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The Effects of Medial Septal Modification

on the Theta Rhythm of the Amygdala

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Abstract

The theta rhythm is a 3-12 Hz electroencephalographic (EEG) oscillatory phenomenon that has been recorded from the hippocampus of small animals during large motor movements, periods of arousal, and memory tasks. Theta denotes the amount of brain synchrony occurring, with higher theta amplitude corresponding to more synchrony among individual neurons. The proposed pacemaker of the hippocampal theta rhythm is the medial septal area (MSA), which consists of neurons that fire rhythmically in frequencies corresponding to the theta rhythm. Despite the proven physical connection between the MSA and the amygdala, there is uncertainty as to whether the MSA serves as a pacemaker for the amygdala theta rhythm as well. To assess the effect of MSA neuronal firing on the amygdala theta rhythm, cholinergic antagonists and GABAergic agonists were infused into the MSA. Eight male Long-Evans rats were anesthetized with a ketamine/xylazine mixture. A guide cannula was inserted into the MSA and a recording electrode was inserted into the amygdala. After one week, rats were re-anesthetized and amygdala EEG was recorded. Baseline recordings were taken for 5 minutes, after which each rat was infused with 0.5 µl of all of the following drug treatments: saline, scopolamine (10 µg or 20 µg), muscimol (30 ng or 60 ng), and ethanol (0.1% or 1.0%). A within-subjects ANOVA was used to analyze pre- and post-infusion differences in theta power. There were main effects for muscimol and ethanol, but no main effect for scopolamine. Subsequent T-tests revealed a significant difference in theta power between the saline treatment and both the high, $t(7) = 4.015$, $p < .05$, and low $t(7) = 2.934$, $p < .05$, doses of muscimol. There was also a significant difference in theta power between the saline and the high, $t(6) = 2.602$, $p < .05$, and low, $t(4) = 3.240$, $p < .05$, doses of ethanol. These results have strong implications for the respective roles of the cholinergic and GABAergic pathways in amygdala theta modulation.

The Effects of Medial Septal Modification on the Theta Rhythm of the Amygdala

In the late 1920s, neuroscientists first observed rhythmic electroencephalographic (EEG) activity from the human scalp. The first rhythm, denoted "alpha", was recorded at frequencies ranging from 8 to 12 Hz (Stewart & Fox, 1990). Soon thereafter, researchers found a distinct brain oscillation that occurred between 4 and 8 Hz in humans and between 3 and 12 Hz in animals. They named this frequency "theta" and noted that it was most readily recorded from the hippocampus of the limbic system (Tesche & Karhu, 2000; Konopacki, 1998). Since then, theta research has focused on discovering how this rhythm affects brain functioning and human behavior, including learning and memory capabilities. Researchers have expanded their experiments to learn about the timing, origin, and maintenance of this phenomenon, and how it is affected by possibly harmful substances. The majority of research on theta has focused on three aspects of the rhythm: effects on cognition and behavior, the neuroanatomical basis of theta, and the neurochemical basis of theta.

The Effects of Theta on Cognition and Behavior

The theta brain oscillation appears during several aspects of human cognition and behavior, all of which are important for proper functioning. It is predominantly present during large motor movements (Whishaw & Vanderwolf, 1973; Oddie, Stefanek, Kirk & Bland, 1996), periods of attention and arousal (Fuentemilla, Marco-Pallarés, Münte & Grau, 2008; Nakashima & Sato, 1992), and working memory tasks (Gevins & Smith, 2001; Lisman & Idiart, 1995; Wilson & McNaughton, 1994; Laukka, Järvilehto, Alexandro & Lindqvist, 1994; Winson, 1978).

Motor movement. The theta rhythm is involved in successful execution of motor movements. Slow, rhythmic theta activity in the rat is present during large, voluntary movements, such as walking, running, forelimb movement, and posture shifts (Whishaw &

Vanderwolf, 1973; Oddie et al., 1996), but it is absent during fast reflex activities, such as scratching, shivering, or chattering the teeth (Whishaw & Vanderwolf, 1973). Specifically, high theta amplitude is associated with large motor movements, and chemical lesions significantly decrease the amplitude of theta and the EEG frequency during voluntary movement (Whishaw & Vanderwolf, 1973; Oddie et al., 1996). Interestingly, hippocampal theta did not disappear neither when the animal repeated the movement nor during the presence of both large and small movements (Whishaw & Vanderwolf, 1973). The presence of the theta rhythm during all movement suggests a strong connection between theta and motor functioning.

