Is the Lateral Septum's Inhibitory Influence on the Amygdala Mediated by GABA-ergic Neurons?

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Previous studies suggest that the lateral septum and amygdala are critical in the production and regulation of anxiety. The lateral septum may express its anxiolytic properties by directly inhibiting the activity of the amygdala. The present study examines whether this connection is primarily mediated by GABA-ergic neurons. Rats were fully sedated and an acute recording electrode was placed in the central nucleus of the amygdala; the lateral septum was stimulated during recording. Picrotoxin was injected intraperitoneally and recording was repeated. The interaction effect of picrotoxin and stimulation support the conclusion that GABA does play a central role in this connection.
## Table of Contents

### Background

Introduction ........................................................................ 3

The Amygdala ................................................................. 8

The Lateral Septum ........................................................... 15

Connections ....................................................................... 20

An Opposing Theory ......................................................... 22

The Project ....................................................................... 25

CeA vs. BLA .................................................................... 27

### Methods

Design ............................................................................. 29

Subjects .......................................................................... 30

Apparatus ........................................................................ 30

Procedure ......................................................................... 30

### Results

Histologies ................................................................. 32

Response to Stimulation (Without Picrotoxin) .............. 34

Response to Stimulation (With Picrotoxin) .................. 40

Interaction Effect .......................................................... 45

Main Effect of Picrotoxin ............................................... 48

Discussion ..................................................................... 48

References ..................................................................... 54
Background

Introduction

Fear is considered to be one of the most basic emotions exhibited by humans. This makes evolutionary sense, given its highly adaptive function, providing the impulse for the fight or flight responses that are necessary to escape predators, avoid natural hazards, and challenge competitors in order to pass on genetic material. As useful as fear may be however, it also comes at a steep price. A highly fearful state is very taxing, both physically and emotionally, and a state of persistent fear can interfere greatly with the normal activities of life, (as demonstrated by anxiety disorders in humans). Thus, as adaptive as the production of fear might be, the ability to ease one’s fear is equally important, both for man and lower animals. As a result of the weighty role that the mechanisms for anxiogenesis (creating fear) and anxiolysis (reducing fear) play in evolutionary history, fear is one of the few emotions that can be effectively modeled in nearly any complex animal. Components of the human fear response, (such as heart pounding, upset stomach, increased respiration, jumpiness, fidgeting, and apprehensive expectation) can be easily mapped back onto animal fear responses, (such as increased heart rate, stomach ulcers, respiration change, increased startle, grooming, and freezing, respectively), (Davis 1992). This has permitted the development of several animal-model approaches for the study of biological mechanisms of fear and anxiety.

The oldest animal model for inducing and studying fear and anxiety is classical conditioning. In this most basic form of this technique, a stimulus that would not normally elicit a fear response (such as a light or a tone), is paired with a naturally aversive stimulus (usually an electrical shock). As the two stimuli are presented in
tandem, the animal comes to “expect” the aversive stimulus whenever the conditioned stimulus is presented; as a result, fear is exhibited whenever the conditioned stimulus is presented, even in the absence of the naturally aversive stimulus. This general framework can be adapted in a variety of ways in order to study specific elements and substrates of the anxiety response. For instance, a third, so-called inhibitory stimulus can be introduced, which is paired with the absence of the aversive stimulus. By doing this, one can study the mechanisms by which fear is diminished. Frequently, this classical conditioning paradigm is employed to test the effects of chemical and neurological manipulations on the fear response. Anxiogenic and anxiolytic drugs may be applied either systemically or directly to individual brain regions; neural structures may be also be stimulated, lesioned, or ablated. The effect of these manipulations is then measured by the degree to which the animal exhibits one or more of the physical correlates of fear. Startle response, heart rate, skin conductance, and ulceration are among the more common variables examined in this type of study. Based on the results of these experiments, as well as its variants, researchers seek to isolate the contribution of each structure, and the role of pharmacology, in anxiety. These tests are popular because they rely on conditioning methods that have been verified for over a century. They also allow for flexibility in terms of the specific physiological or behavioral variable that can be examined. They are somewhat limited however, as they may only be producing a stimulus-specific fear, rather than generalized anxiety; this is a distinction that is neither obvious nor universally accepted, but has been used by some researchers to criticize the results of these studies.
A commonly-used elaboration of the classical conditioning method is a class of setups called conflict experiments. In this type of experiment, a shock is contingent on a behavior that is normally rewarding. For instance, in the water-lick test, animals are chronically deprived of water; as a result, drinking water becomes very rewarding. However, during periods during which a conditioned stimuli is present, the more the animal drinks from a water bottle, the more it is shocked. Thus, the animal is conflicted about whether to pursue the rewarding behavior despite its aversive effects, or to avoid it. The amount that an animal drinks in this situation is thought to be a good index of anxiety, as a more anxious animal will tend to avoid the shock more, and thus drink less. This test seems to closely parallel the common human response to anxiety of freezing up during moments of anxious tension. It also exploits a rat’s natural drive to drink when thirsty, a response which, in itself, does not need to be conditioned. Its drawback is that responses can be construed as a reflection of changes in drive or thirst in general, rather than of anxiety. To counteract this potential confound, the animal’s behavior during a period where it receives no shocks must be examined carefully.

Another model of anxiety is the elevated plus maze. This involves the use of a large cross-shaped platform with two closed arms (walls along the sides) and two open arms (no walls). An animal is naturally inclined to spend more time in the closed arms as there is no risk of falling. In manipulations of general anxiety level however, a more fearful animal should spend even more time in the closed arms, while a less anxious animal should show no preference (since the fear of falling is not present). The advantage of this test is that makes use of an animal’s innate fear of falling and thus does not rely on any sort of artificial conditioning. It does however, rely on the assumptions
that, in this fear-provoking apparatus, anxiety-level is the most important modifier of preference and, inversely, behavioral expressions of preference are the best index of this anxiety level; since these assumption may or may not be valid, it is important to monitor other aspects of the rat’s behavior during the test in order to garner clues about alternative explanations of results.

The social interaction model of anxiety works on a theory that is easily paralleled in humans: the more anxious an animal is, the less it will interact with his peers. In this model, two animals are placed in a closed environment, after which the amount of interaction between a target animal and the partner animal is measured. The relative advantages and disadvantages of this test are similar to that of the elevated plus maze: it is a more naturalistic paradigm, but also relies on anxiety’s primary role in the modification of behavior. Like with the plus-maze, observations must be about several aspects of the animal’s behavior, not just the primary variable of social interaction.

A final behavioral test involves passive avoidance tasks. In this relatively simple model, an animal must either stay in place or avoid a visible probe, in order to prevent receiving a shock. As in the previous tasks, a more anxious animal will be more likely to vigilantly avoid the aversive stimulus, while a less anxious animal will show less caution and be shocked more frequently. The appeal of this paradigm is its simplicity: the shock is an unconditioned aversive stimulus and avoidance of the shock requires no atypical behavior on the part of the animal. The simplicity of test however, allows for multiple interpretations of its results, including the possibility that the animal’s level of avoidance is related to its ability to suppress its behavior, regardless of anxiety level.
In addition to these behavioral models, purely physiological approaches are often used to examine the neural response of brain areas proposed to be involved in the production and modulation of anxiety. Electrophysiological activity of a group of cells in a particular region may be recorded after administration of anxiogenic or anxiolytic chemicals, stimulation of other brain areas, lesioning or ablation of other brain areas, or a combination of several of the above. Anteriograde and retrograde tracing techniques are also used to visually map the neural connections between structures. Generally, this class of techniques is excellent for examining the physiological relationship between neural structures. Strong causal relationships of this type are a necessary condition for a valid theory about the biological substrates of anxiety. Using results for these techniques to make judgments about complex psychological processes is problematic however, since neither behavior nor mental state is considered at any time. These tests, like any of the ones described here, are imperfect and cannot stand on their own; each has its own particular set of advantages and disadvantages. As a result, researchers depend on the results from the full array of techniques in order to make any meaningful conclusions about their data.

Converging evidence from the above techniques has implicated two subcortical, limbic structures, the amygdala and the lateral septum, as having principal influence on the production and modulation of anxiety responses. The amygdala has been proposed to be crucial for anxiogenesis, while the lateral septum is though to have a primary role in anxiolysis. Although these two areas are slightly different in humans than in rats, cats, and lower primates, (owing in part to more extensive connections to our greatly expanded neocortex), they are generally well preserved in mammalian species. As Davis, Rainnie,
and Cassell (1994) state, the human amygdala “contains almost all the nuclear groups present in the primate amygdala [and] its extrinsic and intrinsic connections and neurochemistry are also remarkably similar…” As for the lateral septum, Fried (1973) confirms that “the differentiation of various nuclei within the septum has remained relatively consistent in all mammals.” Consequently, the results of physiological experiments on these areas are largely comparable across species, and applied to the study of anxiety in humans.

