in determining whether the increased response we saw in the rats that received MK-801 was due to disinhibition of neural output caused by decreased activation of GABAergic cells (due to NMDA receptor blockage) relative to glutamatergic cells.

Lastly, an additional study could look at how the mPFC modulates stress response habituation and whether this modulation is dependent upon NMDA receptor activity. To accomplish this we will repeat the experimental design of experiment 3, but will utilize microinfusions of MK-801 into the mPFC instead of peripheral injections. This will allow us to look at the effects of NMDA receptor blockage specifically in the mPFC. If we do not see an effect of MK-801 treatment on habituation in this experiment, then we could conclude that NMDA receptor
activation at the level of the mPFC is not essential for the development of habituation.

Possible Sources of Error

One limitation of our studies is that cFos mRNA, which we used as a measure of neural activity, is ambiguous regarding excitation vs. inhibition. That is, we cannot tell from looking at cFos expression whether that cell has excitatory or inhibitory effects on other cells. In order to differentiate between the activity of excitatory vs. inhibitory cells we could perform double-label in situ hybridization (see future directions). Next, the animals in the “no stress” condition may have inadvertently been exposed to stressors. Although the rats were exposed to human handling for three days prior to experimentation, there is still a chance that certain aspects of experimentation could have activated their stress response. This could have occurred during drug injection, or when the rats were moved from their home cage to the decapitation platform. However, the no-stress rats that received only vehicle had relatively low levels of our stress response measures so it appears as if this effect, if present, was minimal.

Conclusion

NMDA receptor activation immediately after initial exposure to a stressor appears at the very least to be an important factor contributing to stress response
habituation. This is consistent with our expectations. However, the results of our studies should not be taken as definitive evidence owing to an unexpected, apparently long-lasting excitatory effect on the stress response of our drug, MK-801. Intriguing explanations exist for why this drug effect may have occurred, and future studies should test for these alternative hypotheses as well as attempt to solidify the conclusions we have made here.

If future studies are able to confirm that NMDA receptors are indeed important to the development of stress response habituation, then there could be great implications for the treatment of certain psychiatric disorders in humans. It could be possible that, in some cases of PTSD, major depression, or other illnesses resulting from a maladaptive response to chronic stress, the symptoms are due at least in part to dysfunction of the NMDA receptors in the brain. This presents a possibility for the development of medications that act on NMDA receptors in these patients. Such medications would aim to restore optimum level of NMDA receptor activity, thus alleviating symptoms related to previously maladaptive responses to chronic stress. We can already see an example of this in the field of psychiatry as an NMDA receptor antagonist, ketamine, continues to be an important new remedy for major depression (though we are not yet certain as to the exact mechanism by which it has its therapeutic effects). Due to this obvious potential for improved treatment of various illnesses, this area of research should continue to be the subject of investigation in the future.
References