Attention and arousal. Theta also regulates attention and arousal levels, particularly wakefulness (Dalle-Lucca & Timo-Iaria, 1992), detection of stimulus change (Fuentemilla et al., 2008), novel object recognition (Sambeth et al., 2007), and orientation (Dietl et al., 1999). Specifically, enhancement of theta amplitude, also known as power, is correlated with higher cognitive operations and longer sustained attention levels (Dietl et al., 1999, Klimesch et al., 2001). Theta has been linked to increased awareness during REM-sleep (Klimesch et al., 2001) and heightened attention levels during meditation (Aftanas & Golochiekine, 2001). Increased levels of attention and self-awareness during these episodes were correlated with higher theta synchronization. Clearly, theta is active during periods of internal awareness and during changes in levels of attention and arousal.

Memory. Attention and concentration are particularly important in successful completion of memory tasks. The theta rhythm has been associated with several aspects of memory, such as acquisition, encoding, and storage (Wilson & McNaughton, 1994; Destrade, 1981; Tesche & Karhu, 2000; Sederberg et al., 2003). Recordings from human scalp EEG show that theta power increases during encoding and retrieval of episodic memory (Klimesch et al., 2001). The theta

rhythm is important working memory (Lisman, 1995; Givens, 1996; Givens & Olton, 1990) and particularly spatial memory (Ekstrom et al., 2005, Winson, 1978), which is a type of working memory. It is also recorded during periods of recognition (Klimesch et al., 2001; Burgess & Gruzelier, 1997).

The theta rhythm is particularly important in working memory (Givens & Olton, 1990; Givens, 1996), which involves encoding and associating pieces of information during a short period of time (Tesche & Karhu, 2000). In a memory task, theta activity was prominent during initial acquisition of word pairs and triplets. The observed theta rhythm indicates effective association and learning (Caplan & Glaholt, 2007). Both high difficulty (Gevins & Smith, 2000) and extended duration (Tesche & Karhu, 2000) of working memory tasks are correlated with higher theta power (Sederberg et al., 2003). Lesions to the hippocampus and damage to the theta rhythm cause significant increases in learning rate and impairments in recognition ability, and performance in working memory tasks (Nokia, Penttonen, Korhonen & Wikgren, 2008).

Spatial memory, a type of working memory, is also associated with the theta rhythm. Hippocampal theta is recorded at 6-10 Hz during spatial movement in rats (Itskov, Pastalkova, Mizuseki, Buzsaki & Harris, 2008) which is consistent with other findings on theta frequency (Tesche & Karhu, 2000; O'Keefe & Burgess, 1999; Oddie et al. 1996). Amplitude of theta in the hippocampus and the frontal cortex increases during active phases of virtual navigation tasks, in which participants navigate throughout a virtual city to identify stores, make deliveries, and locate other participants (Ekstrom et al., 2005). Successful completion of this type of task is the result of theta's constant organization of information during perception (Laukka et al., 1994). Depletion of the theta rhythm decreases ability to detect and integrate sensorimotor information and causes deficits in spatial memory tasks (Maho, Dutrieux & Ammassari-Teule, 1988;

Winson, 1978). These results suggest that neurons firing in the theta frequency are often involved in memory task completion.