The Amygdala

The amygdala is a small bilateral group of nuclei located within the inferior medial anterior temporal lobe. One of the few nearly universally agreed upon notions in the study of the biological bases of anxiety, is that this structure plays an important, if not central, role in anxiogenesis. This idea came originally from early observations of psychiatric patients. Chapman et al. (1954) electrically stimulated the amagdalae of five epileptic patients in their care. Upon stimulation, four out of the five patients reported subjective feelings of fear and anxiety. In addition, stimulation elicited dramatic increases in heart rate and blood pressure, two of the primary physiological correlates of anxiety, as described above.

This dramatic finding, and others like it, led to a great deal of attention being paid to the amygdala. A more recent human study, Bechera et al. (1995), looked at patients with localized brain damage, and their ability to learn a conditioned fear response. These patients, one with selective bilateral damage to the amygdala and one with selective bilateral damage to the hippocampus, were conditioned to expect an air horn blast
whenever shown a particular monochrome slide. Skin conductance was measured throughout the training and extinction periods. Strikingly, although the patient with amygdala damage was able to verbally articulate the pattern in which the horn was paired with the slide, he did not demonstrate any elevation in skin conductance as a result of this pairing. In other words, although he had conscious knowledge of the stimulus that ought to have elicited an autonomic fear response, the amygdala damage somehow prevented actual fear-learning and expression. Incidentally, the exact opposite pattern was found in the hippocampus-damaged patient, who was unable to describe the pattern, but showed the same pattern of fear-related skin conductance as controls. The results of these human stimulation and ablation studies clearly link the amygdala to anxiety in some way; they do not make clear however, what exactly that link is. Does the amygdala have direct and primary control of all facets of anxiety or only some? Or, might the effects found in the studies be due to the stimulation or destruction of axons leading to or from other areas, making the amygdala’s role in anxiety merely incidental?

Researchers proposed a series of requirements for determining whether or not the amygdala had necessary attributes to be the locus of anxiogenesis. One such requirement was that the amygdala be intimately linked with the other brain structures that have been found to be directly linked with each specific physical and behavioral correlate of anxiety. A series of studies, as summarized by Davis (2000) indicate that this requirement is fully satisfied. Neurons originating in the central nucleus of the amygdala have direct connections to the lateral hypothalamus, which seems responsible for many of the physical correlates of fear, including increased heart rate and blood pressure, pupil dilation, and paleness. In addition, it has projections to the parabrachial nucleus, which is
related to panting and respiratory distress. On the behavioral side, this nucleus has neurons leading to the central grey (implicated in freezing, and conditioning and conflict behavior), to the nucleus ambiguous and dorsal motor nerve of vagus (linked to ulcers, urination, and defecation), to the nerve reticularis pontis caudalis (associated with increased startle), to the to the motor neurons responsible for facial expressions, and to several other areas which seem responsible for increased arousal, vigilance, and attention. Finally, and most importantly, perhaps, for the subjective sensation of anxiety, the central nucleus of the amygdala has strong connections to the paraventricular nucleus of the hypothalamus, the structure that controls the secretion of corticosteroids that induce the stress response. No less essential than all these outputs though, is the fact that the amygdala receives direct inputs from sensory cortex and thalamus, allowing for a rapid activation of the fear response that is articulated by the neural network described above, (Pitkanen, 2000).

These physiological links were tested functionally by LeDoux et al. (1988). In this experiment, rats were lesioned either in the central grey (CG), lateral hypothalamus (LH), or the stria terminalis, then classically conditioned to associate a tone with a shock. The researchers found that, while all of the rats were successfully conditioned, the CG-lesioned rats failed to demonstrate freezing at the presentation of the tone, while the LH-lesioned rats showed no change in arterial pressure. Thus, not only are these regions capable of producing the specific physiological correlates of anxiety, but, in their absence, anxious animals fail to show their particular responses. In the light of these results, it would seem most parsimonious to place the amygdala at the center of a theory of anxiogenesis. Parsimonious or not however, this data is insufficient to make any firm
conclusions about the anxiety system as a whole. Before one can do that, one would have
to subject the amygdala itself to a battery of tests to see whether it is necessary for
anxiogenesis and whether it conforms to other models of anxiety.

One approach in this pursuit has been to examine how electrical stimulation of the
amygdalae of animals changes their performance in one or more measure of anxiety. In a
relatively early study, Delgado, Rosvold, and Looney (1956) conditioned monkeys to
reach for one of two cups, depending on the frequency of a tone; during a high frequency
tone, the monkeys were shocked if they did not quickly select the correct cup, thus
producing anxiety. After the monkeys were fully conditioned, stimulating electrodes
were placed in several areas of the brain. Finally, the experimenters compared how the
monkeys responded to the conditioned stimuli against how they responded to electrical
stimulation in each area of the brain. They found that, while stimulation of several brain
areas either had an inhibitory effect or no effect at all, stimulation of amygdaloid nuclei
resulted in a behavioral response similar to the response during the high-frequency tone.
In a related study, Rosen and Davis (1988) studied the effect of amygdala stimulation on
acoustic startle in rats. Foregoing the frequently used conditioning paradigm, these
researchers relied on the natural reaction of rats to startle at the presentation of a sudden
loud noise. The results of this study indicated that electrical stimulation of amygdaloid
nuclei enhances at least one of the rats’ natural fear reactions, even in the absence of
conditioning. These two experiments take the results of the previous ones a step further:
not only does the amygdala possess the physical attributes required of an anxiogenic
structure, but amygdaloid activity also seems sufficient for anxiogenesis.
A third method for the study of the amygdala’s role in anxiety has been to examine the behavior of animal that has had its amygdala destroyed. If the amygdala is both *sufficient* and *necessary* for anxiogenesis, then the effect of an amygdaloid lesion should be the opposite of amygdaloid stimulation. Slotnick (1973) used the passive avoidance paradigm to test this effect. In this experiment, rats were placed in a cage with a bottom that consisted of four metal plates; in order to avoid shock, rats simply needed to stay on a single plate. As expected, rats with amygdaloid lesions moved around much more than controls and, consequently, were shocked more often. This so-called passive avoidance deficit was thought to be indicative of an abnormally low level of anxiety; without fear of shock to guide the rats’ behavior, they had no impetus to avoid moving to the other plates. Hitchcock and Davis (1987) tested the effect of amygdaloid lesions on fear-potentiated startle. Rats were trained to expect a shock during the presentation of a tone or a light. After surgery, the rats without functional amygdalae had a greatly diminished startle reaction as compared to the rats that had sham surgeries. This was true regardless of sensory modality (tone or light). Although the testing methods differ somewhat, the results of these experiments do appear to be the opposite of the stimulation experiments.

Taken together, these studies are highly suggestive of a primary role of the amygdala in anxiogenesis; the amygdala seems, at least to a large extent, to be both sufficient and necessary for anxiety, as demonstrated in these animal models. At this point however, the question of how it works pharmacologically remains unanswered. An additional line of research has been conducted to answer this question. Accompanying these experiments is the larger question of whether the amygdala can be considered the
seat of anxiety if it does not respond appropriately to contemporary pharmacological treatments of anxiety disorders in humans. The most widely used class of anxiolytic drugs on the market is benzodiazepines. Among the more commonly prescribed anxiety treatments in this class are Valium (diazepam), Ativan (lorazepam), and Xanax (alprazolam). The pharmacological action of BDZs are highly related to that of the neurotransmitter gamma-aminobutyric acid (GABA). The GABA_A receptor complex contains not only receptors for GABA, but also contains several other receptors, often including those for BDZs. When a BDZ molecule binds to its receptor, the GABA receptor in the same complex exhibits an increased affinity for the GABA molecule. This means that GABA molecules released into the synapse bind more frequently to their receptors. Thus, the administration of BDZs potentiates the action of GABA. Because GABA is a universally inhibitory neurotransmitter, BDZs tend to further inhibit activity in the neurons to which GABA binds. If the amygdala is regarded as the locus of anxiogenesis, then it ought to be affected directly by BDZs. Given the pharmacological profile of BDZs, one would expect to find an abundance of GABA_A (and, by association, BDZ) receptors on any structures which show an effect of BDZ application. Thus, one could only say that the amygdala is crucial for anxiogenesis if it meets this criterion.

Providing support for theorists who place the amygdala at the center of anxiogenic activity in the brain, it has been demonstrated that there is a high concentration of BDZ receptors in the amygdala. Niehoff and Kuhar (1983) used fine light microscopic techniques to actually count the distribution of BDZ receptors. This study confirmed that, although the receptors are not evenly distributed, all areas of the amygdala support a high degree of BDZ binding. Using this as a physiological
framework, Shibata et al. (1989) conducted a multi-part experiment using the water-conflict paradigm to test anxiety after a variety of manipulations to the amygdala. In the first experiment, they lesioned rats in various areas of the amygdala and found that many lesion locations result in increased water consumption, the anti-conflict (reduced anxiety) response; this parallels the results of the lesioning studies described above. In experiment two, they directly injected several types of BDZs (diazepam, zopiclone, lormetazepam, flurazepam, and phenobarbital) into the areas of the amygdala that produced anticonflict behavior in experiment one. Given the inhibitory action of BDZs, one would predict that, if BDZs affect these areas, direct application would mimic the results of lesioning. In line with this, the results demonstrated a dose-dependent, anti-conflict effect. This indicates that the anxiolytic properties of BDZs result, at least in part, from their inhibition of amygdaloid activity. In a social interaction experiment, Sanders and Shekhar (1995) attempted to extend these results by looking at the effect of GABA$_A$ antagonists (bicuculline methiodide and picrotoxin) in the amygdala. In order to establish a bi-directional role of the GABAergic neurons in this structure, a blockade of GABA action (and the resulting disinhibition of the post-synaptic neurons) would need to prove anxiogenic. They demonstrated that, as expected, injecting these GABA antagonists into at least one of the amygdaloid nuclei caused rats to interact significantly less than controls. This apparently anxiogenic consequence of amygdaloid disinhibition echoes an earlier study by the same researchers, which showed an increase of heart rate and blood pressure as a result of bicuculline methiodide injection into the amygdala, (Sanders and Shekhar, 1991); it also closely parallels the result of amygdala stimulation
experiments, presumably because GABA antagonists cause an increase in overall amygdaloid neural activity.

The evidence from this wide array of experiments suggests that the amygdala plays some major role in anxiogenesis. The stimulation studies show that an increase in activity in this structure is strongly correlated with an increase in anxiety behavior and physical response. Lesion studies show that, in the absence of amygdaloid influence on other structures, anxiety is dramatically reduced. Finally, manipulations of GABA receptors in the amygdala suggest that the structure is a major site of activity of drugs that produce subjective feelings of anxiolysis in humans, and diminish anxiety-correlated behavior in animal models. Some researchers disagree that the amygdala is the most important structure in anxiogenesis, but few deny that it is extremely important for this function.

**Lateral Septum**

On first glance, an additional mechanism for the modulation of anxiety might seem unnecessary. If chemicals such as BDZs could suppress amygdaloid activity, thus reducing anxiety, why couldn't there be similar, endogenous chemicals within the amygdala itself? In other words, why couldn't the amygdala be self-regulating? In theory, especially given the heterogeneity of its component nuclei, it could. Several recent studies however, suggest that in actuality, it does not. Yadin et al. (1991) lesioned the central and basolateral nuclei in the amygdala of rats, then subjected the rats to a water-conflict test. As expected, the lesions resulted in a significant increase in punished licking; this finding was nothing new beyond the lesion studies described previously. In
the next step of the experiment though, they injected the lesioned rats with chlordiazepoxide, a BDZ, systemically. If the amygdala was the only site of the anxiolytic action of BDZs, then the drug should have no effect in these rats. Instead, not only did the chlordiazepoxide have an additional effect, but this effect was actually greater than the effects found of chlordiazepoxide administration in unlesioned rats. These results encouraged researchers to find a second anxiety-modulating structure upon which BDZs might be working. Specifically, many began to examine candidates for a complementary, anxiolytic structure with which the amygdala might be in a kind of autonomic balance. One structure proposed to fill this role is a bilateral group of subcortical nuclei in the medial telencephalon, the lateral septum.

Some of the earlier studies that suggested the lateral septum might influence anxiety came from the study of the behavior of animals with septal lesions. Brady and Nauta (1953) found that when rats had their septums ablated, they began to exhibit a set of behaviors known as septal rage. That is, they had an extremely exaggerated startle response, increased vigilance, and frequent defensive attacks on nearly any object placed in their cage. Although termed a type of "rage," it was noted by researchers that this set of responses bears a striking resemblance to those related to extremely high levels of anxiety. They reasoned that, if septal ablation results in a heightened state of anxiety, then this region might normally be involved in anxiolysis. Physiological studies hint that this might be true. Malmo (1961) showed that stimulation of the septum lowers the heart rate of rats. Covian, Antunes-Rodrigues, and O'Flaherty (1963) demonstrated that this procedure lowers the blood pressure and slows respiration in cats. Finally, Yadin and Thomas (1996) suggest that chronic septal stimulation can decrease stress-induced
ulceration. These responses, being the reverse of some of the correlates of anxiety, demonstrated that the septum had the ability to counteract some of the anxiogenic outputs of the amygdala. However, it is not clear from these studies alone that it is anxiety per se that the lateral septum is influencing in these studies; in order to prove that, one would need to test septal manipulations in established animal models of anxiety.

One manner by which researchers have examined septal functioning is studying how stimulation can be used during conditioning. In one of the original studies of this type, Olds and Milner (1954) used the intracranial self-stimulation (ICSS) paradigm to test the reward potential of septal stimulation. In this type of design, stimulating electrodes are placed in a specific region of the brain (in this case, the septum). The animal is placed in a Skinner box containing a bar that it can press. Each time it presses the bar, the brain region is stimulated. In this study, the rats quickly became trained to press the bar, and did it often, suggesting that it is rewarding. But what exactly about the septal stimulation did the rats find rewarding? A follow-up study by Grauer and Thomas (1982) shed additional light on this question. They compared ICSS in the medial forebrain bundle to ICSS in the septum, either in the presence or the absence of a conditioned fear stimulus. The rats that were stimulating the medial forebrain bundle showed a significant decrease in bar-pressing during the fear condition, as compared to the baseline condition. The rats that were stimulating the septal area exhibited no such effect. The results of this study hint that, since it did not have a negative effect on septal ICSS, fear (and the alleviation thereof) might be related to the reason that septal stimulation is rewarding in the first place. This idea was tested directly by Thomas and

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1 Classically, this is done with electrodes implanted in the medial forebrain bundle, the proposed pleasure center of the brain; in this case, male rats will self-stimulate to the point of physical exhaustion, even ignoring the presentation of food, water, or female rats in favor of the bar.
Evans (1983), who trained cats to either press a pole or jump onto a platform in order to receive septal stimulation. These tasks are much more effortful than the bar-pressing of the previous experiment and are thus less likely to be performed unless the consequence is extremely rewarding. Like in the previous experiment, the experimenters also applied an aversive stimulus (hypothalamic stimulation) as a means of comparison. In the non-fear condition, septal stimulation was not found to be an effective tool for reinforcing behavior; during hypothalamic stimulation however, the cats performed whatever task was necessary to receive septal stimulation. If behavior motivated by septal stimulation is only exhibited in response to an aversive condition, then it is reasonable to conclude that the rewarding properties of this stimulation are also dependent upon this condition. In the context of this experiment, the only possibility is that this reward derives from inhibiting the impact of the aversive stimulus. Since it has been demonstrated that septal stimulation does not produce analgesia (Grauer and Thomas, 1982), it must specifically be the aversive emotions (in other words, fear) that are inhibited.

Yadin et al. (1993) extended the findings of these ICSS studies by examining the effects of lateral septum stimulation or lesion in a more direct model of anxiety, the water-lick test. If the lateral septum is truly an anxiolytic structure, than stimulation should be anxiolytic, resulting in more punished licking, while lesioning should be anxiogenic, resulting in less, (in essence, the exact opposite pattern as in the amygdala). In fact, the results of the experiment conformed to exactly this pattern. Stimulating the lateral septum caused rats to lick significantly more than their baseline amount, while stimulating in the medial forebrain bundle had no significant effect. As a means of demonstrating that the water-lick test is a valid model of anxiety, rats were given BDZs
systemically; their performance in the test mirrored that of the rats who received septal stimulation. The results of the lesion component of the experiment also proved faithful to the notion that the lateral septum is anxiolytic. In testing of previously conditioned rats, both one-week and two-weeks after administration of lateral septal lesions, the lesioned rats licked significantly less than controls.