Theta resetting. One explanation for how theta specifically enhances cognition is through a resetting of the rhythm as new information enters the brain. This involves neuronal synchronization, which means that all neurons in a particular group are firing at the same time. When they fire in synchrony, they create neuronal oscillations, which are consistent bursting patterns of neurons in the cerebral cortex (Givens, 1996). Consistent, oscillatory firing of neurons in a particular area of the brain is vital for perception (Jacobs, Kahana, Ekstrom & Fried, 2007), learning (Ponomarenko et al., 2008; Otten, Henson & Rugg, 2002) and sensorimotor integration (Bland & Oddie, 2001). The theta rhythm contains phases during which a larger number of neurons fire in synchrony (Jacobs et al., 2007). When a new piece of information enters the brain, the theta rhythm temporarily stops and resets itself in order to let the new stimuli enter during a phase of high synchronization. To retain an item of information in memory, the item must enter memory when the theta rhythm resets (Lisman, 1995; Ponomarenko et al., 2008; Buño & Velluti, 1977). The phase of high synchronization may be at different phases of the oscillation—at the peak, at the trough or anywhere along the wave (Jacobs et al., 2007; Givens, 1996). This phenomenon is called "phase-locking" with the neuronal oscillation (Tesche & Karhu, 2000; Givens, 1996). Studies have supported this phase-locking, oscillatory theory by observing theta reset during spatial (Sederberg et al., 2003; Raghavachari et al., 2001) and episodic (Buszaki, 2005; Burgess & Gruzelier, 1997) memory tasks.

Theta and LTP. Theta is physiologically associated with long-term potentiation (LTP), which is the molecular process by which information is stored and retained (Bliss & Collingridge, 1993; Greenstein, Pavlides & Winson, 1988). It is rapid, synchronous firing of

neurons that communicate with each other via neurotransmitters that travel across the neuronal synapse (Bliss & Collingridge, 1993). The firing of neurons causes an excitatory postsynaptic potential which leads to a depolarization of the synaptic membrane and continuation of the impulse to the subsequent neurons. Synapses can be weak or strong, and the strength of the synapse is determined by the amount of neurotransmitter released into the synaptic cleft (Bliss & Collingridge, 1993).

Higher depolarization of the neuronal membrane is associated with higher theta amplitude (Núñez, García-Austt & Buño, 1990). Moreover, LTP enhances hippocampal activity, thereby improving working memory and mnemonic ability. Researchers found that the strongest LTP occurred in the dentate gyrus and CA1 region of the hippocampus approximately 200 ms after the stimulus. This time-interval correlates directly to the human theta-wave rhythm (5 Hz), confirming the link between LTP and natural brain rhythms, such as theta (Greenstein et al., 1988).

Neuroanatomical Basis of Theta

Research on the neuroanatomy of theta has revolved around three structures that have a significant role in cognitive processing. These areas include the hippocampus (Scarlett, Dypvik & Bland, 2004; Sainsbury, 1998; Rawlins, Feldon & Gray, 1979), the prefrontal cortex (Jensen & Tesche, 2002; Laukka et al., 1995; Nakashima & Sato, 1992), and the amygdala (Paré, Collins & Pelletier, 2002; Pape et al., 2005). The majority of research has been done on the hippocampal theta rhythm, while significantly less has been done on theta in the prefrontal cortex and the amygdala.

Hippocampus. The majority of theta research has been done on the hippocampus and its surrounding structures. The hippocampus is one of the main limbic cortical regions in the brain

and it is involved in the acquisition and storage of long- and short-term memories (Olton, 1977; Squire & Zola-Morgan, 1991). Most commonly, the theta rhythm has been recorded from various hippocampal structures, including the dentate gyrus (Givens, 1996; Maho et al., 1988; Winson, 1978), the CA1 nucleus (Buszaki, 2002; Stewart & Fox, 1990; Ponomarenko et al., 2008), and the entorhinal cortex (Konopacki, 1998). These areas are capable of generating independent theta rhythms (Konopacki, 1998). On a cellular level, theta is produced by granule cells of the dentate gyrus (Givens, 1996) and pyramidal cells (Ponomarenko et al., 2008; Buszaki, 1996) of the remaining areas of the hippocampus.

The hippocampus contains direct physical inputs from the medial septal area, the proposed pacemaker of the theta rhythm (Leung, Martin & Stewart, 1994; Alonso, Gaztelu, Buño & García-Austt, 1986). Specifically, the medial septum provides a major source of cholinergic and GABAergic synaptic activity to neurons and interneurons in the hippocampus (Cobb & Davies, 2005) via a structure called the fimbria-fornix (Winters & Dunnett, 2004). To receive these inputs from the medial septum, the hippocampus contains cholinergic and GABAergic receptors (Cobb & Davies, 2005). The receptors control incoming currents with the help of various voltage-gated ion channels and blockers (Cobb & Davies, 2005). By doing so, the receptors help modulate hippocampal theta and, in turn, memory capability and arousal levels.