Behavioral evidence for the classification of the lateral septum as an anxiolytic structure seems strong based on the studies presented thus far. A strong claim about its function also depends however, on the results of experiments that examine the lateral septum at the level of its individual neurons. Yadin and Thomas (1981) took single-cell recordings from the lateral septums of rats during classical conditioning experiments, both of the inhibitory and of the excitatory type. In the training session, either a tone or a light was associated with a shock, and as a result was treated as an aversive stimulus (CS+). Simultaneously, the stimulus (tone or shock) that was not being used as the CS+ became paired with the absence of a shock, and thus became inhibitory in its effect (CS-). The researchers found that the activity of lateral septal neurons increased during the presentation of the CS- (when the rats were presumably less anxious), but decreased during the presentation of the CS+ (when the rats were presumably more anxious). These results directly correlate normal lateral septal activity with anxiolysis. In a follow-up study, the researchers injected chlordiazepoxide systemically to see the effect of anxiolytic drugs upon lateral septum functioning during these same manipulations, (Yadin, Thomas, and Vaughan, 1986). Compared to non-drugged rats, the lateral septal neurons of the BDZ-injected rats showed increased activity in the presence of the CS- and during the absence of a conditioned stimulus, while the normal suppressive effects of
the CS+ were blocked. This is exactly the set of results expected if the lateral septum is both an anxiolytic structure and a site of BDZ action.

Connections

Although complementary evidence from studies of the lateral septum and amygdala in isolation are important for establishing that each structure has some role in anxiety, these results say little about their relationship. Specifically, this type of evidence not sufficient to support the theory that these two structures reciprocally modulate each other’s activity. The two structures could, for instance, have their opposing effects further downstream, exerting their competing influence directly on the structures more directly associated with the physiological and behavioral correlates of anxiety. Strictly speaking, in that model, the lateral septum and amygdala would not need to interact at all. Proving a more intimate link is dependent upon providing evidence of strong and rapid neural connections between the two structures. Unfortunately, this is the very aspect of the anxiety system that is least extensively researched. Volz et al. (1990) chemically traced a few neurons that connected directly from the lateral septum to the central nucleus of the amygdala to the in cats. Jakab and Leranth (1995) suggest that there may also be a connection to from the lateral septum to the basolateral nucleus of the amygdala, via a synapse in the bed nucleus of the stria terminalis. Neither of these studies suggests however, that these connections are particularly strong, nor do the studies speak to their functional or pharmacological nature. Consequently, the best available research on the relationship between the lateral septum and the amygdala is much more correlational in nature.
King and Meyer (1958) looked at the effect of amygdaloid lesions on the septal rage syndrome that had been observed in animals that had undergone septal ablation. They found that lesions of the amygdala returned the emotionality of septal ablated rats to near-normal levels. Melia, Sananes, and Davis (1991) also examined the relationship between lesions of the two structures, but measured the changes with the much more restricted, and objective, variable of acoustic startle. They found that, whereas septal ablation resulted in a greatly exaggerated startle reflex, the combined lesions of the septum and amygdala resulted in a slight reduction of startle behavior. In fact, the effect of the combined lesions was almost identical to that of amygdaloid lesions alone. Thus, the results of both this study and King and Meyer (1958) cannot result merely from the summed canceling out of the inhibitory effects of amygdaloid lesions and the excitatory effects of septal lesions. Since the excitatory impact of septal lesions are blocked by amygdaloid lesion (and not the other way around), the septum’s normal anxiolytic properties are likely to be a result of its influence on the amygdala itself. The amygdala may also reciprocally influence septal activity; this would serve to modulate septal inhibition of the amygdala itself though (as in a negative-feedback loop), rather induce direct septal influence on other structures.

Some of the most recent attempts to prove a causal link between activity in the lateral septum and activity in the amygdala have been electrophysiological experiments, many of which have been conducted in Earl Thomas’ lab at Bryn Mawr College. Thomas and Sancar (2001) either stimulated or injected chlordiazepoxide directly into the lateral septum. Both of these manipulations resulted in inhibition of neural activity in the central nucleus of the amygdala. The first of these treatments proves that there is,
directly or otherwise, a physiological link between these two areas. Specifically, as hypothesized, increased activity in the lateral septum suppresses activity in the amygdala. The second of these manipulations suggests that the anxiolytic action of BDZs is connected to this relationship. This is perhaps the strongest evidence collected that the lateral septum’s role in anxiolysis is performed by directly modulating the anxiogenic activity of the amygdala. A thesis experiment conducted in Dr. Thomas’ lab last year, deWolfe (2003), demonstrated that stimulation of the central nucleus of the amygdala had a profoundly excitatory effect on lateral septal activity. This was the final link required to reveal the reciprocally modulatory relationship of the amygdala and the lateral septum.

**An Opposing Theory**

So far, the evidence I’ve presented is fairly clean in its support of the theory that the two primary mechanisms of anxiety production and modulation are the amygdala and the lateral septum. There are however, several alternative theories about the biological substrates of anxiety. The most prominent of these opposing theories has been developed by Jeffery Gray and expanded by Dallas Treit and Christine Pesold. In his book, *The Neuropsychology of Anxiety* (Gray and McNaughton, 2000), Gray outlines his idea that the septohippocampal system is the primary axis of anxiety regulation within the brain. The differences between that theory and the one outlined thus far are not subtle. Firstly, in Gray’s model, the amygdala is only important for conditioned fear, and not for anxiety as a whole. As stated in the introduction, the difference between fear and anxiety is not easily distinguished, especially in animal models which must rely only behavior rather than mental states. In this instance however, Gray seems to be indicating that there is an
essential disparity between fears that are learned through classical conditioning to a specific aversive stimulus, and those that are either more generalized or innate. As a result of this notion, Gray rejects data that comes from tests that rely on classical conditioning of any sort, immediately weakening the claims that the amygdala is important for anxiety as a whole. As one can see by the data presented earlier however, these classical conditioning studies are not the only technique that has been employed in the study of the amygdala’s role in anxiety. Some of the human studies, social interaction tests, non-conditioned startle measures, and passive avoidance tests do not involve conditioning, but are also sensitive to manipulations of the amygdala.\textsuperscript{2} Thus, regardless of whether there is a meaningful difference between fear and anxiety (either in terms of function or biological systems) the amygdala does seem to play a vital role in both.

The second major difference between the lateral septum-amygdala theory of anxiety and Gray’s, is his proposal that the lateral septum is actually an anxiogenic structure. This notion is borne partly out of early findings of passive avoidance deficits in mice with septal lesions, such as Slotnick and McMullen (1973). In this study, the researchers found that, like mice with amygdala lesions, mice with septal lesions were unable to avoid shocks that were contingent on a failure to stay in place. This suggested to Gray and his followers, that these mice were less anxious following septal damage. As the logic for all studies of this genre goes, if a lesion causes a reduction in anxiety, then the affected structure must normally be anxiogenic. This line of reasoning seems to be

\textsuperscript{2} FMRI studies, such as Morris and Dolan (2001) and Dolan and Morris (2000) have suggested that the amygdala has a role in the processing and recognition of fearful facial expressions in humans. As with anxiogenesis, a process to which fear processing is related, these studies suggest that the amygdala is relatively active in both conditioned and unconditioned manipulations.
supported by the research of Treit and Pesold. Treit and Pesold (1990) subjected septally lesioned rats to two tests of anxiety, the elevated plus maze and the shock-probe test (a type of passive-avoidance task). In accordance with their theory, the lesioned rats seemed to exhibit reduced anxiety during the plus maze task, spending significantly more time on the open arms than unlesioned rats. They had similar results on the passive-avoidance task, with the rats receiving many more shocks than controls. Next, Treit and Menard (2000) attempted to verify these tasks as true tests of anxiolysis by examining how BDZ affected the rats performance. In a finding that seems to support their theory, (but also supports the theory outlined more fully in this paper), BDZs injected into the septum results in behavior that closely parallels the test results of rats with septal lesions. If these studies are to be taken at face value, the amygdala-lateral septum theory of anxiety faces a major challenge. In the opinion of the author, as well as that of Dr. Thomas, these results can be explained either by a lack of precision in methodology or by poor inferences from the data.

To address these apparently conflicting findings, Thomas (1988) emphasizes the importance of distinguishing the two major areas of the septum, the lateral and the medial. Whereas Gray and his followers appear to treat the septum as functionally and anatomically homogeneous, Thomas cites a variety of studies showing the differential impact of stimulating its two sub-areas. Thus, the results of these studies could conceal the underlying variable of the precise location (lateral or medial) of the lesions, and may not contradict Thomas’ idea that the lateral septum is the primary locus of anxiolysis.

Even if this failure to distinguish the two areas is not a factor however, there is a second way of interpreting the results of these studies that is not considered by their
authors. Both the plus-maze and passive avoidance tasks use avoidance of an aversive stimulus as a primary index of anxiety; in the plus-maze, this stimulus comes in the form of the danger of falling, while in the passive avoidance task, it comes in the form of a shock. What if however, the rats’ baseline anxiety level were so high that these stimuli provided no additional aversive impact? Would the rats still avoid the shocks or open cliff if they feared the non-shock and closed cliff just as much? Essentially, the proposed resolution of this challenge is that these avoidance tasks have a ceiling effect: the anxiety level of the septally lesioned rats is so high that they fail to discriminate between aversive and non-aversive stimuli. In lower-levels of anxiety, these tests may be valid measures, but when the level of anxiety is high such that being shocked or falling induces no additional fear, these tests become insensitive. Thus, while it is true that septal-lesions have the same testing outcome as intra-septal injection of BDZs, the reasons for these outcomes may be completely different. Observation of rat behavior during these tasks supports this idea: defecation, urination, emotional reactivity increases as a result of septal lesions, but decreases as a result of BDZ administration, (Thomas, personal communication).

The Project

My project seeks to explore some of the major questions that remain unanswered within this model of anxiety. Studies such as Sanders and Shekhar (1995) have found that GABA$_A$ agonists (in that case, muscimol) are anxiolytic when applied directly to the amygdala. This suggests that GABA is a major, if not the primary, neurotransmitter for inhibition of the amygdala’s anxiogenic action. Since BDZs work on the GABA receptor
complex, studies like Shibata et al. (1989) bolster this notion. Thomas and Sancar (2001) then demonstrated that stimulation of the lateral septum, as well as intra-septal injection of BDZs, inhibits activity in the central nucleus of the amygdala. This suggested that part of the action of BDZs is amplifying the inhibitory effect of the lateral septum on the amygdala. It does not answer however, what exactly is happening on the amygdala’s side of this connection. Do the projections from the lateral septum (or intermediate structures) act on the amygdala’s GABA_A receptors? This question represents a major gap in the theory. If the lateral septum does act in this way, it provides crucial evidence for its role as source of endogenous GABA, further strengthening the notion that this structure has an important role in anxiolytic modulation of the amygdala. If the projections do not act in this manner, then the current theory may need to be amended significantly. My experiment will attempt to answer this question by examining the effect of lateral septum stimulation during a blockade of GABA_A receptors.

First, I will attempt to replicate one of the findings of Thomas and Sancar (2001) by looking for inhibition in the central nucleus of the amygdala in response to stimulation of the lateral septum in rats. I will then administer picrotoxin, a GABA_A antagonist systemically. Once the drug has taken effect, I will observe the changes, if any, in the effects of lateral septum stimulation on recorded activity in the central nucleus of the amygdala. If the projections that cause inhibition in the non-drug condition are GABA-ergic, then, after administration of picrotoxin, activity in the amygdala should no longer be inhibited by septal stimulation. In addition to answering the major question posed by the study, this result might suggest that BDZs have their anxiolytic effect not only by increasing the activity of the lateral septum itself, but also by amplifying the effect of the
lateral septum’s projections at their amygdaloid synapses. This would enrich the understanding of the mechanism of action of current anxiety treatments, possibly leading to the development of more selective, improved drugs. A contrary result could indicate that a different, non-GABA-ergic, system is involved in anxiolysis or that an intermediate structure helps to mediate the lateral septum’s inhibition of the amygdala.

**CeA vs. BLA**

Although I have alluded to different nuclei within the amygdala, I have thus far treated the structure as being somewhat homogenous in form and function; like with the septum however, this is far from the case. Although it is clear that the majority of the amygdala’s efferent projections originate from the central nucleus (CeA), the other nuclei may also play important, possibly distinct, roles in anxiogenesis. In fact, it has been demonstrated that another amygdaloid nucleus, the basolateral (BLA), actually has a much higher concentration of BDZ receptors than the central nucleus, (Niehoff and Kuhar, 1983). The relative importance of these two nuclei is a matter of heated debate within the anxiety literature. Measuring acoustic startle, Rosen and Davis (1988) concluded that the two areas produce *equivalent* behavioral effects in response to stimulation; Shibata et al. (1989) supported this, finding water-lick conflict behavior equally responsive, both to lesioning and direct administration of BDZs, in either of the areas. Shibata et al. (1982) however, found that administration of BDZs to the CeA did produce anti-conflict behavior in the water-lick test, administration to the BLA did *not* produce such a response; somehow, Scheel-Kruger and Petersen (1982) found the exact *opposite* pattern using the same conflict test. Corroborating this second pair of findings,
Green and Vale (1992) found that BLA-administered BDZs reduced anxiety in the elevated plus-maze paradigm, but CeA-administered BDZs provoked no response. Sanders and Shekhar (1991) also found that GABA_A antagonists elicited changes in heart rate and blood pressure only when applied to the BLA, and not the CeA. Finally, as if the situation were not complicated enough, Pesold and Treit (1995) found a double-dissociation in shock-probe performance and plus-maze performance: only the CeA had anxiolytic action in response to BDZs during the first task, while only the BLA responded during the second. On surface, this debate seems to introduce a major confound into my experiment: recording from the central nucleus might obscure the role of the BLA. For instance, although there is some evidence of a direct connection from the lateral septum to the CeA, there might exist a stronger pathway that synapses first in the BLA. If this turns out to be the case, it seems on the surface that my experiment would fail to document the response of this nucleus, resulting in falsely negative findings. Given its relatively high concentration of BDZ receptors and its more extensive afferent connections, the BLA might seem to be a better candidate to receive anxiolytic inputs, so shouldn’t I be recording from this nucleus instead of the CeA? Though these are important considerations, the internal structure of the amygdala allows me to record from the CeA with confidence that BLA activity is also factored.

As Davis, Rainnie and Cassell (1994) explains, the BLA has strong, unilateral, excitatory projections to the CeA. As a result, inhibition or disinhibition of the BLA should have an equivalent effect on activity of the CeA. Thus, while my experiment does not eliminate the confounding variable of whether the lateral septal stimulation (and the proposed blockade thereof) has its effects on the CeA directly or via a synapse in the
BLA, my data should not be affected. With this in mind, it is preferable to record from the CeA than from the BLA, because while recording probe in the CeA would also be sensitive to activity in the BLA, the reverse is not true. Secondly, since all previous electrophysiological studies conducted in Dr. Thomas’ lab have probed the CeA, I have an available pool of data against which I can compare my results; that the first step of my experiment has been proven valid in the same lab also increases the likelihood of positive results. Finally, the majority of anxiogenic efferent projections from the amygdala originate in the CeA, this nucleus has a stronger relationship with the somatic correlates of anxiety; thus, recording here would better allow me to better generalize the conclusions I draw from my data.

**Method**

**Design**

The experiment was a 2 X 2 within-subjects design. The independent variables were whether or not picrotoxin has been administered at the time of recording and the time period during which neural activity is recorded (either one second prior to stimulation or one second following stimulation). The dependent variable was the rate of neural firing.

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3 As originally planned, there would have been a third variable, the pattern of electrical stimulation, of which there would have been three conditions: five pulses at 100Hz, five pulses at 20Hz, or one pulse. The 100Hz pulse would simulate maximal temporal summation, and thus would have produced the greatest inhibitory response; the 20Hz pulse best mimics the normal rate of neural firing and would show how the connections function in somewhat more natural conditions; the single pulse would have been used to measure length of latency, which would then be analyzed to determine the number of synapses involved in the neural connection. However, due to equipment failure, all rats except one (#M25) received no variability in stimulation pattern. Consequently, stimulation pattern was dropped as a variable of interest.
Subjects

The subjects were eight male Sprague-Dawley albino rats from Harlan. They were housed in metal cages with grated bottoms. They were given food pellets and water *ad libitum*; their bedding was changed semiweekly and they were handled regularly.

Apparatus

A standard stereotaxic frame was used to secure the heads of the rats and to insert the electrodes. Rats were stimulated using a bipolar electrode composed of two twisted stainless steel wires connected to a WPI A365 stimulus isolator and controlled by an A.M.P.I Master-8 stimulator. Data was recorded using a Frederick Haer and Co. tungsten electrode, grounded and connected to an A-M systems microelectrode AC amplifier, model 1800. After data from the amplifier was fed through a Frederick Haer and Co. window discriminator, it was sent both to an Onkyo standard audio tape recorder and a Hitachi digital oscilloscope (VC-6050). From the oscilloscope, data flowed to a Frederick Haer and Co. audio analyzer before being sent to a Dell Optiplex GXa for digital recording with the Datawave Discovery software package. Concurrent with this, data from the stimulator was sent both to the computer and the tape recorder.