Through the fimbria-fornix, the hippocampus is also connected to other cortical and subcortical structures, such as the locus coeruleus, hypothalamus, and prefrontal cortex (Winters & Dunnett, 2004). Therefore, lesions to the fimbria-fornix cause decreases in cholinergic projections to these brain areas. This chemical disconnection results in severe impairments on working and spatial memory tasks (Winters & Dunnett, 2004).

Prefrontal cortex. Prefrontal recordings suggest that the theta rhythm not only modulates memory formation, but also higher-level cognitive functioning. The prefrontal cortex is associated with mental task execution (Aftanas & Golocheikine, 2001), motor and speech integration (Fuster, 1990), and attentional processing (Ishii, Shinosaki, Ukai, Inouye, Ishihara, Yoshimine, Hirabuki, Asada, Kihara, Robinson & Takeda, 1999). Specifically, theta is present in the frontal region during higher cognitive tasks such as calculating, navigating through a maze, and counting objects in a three-dimensional visual field (Ishii et al., 1999; Inouye, Shinosaki, Iyama, Matsumoto, Toi & Ishihara, 1994). It is strong during periods of high concentration in spatial memory tasks (Laukka et al., 1994) and working memory tasks (Jensen & Tesche, 2002). Research with rats and monkeys shows that prefrontal neuron activity increases during periods of high arousal, such as when anticipating a reward (Gill, Sarter & Givens, 2000).

Some research has been done on the physical connections from the medial septal area to the prefrontal cortex. Rodent studies show that cholinergic fiber bundles project from the prefrontal cortex to the medial septal area, nuclei in the diagonal band of Broca's area, and the magnocellular basal nucleus (Gaykema et al., 1991). Additionally, there are direct anatomical connections between the prefrontal cortex and the hippocampus, suggesting a relation between the theta rhythms in each structure. Despite this known physical connection, further research is necessary to confirm that the medial septal area is the pacemaker of the prefrontal theta rhythm.

Amygdala. Little progress has been made on the theta rhythm of the amygdala and how it is maintained. The amygdala is the main center for emotional memory consolidation and arousal regulation in the brain (Paré, Collins & Pelletier, 2002). It is evident that the rhythm exists in the amygdala during emotional arousal (Paré, Collins & Pelletier, 2002; Sanhueza & Bacigalupo, 2003), long-term fear memory (Narayanan, Seidenbecher, Kluge, Bergado, Stork & Pape, 2007;

Pape et al., 2005), and changes in sleep patterns (Hedge et al., 2008; Paré, Collins & Pelletier, 2002). The amygdala also controls fear startle response (Pissiota, Frans, Michelgård, Appel, Långström, Flaten & Fredrikson, 2003) and stress regulation (LeDoux, 1996) during traumatic or stressful situations. During a classical conditioning experiment, cats that were anticipating a noxious stimulus showed increased rate of amygdala neuronal firing that was synchronized to the theta frequency (Paré & Collins, 2000).

The amygdala receives sensory input from all parts of the brain, and it stimulates cortical regions that are involved in memory formation, perception, and behavior control (Paré et al., 2002). The amygdala receives the majority of these sensory inputs in the basolateral complex, where cortical and thalamic signals end (Paré, Collins & Pelletier, 2002; Paré & Gaudreau, 1996). There is evidence that the amygdala receives neuronal inputs from the medial septal area (Paré, Collins & Pelletier, 2002). However, little research exists on the pacemaker of the amygdala theta rhythm. Therefore, the current project will assess whether the medial septal area is the pacemaker of the amygdala theta rhythm.