Procedure

Each rat was first anaesthetized with urethane (1.4g/kg) intraperitoneally. After waiting 5-10 minutes for the anesthesia to take effect, the dorsal surface of rat’s head was shaved. The rat was then placed in the stereotaxic frame. An incision was made longitudinally, along the medial dorsal surface of the scalp. The scalp was pulled and
held apart using hemostats and the surface of the skull was cleared, cleaned, and dried
using a spatula, water, an air-blower, gauze, and cotton swabs. Next, the recording and
stimulating electrodes was zeroed at bregma. Using coordinates specified by Paxinos and
Watson (1982), the recording electrode was moved 4.0 mm laterally and 2.2 mm
posterior and the stimulating electrode was moved 0.7 mm ipsilaterally and 0.3 anterior.
Holes were drilled at these spots and the stimulating electrode was lowered into the
lateral septum, 6 mm below the surface of the skull. The recording electrode was
lowered into the central nucleus of the amygdala, between 6.6 and 7.4 mm below the
dorsal surface of cortex. The determination of the final placement of the recording
electrode was based on neural activity found.

Once the recording electrode was in place, neural activity was recorded onto
audiotape and on the Datawave Discovery computer program. Next, each received a
series of 50 electrical pulses, each approximately 10 milliseconds in duration. On the
completion of this round of stimulation, picrotoxin, a GABA<sub>A</sub> antagonist, was injected
intraperitoneally at 1g/kg, a dose guided by the results of Dalvi and Rodgers (1996).
After waiting fifteen minutes for the picrotoxin to achieve maximal efficacy, a second
round of stimulation, identical to the one described above, commenced. Each rat was
then be sacrificed by overdose of sodium pentobarbital and decapitation. The brains were
removed and placed in formalin for preservation; a histology was performed after a
period of at least one day.

I looked primarily for an interaction between picrotoxin administration and time
period in order establish the drug’s effect on inhibition, if any. For analysis, neurons

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4 Unlike all other rats, #M25 received the three patterns of stimulation that were originally planned. For
this rat, all data will be in reference to the first pattern (50 trains of five 100Hz pulses).
were isolated from the recorded data using the Datawave Discovery software package. Data from these neurons were plotted on a peri-event time histogram (PETH), wherein the rate of firing for each neuron during the one-second period prior to each electrical stimulus was compared to the rate of firing during the one-second period following each electrical stimulus. It was determined that stimulation of the lateral septum inhibited activity in the recorded neurons of the central nucleus of the amygdale, if the average rate of firing in the “before” periods was significantly greater than during the “after” periods. At the end of the experiment, ANOVA tests were performed to determine whether picrotoxin mitigates the inhibitory effects of lateral septal stimulation on the activity of central amygdaloid neurons.

Results

Histologies

Of the 34 rats that were subjected to the procedure, eight (#F05, #F12, #M04, #M16, #M18, #M19, #M23, #M25) yielded units with amplitudes and frequencies high enough to permit recording. Histologies confirmed accurate placement of the stimulating electrode in all subjects, (for example, see Figure 1). They also indicate accurate placement of the recording electrode in #F05, #M04, #M18, and #M19, (for example, see Figure 2). Slides taken of #F12 and #M23 suggest, but do not fully confirm accurate placement of the recording electrode. The recording electrode was slightly too medial in #M16, likely having been placed in the medial amygdaloid nucleus rather than the central nucleus; it was slightly too ventral in #M25, and probably recorded the activity of a neuron in the basomedial nucleus of the amygdala.
Figure 1: Slide of accurately placed stimulating electrode (from rat #M18)
Response to Stimulation (Without Picrotoxin)

For each rat, action potentials were recorded and plotted onto peri-event time histogram (PETH) charts, (see figures 3-10). T-tests were then performed on the resulting data, with the mean number of spikes recorded in the 100 ten-millisecond intervals prior to stimulation being compared to the mean number of spikes recorded in the 100 ten-millisecond intervals after stimulation. Analysis of the data shows that, in six of the eight rats, electrical stimulation of the lateral septum resulted in some degree of inhibition of the rate of neural firing in the amygdala. Three of these rats, #F05 \( t(174.220) = 5.023, \ p<.001 \), #F12 \( t(139.688) = 6.155, \ p<.001 \), #M04 \( t(182.145) = 9.174, \ p<.001 \), showed statistically significant levels of inhibition as a result of stimulation. Because of the effect of strong post-inhibitory rebound, #M16 did not
display statistically significant inhibition in the analysis of the 1-second periods before and after stimulation \( t(171.100) = -2.513, p<.02 \); limiting the analysis to the .5-second periods before and after stimulation, however, does reveal inhibition at a statistically significant level \( t(56.018) = 4.547, p<.001 \). Two rats, #M19 \( t(165.751) = 1.294, p<.2 \) and #M23 \( t(197) = 1.098, p<.28 \), show some inhibition, but not at a significant level. Both of the remaining rats, #M18 \( t(156.508) = -2.131, p<.04 \) and #M25 \( t(122.3) = -4.369, p<.001 \), exhibited a statistically significant increase in the rate of neural firing due to stimulation.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Mean (before stimulation)</th>
<th>Mean (after stimulation)</th>
<th>degrees of freedom</th>
<th>T-score</th>
<th>P-value</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>F05</td>
<td>1.796</td>
<td>.746</td>
<td>174.220</td>
<td>5.023</td>
<td>0.000</td>
<td>Inhibition</td>
</tr>
<tr>
<td>F12</td>
<td>1.200</td>
<td>.593</td>
<td>139.688</td>
<td>6.155</td>
<td>0.000</td>
<td>Inhibition</td>
</tr>
<tr>
<td>M04</td>
<td>2.370</td>
<td>1.081</td>
<td>182.145</td>
<td>6.865</td>
<td>0.000</td>
<td>Inhibition</td>
</tr>
<tr>
<td>M16</td>
<td>.810</td>
<td>1.273</td>
<td>171.100</td>
<td>-2.513</td>
<td>0.013</td>
<td>Not valid</td>
</tr>
<tr>
<td>M16 (.5 secs)</td>
<td>.760</td>
<td>.080</td>
<td>56.018</td>
<td>4.547</td>
<td>0.000</td>
<td>Inhibition</td>
</tr>
<tr>
<td>M18</td>
<td>.515</td>
<td>.848</td>
<td>156.508</td>
<td>-2.131</td>
<td>0.035</td>
<td>Facilitation</td>
</tr>
<tr>
<td>M19</td>
<td>3.833</td>
<td>3.394</td>
<td>165.751</td>
<td>1.294</td>
<td>0.198</td>
<td>Some Inhibition</td>
</tr>
<tr>
<td>M23</td>
<td>.370</td>
<td>.273</td>
<td>197.000</td>
<td>1.098</td>
<td>0.274</td>
<td>Some Inhibition</td>
</tr>
<tr>
<td>M25</td>
<td>1.120</td>
<td>2.586</td>
<td>122.300</td>
<td>-4.369</td>
<td>0.000</td>
<td>Facilitation</td>
</tr>
</tbody>
</table>

Response to Stimulation (With Picrotoxin)

The PETH charts from the data recorded after picrotoxin was administered were created in the same way as in the pre-drug condition, (see figures 11-18); t-tests were also performed in the same manner. Only the data from rats that showed some amount of inhibition in the pre-drug condition can be used to address my primary hypothesis; if stimulation does not cause inhibition before picrotoxin is administered, then we cannot logically examine whether the drug eliminates or diminishes this effect. With this in mind, only one of the rats that exhibited a significant level of inhibition in the pre-drug
condition, #F05, showed no inhibition in the post-drug condition \([t(193) = .056, p > .95]\). The other three rats with statistically significant inhibition in the pre-drug condition continued to display statistically significant inhibition in the post-drug condition, (#F12 \([t(162.996) = 5.057, p<.001]\), #M04 \([t(130.927) = 0.174, p<.001]\), and #M16 \([t(52.689) = 3.621, p<.002]\)\(^5\)). Of the rats with non-statistically significant levels of inhibition in the pre-drug condition, #M19 did display statistically significant levels of inhibition in the post-drug condition \([t(189) = 2.807, p<.008]\), while #M23 ceased to show any effect of stimulation \([t(191.194) = -.379, p>.7]\). Though it is not directly related to the hypothesis, it is interesting to note that one of the two rats that displaying a significantly facilitating effect of stimulation in the pre-drug condition, #M18, ceased to exhibit any effect of stimulation in the post-drug condition \([t(194) = .027, p>.97]\).

**Table 2: T-tests on data in post-drug condition**

<table>
<thead>
<tr>
<th>Rat</th>
<th>Mean (before stimulation)</th>
<th>Mean (after stimulation)</th>
<th>degrees of freedom</th>
<th>T-score</th>
<th>P-value</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>F05</td>
<td>.722</td>
<td>.714</td>
<td>193.000</td>
<td>0.056</td>
<td>0.956</td>
<td>No effect</td>
</tr>
<tr>
<td>F12</td>
<td>.798</td>
<td>.265</td>
<td>162.996</td>
<td>5.057</td>
<td>0.000</td>
<td>Inhibition</td>
</tr>
<tr>
<td>M04</td>
<td>1.940</td>
<td>.485</td>
<td>130.927</td>
<td>9.174</td>
<td>0.000</td>
<td>Inhibition</td>
</tr>
<tr>
<td>M16 (.5 secs)</td>
<td>.400</td>
<td>.020</td>
<td>52.689</td>
<td>3.621</td>
<td>0.001</td>
<td>Inhibition</td>
</tr>
<tr>
<td>M18</td>
<td>.707</td>
<td>.697</td>
<td>194.000</td>
<td>0.027</td>
<td>0.978</td>
<td>No Effect</td>
</tr>
<tr>
<td>M19</td>
<td>2.485</td>
<td>1.677</td>
<td>189.000</td>
<td>2.807</td>
<td>0.006</td>
<td>Inhibition</td>
</tr>
<tr>
<td>M23</td>
<td>.760</td>
<td>.808</td>
<td>191.194</td>
<td>-0.379</td>
<td>0.705</td>
<td>No Effect</td>
</tr>
<tr>
<td>M25</td>
<td>.630</td>
<td>1.596</td>
<td>125.334</td>
<td>-4.073</td>
<td>0.000</td>
<td>Facilitation</td>
</tr>
</tbody>
</table>

\(^5\) Here, and with all further analyses, the tests on #M16 will only include data collected from the .5 second periods before and after stimulation.
Figure 3: F05 without picrotoxin

Figure 11: F05 with picrotoxin
Figure 4: F12 without picrotoxin

Figure 12: F12 with picrotoxin
Figure 5: M04 without picrotoxin

Figure 13: M04 with picrotoxin
Figure 6: M16 without picrotoxin

Figure 14: M16 with picrotoxin
Figure 7: M18 without picrotoxin stimulation

Figure 15: M18 with picrotoxin stimulation
Figure 8: M19 without picrotoxin

Figure 16: M19 with picrotoxin
Figure 9: M23 without picrotoxin stimulation

Figure 17: M23 with picrotoxin stimulation
Figure 10: M25 without picrotoxin

![Graph showing spikes without picrotoxin stimulation.](image)

Figure 18: M25 with picrotoxin

![Graph showing spikes with picrotoxin stimulation.](image)
Interaction Effect

Although performing a t-test on the post-drug condition is important for a categorical evaluation of the effect of picrotoxin, its quantitative effect cannot be derived in this manner. To achieve this, 2X2 ANOVA tests were performed on the data from each rat, with the two independent variables being stimulation (before or after) and picrotoxin (before or after administration). From these analyses, two of the nine rats showed a statistically significant interaction effect of picrotoxin and stimulation in the direction predicted by the hypothesis. As would be expected from the t-tests, the data from #F05 revealed that the stimulation had significantly less of an inhibitory effect after picrotoxin was administered than it had prior to administration [F(1,387) = 15.660, p<.001]. Though there was still statistically significant inhibition in the post-drug condition of #F12, the ANOVA test revealed a similar interaction in this rat as well [F(1,385) = 3.884, p<.05]; while the stimulation effect was still apparent in the post-drug condition, the magnitude of the effect was significantly less than in the pre-drug condition. A third rat, #M16 also exhibited this type of interaction, though not at the level of significance, [F(1,196) = 2.696, p<.11]. The fourth, and final, rat that showed significant inhibition in the pre-drug condition, #M04, showed no interaction effect of the drug at all [F(1,394 = .454, p>.5]; neither of the two rats which had non-significant levels of stimulation-induced inhibition in the pre-drug condition, (#M19 [F(1,377) = .432, p>.51] and #M23 [F(1,394) = .883, p>.34]) showed any such effect either. In both #M18 [F(1, 386) = 2.463, p<.12] and M#25 [F(1,394) = 1.492, p<.23], picrotoxin reduced the facilitating effect of stimulation, though not at statistically significant levels.
**Table 3: Interaction Effect**

<table>
<thead>
<tr>
<th>Rat</th>
<th>degrees of freedom</th>
<th>F-score</th>
<th>P-value</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>F05</td>
<td>1, 387</td>
<td>15.660</td>
<td>0.000</td>
<td>Significant reduction in inhibition</td>
</tr>
<tr>
<td>F12</td>
<td>1, 385</td>
<td>3.884</td>
<td>0.049</td>
<td>Significant reduction in inhibition</td>
</tr>
<tr>
<td>M04</td>
<td>1, 394</td>
<td>0.454</td>
<td>0.501</td>
<td>No effect</td>
</tr>
<tr>
<td>M16</td>
<td>1, 196</td>
<td>2.696</td>
<td>0.102</td>
<td>Non-significant reduction in inhibition</td>
</tr>
<tr>
<td>M18</td>
<td>1, 386</td>
<td>2.463</td>
<td>0.117</td>
<td>Non-significant reduction in facilitation</td>
</tr>
<tr>
<td>M19</td>
<td>1, 377</td>
<td>0.432</td>
<td>0.511</td>
<td>No change</td>
</tr>
<tr>
<td>M23</td>
<td>1, 394</td>
<td>0.883</td>
<td>0.348</td>
<td>No change</td>
</tr>
<tr>
<td>M25</td>
<td>1, 394</td>
<td>1.492</td>
<td>0.223</td>
<td>Non-significant reduction in facilitation</td>
</tr>
</tbody>
</table>

**Figure 19: Plot of Interaction Effect in #F05**

**Figure 20: Plot of Interaction Effect in #F12**

**Figure 21: Plot of Interaction Effect in #M04**

**Figure 22: Plot of Interaction Effect in #M16**
Main Effect of Picrotoxin

A secondary finding in these ANOVA tests was that picrotoxin also had a main effect in all rats except for #M18. Furthermore, its effect was negative in six of these cases, #F05, #F12, #M04, #M16, #M19, and #M25.

Table 4: Main Effect of Picrotoxin

<table>
<thead>
<tr>
<th>Rat</th>
<th>Pre-Drug Mean</th>
<th>Post-Drug Mean</th>
<th>degrees of freedom</th>
<th>F-score</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>F05</td>
<td>1.311</td>
<td>.718</td>
<td>1, 387</td>
<td>30.010</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>F12</td>
<td>.999</td>
<td>.292</td>
<td>1, 385</td>
<td>6.688</td>
<td>&lt;.02</td>
</tr>
<tr>
<td>M04</td>
<td>1.725</td>
<td>1.212</td>
<td>1, 394</td>
<td>17.337</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>M16</td>
<td>.420</td>
<td>.210</td>
<td>1, 196</td>
<td>5.285</td>
<td>&lt;.03</td>
</tr>
<tr>
<td>M18</td>
<td>.696</td>
<td>.699</td>
<td>1, 386</td>
<td>.001</td>
<td>&gt;.97</td>
</tr>
<tr>
<td>M19</td>
<td>3.613</td>
<td>2.125</td>
<td>1, 377</td>
<td>49.242</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>M23</td>
<td>.321</td>
<td>.784</td>
<td>1, 394</td>
<td>35.781</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>M25</td>
<td>1.853</td>
<td>1.113</td>
<td>1, 394</td>
<td>13.074</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>
Discussion

Despite the small sample size and the impossibility of aggregating the data collected from each rat, the results of the experiment still bore several pieces of evidence that support my primary hypothesis, that neural projections from the lateral septum act on the amygdala’s GABA$_A$ receptors.

First, the data confirmed that stimulating the lateral septum results in inhibition of neural activity in the central nucleus of the amygdala; this replicates the findings of Thomas and Sancar (2001). Four out of the eight rats exhibited such inhibition at a significant level; two more displayed inhibition at non-significant levels. Without data from the single short-pulse pattern of stimulation to which the rats were originally to be subjected, one cannot determine the latency of this effect; consequently, no judgment can be made about the number of intermediate neurons involved in the connection from the lateral septum to the neurons from which data was recorded. Regardless of the nature of the connection however, confirming both that it exists and that it tends to exert an inhibitory influence permitted evaluation of the effect that most directly addresses the hypothesis; only after confirming that inhibition actually occurs could one logically test whether picrotoxin mitigates this effect.

Although t-tests suggested that only one rat, (#F05), responded significantly to picrotoxin, ANOVA tests revealed that two others (#F12 and #M16) also exhibited the expected effect. After blocking the activity of GABA$_A$ receptors in #F05, stimulation failed to exert any effect on activity of the neuron being recorded, (the ANOVA test confirmed this highly significant interaction effect). This suggests unambiguously that the lateral septum’s inhibitory influence on this amygdaloid neuron was primarily
mediated by GABA-ergic projections. Though rat #F12 did not respond quite as robustly, (still exhibiting significant levels of inhibition in the post-drug condition), it did show a statistically significant interaction effect. There are several potential reasons why picrotoxin might have significantly reduced the stimulation-induced inhibition, while not fully eliminating it in #F12. First, it is possible, (if not likely), that the picrotoxin did not fully block all GABA receptors. I chose to administer a dose of 1g/kg because Dalvi and Rodgers (1996) demonstrate that this amount elevates the anxiety level, as measured by behavioral indicators, without inducing seizures. Thus, at this dose, the treatment better mimics natural anxiety than would a higher dose. This also means however, that this dose is not likely to induce the maximal effect. The potential for technical error also exists, as it is possible I injected the picrotoxin incorrectly, conceivably causing absorption of an amount not equal to the full dose. Without a clear histology showing the exact placement of the recording electrode in #F12, the possibility also remains that I missed the central nucleus, and was instead recording from an adjacent amygdaloid nucleus. If this is the case, this neuron may be responding to a slightly different pathway than the neuron in #F05; it may be that in this pathway, GABA is only one of multiple neurotransmitters that create the inhibitory effect. These explanations may also be applicable to #M16. Here, the histology indicates that the recording electrode was improperly placed in the medial amygdaloid nucleus, but the interaction effect still approached significance.

Next, data from the two rats that initially exhibited stimulation-induced facilitation may provide important insight into the nature of the pathway from the lateral septum to the amygdala. Firstly, it is intriguing that this connection, which I predicted
would primarily be inhibitory, could also have a excitatory effect. The most obvious explanation is that there might be parallel pathways between the two structures, one inhibitory and one excitatory. If this were the case, it would significantly complicate the model of the lateral septum serving as an anxiolytic modulator of the amygdala. There is an alternative explanation for the facilitation that I observed, though: it may have been the consequence of neural disinhibition. This would occur if the neurons being recorded received strong inputs from one or more amygdaloid neurons that normally exert an inhibitory influence themselves; if these “input” neurons were inhibited by stimulation, then their influence would be weakened, and the neurons from which I recorded would be more likely to exhibit an increased rate of firing. The finding that, in both rats #M18 and #M25, picrotoxin reduced the effect of stimulation at near-significant levels suggests that GABA is involved in this facilitation in some way. Since GABA is exclusively an inhibitory neurotransmitter, this makes this second scenario more likely. If this is the case, then the amygdaloid “input” neurons are responding in the manner predicted, even if the neurons that were recorded did not; though this evidence is not as strong as that derived from rats #F05, #F12, and #M16, it still supports the hypothesis.

Rats #M04, #M19, and #M23 failed to exhibit any interaction effect despite significant, or near-significant, inhibition in the pre-drug condition. This result could have been a consequence of any of the reasons described previously. However, in studies of this type, it is not necessary that all, or even the majority, of the cells are responsive in the manner expected. Merely demonstrating that many cells do respond as such suggests that the system operates, at least partly, as indicated by the theory. Cells, even within a given neural structure, are not functionally homogenous; they might not all play the same
“role” in the system. Furthermore, it is to be expected that the response of in vivo neurons will be somewhat idiosyncratic, (even among those who play similar “roles”), as each occupies in a slightly different place within the neural web. Given all the potential sources of error and variability inherent to in vivo recording, it is not surprising that some cells do not respond in any significant way. Thus, although the cells in rats # M04, #M19, and #M23 did not respond as expected, they do not considerably dampen the inferences that one can make from the ones that did.

Although the bulk of the data does seem to support my hypothesis, the finding that picrotoxin tended to have an overall negative effect on neural activity sheds a somewhat ambiguous light on my conclusions. Since picrotoxin blocks the effect of an inhibitory neurotransmitter, the a priori expectation might be that the drug would increase neural activity overall. This loomed as a potential confound in my experiment, since a greater rate of firing might mask inhibition for reasons owing nothing to the neural pathway being studied. That this positive effect did not occur might make my conclusion more credible. However, the great significance of the negative effect might be just as troubling, if only for the questions it raises about why it occurred, and how these unidentified causes might interact with the other data. For instance, if picrotoxin itself has a suppressing effect on amygdaloid neural activity, then perhaps its administration lowers the rate of firing to the point where stimulation is less able to have an effect; this chain of events could mirror my findings, but would have nothing to do with the subject of inquiry. It is also possible that this effect is due not to picrotoxin itself, but instead due to the passage of time. It was necessary to record the neuronal activity without picrotoxin first because once it is injected, the drug stays in the rat’s
system for an indefinitely long time period; a counter-balanced condition, wherein one tested the non-drug condition after the drug condition is not possible in an acute set-up, such as this. Thus, in this type of experiment, time and order must always be co-occurring variables. If the neurons simply slowed their rate of firing as a consequence of time though, it is also possible that their susceptibility to the effects of stimulation were not similarly reduced. Additionally, since there is such a strong expectation that picrotoxin would increase the rate of firing, this effect could draw questions about the potency and/or purity of the drug itself. This, coupled with the small number of rats used in this study, renders it even more critical that this experiment be reproduced on a larger scale. However, it may be observed that #M19, which exhibited the largest main effect of picrotoxin, had the lowest interaction effect. This does not fully allay the doubts created by the picrotoxin’s main effect, but does somewhat ease them.

Although more data needs to be collected in this experiment before any conclusions can be made confidently, these initial results do suggest that the connection between the lateral septum and the amygdala is GABA-ergic. This supports the theory that the lateral septum acts as an inhibitory modulator of the amygdala’s anxiogenic activity. The results also provide an insight into why septal stimulation (as described by Yadin et al. (1993)) and intra-amygdaloid administration of picrotoxin (as described by Sanders and Shekhar (1995)) yield opposite behavioral effects. Finally, these findings suggest an additional mechanism by which anxiolytic drugs, especially BDZs, may operate in the body, working on the lateral septum-amygdala connection, and not just on the amygdala itself.
Several modifications could be made to this experiment in order to further bolster the theory, as well our understanding of the anxiety system as a whole. First, the experiment could be rerun with a higher dose of picrotoxin; the scenario would be less “natural,” but the effects could be more robust. Secondly, the drug could be injected directly into the amygdala, as was done by Sanders and Shekhar (1991 and 1995). This could add significant nuance to the data, as one could try adding the picrotoxin to different amygdaloid nuclei or to afferent neurons. With studies such as Niehoff and Kuhar (1983), which detail the variable distribution of BDZ receptors, (an imperfect correlate of GABA<sub>A</sub> receptors), one could compare the results and determine whether there is an association between receptor-density and responsiveness. One could also perform this experiment with BDZs instead of GABA<sub>A</sub> antagonists. If BDZs were to fail to significantly inhibit amygdaloid activity, beyond the effects of septal stimulation, then it would greatly support one possible inference from my experiment: BDZs primarily work by mimicking the anxiolytic effect of septal activity.

It would also be of interest to run the same experiment, but instead record from downstream structures, such as the central gray or the lateral hypothalamus, (as suggested by LeDoux et al. (1988)); if there is a similar effect in these structures, then the behavioral implications of this study are even stronger. Alternatively, the behavioral impacts of these manipulations could be tested directly if one employed a “chronic” treatment, implanting permanent recording and stimulating electrodes. This would be somewhat disadvantageous, as one could not move the recording electrode in order to search for active neurons, but would allow one to test the behavioral correlates of electrophysiological results. A chronic setup could also permit a counterbalance for the
time variable, since the drug and non-drug conditions would not need to be tested on the same day.

Finally, a similar experiment needs to be conducted to examine the other side of this loop, the connection from the amygdala to the lateral septum; by doing this one could determine which neurotransmitter is responsible for the amygdala’s regulation of septal anxiolysis, and could permit the development of a new class of anxiolytic drugs.
References