Medial septal area. The medial septal area is a region of neurons that is physically connected to the hippocampus, the prefrontal cortex and the amygdala via cholinergic fiber bundles (Gaykema et al., 1991). Neuronal cells of the medial septal area, also called the medial septum, fire in rhythmic bursting patterns that correlate to the frequency of theta (Jackson & Bland, 2006; Givens, 1996). These signals travel to the hippocampus and modulate the theta rhythm. In a study on alert rabbits, theta disappeared in all areas of the hippocampus in which medial septal input was blocked (Vinogradova, Kitchigina & Zenchenko, 1998). Therefore, hippocampal theta requires multiple systems of individual neurons of the medial septal area that act in synchrony to produce consistent stimulation (Smythe, Colom & Bland, 1992).

To fully understand the role of the medial septal area in theta regulation in the amygdala, the relation of the firing of its neurons must be clear. The medial septal cells fire on a phase-locked rhythm with those of the hippocampus (Alonso, Gaztelu, Buño & García-Austt, 1986). Therefore, the neurons of the medial septum reach the highest amplitude at the same time the neurons of the hippocampus reach their highest amplitude. In turn, lesions to the medial septum cause depletion of hippocampal theta (Topchiy & Kocsis, 2007; Goutagny et al., 2008). One study found that lesions to the medial septum caused significant permanent damage to the hippocampal theta rhythm (Monmaur, Frankel-Kohn, Sharif, Gratio & M'Harzi, 1996; Partlo & Sainsbury, 1995). It is yet unclear whether damage to the medial septal area leads to destruction of theta in the amygdala as well.

The medial septal area is not the only structure involved in regulation of hippocampal theta. There are a number of other structures that are also physically connected to the amygdala that may play a role in modulation of amygdala theta power. The perirhinal cortex (Liu & Bilkey, 1996), the entorhinal cortex (Höistad & Barbas, 2008), and the median raphe nucleus (Partlo & Sainsbury, 1995) each have neuronal connections to the hippocampus. Lesions to these areas cause decreases in hippocampal theta power. Due to their effect on hippocampal theta, it is possible that it also affects amygdala theta as well.

Neurochemical Basis of Theta

The constant, oscillatory firing of neurons in the medial septum is regulated by the release of various neurotransmitters into the postsynaptic cleft. The medial septum employs two neurotransmitter pathways for regulating neural cognition and behavior: cholinergic and GABAergic (Vinogradova, Brazhnik, Kitchigina & Stafekhina, 1993; Givens & Olton, 1990). Cholinergic synapses are excitatory and utilize acetylcholine (ACh) as the neurotransmitter, and

GABAergic synapses are inhibitory and use gamma-aminobutyric acid (GABA) as the neurotransmitter (Givens & Olton, 1990; Gaykema et al., 1991). These two systems are involved in transmission of impulses from the medial septal area to other brain structures, such as the hippocampus, amygdala, and prefrontal cortex. ACh and GABA work in parallel fashion to maximize neuronal synaptic activity and, in turn, increase memory functioning and motor capabilities.

ACh. ACh serves as a neuromodulator of the theta rhythm in several neuronal memory systems of the brain, including the hippocampus (Monmaur et al., 1996) and the striatum (Gold, 1993). After lesioning of the fimbria-fornix, degenerating fibers in the medial septum were determined to be cholinergic, suggesting an important role of ACh in the maintenance of the hippocampal theta rhythm (Monmaur et al., 1996). Researchers have extensively studied the effects of various drugs on ACh neural regulation to evaluate the effects on theta. The memory deficits associated with decreased neuronal ACh are actually similar to those of individuals with Alzheimer's disease (Vinogradova, Brazhnik, Kickigina & Stafekhina, 1996). Alzheimer's patients show drastically reduced levels of cholinergic neurons, indicating an inverse correlation between the degree of disease and the amount of the neurotransmitter present (Winkler, Thal, Gage & Fisher, 1998). A likely cause of this disease is the loss of cholinergic cells in the medial septum (Brazhnik & Vinogradova, 1988; Beninger et al., 1989).

Administration of ACh antagonistic drugs, such as scopolamine, to the medial septum blocks sustainment of the theta rhythm of hippocampal neurons (Givens & Olton, 1990) and has detrimental effects on learning and memory tasks in rats (Allen & Crawford, 1984; Sambeth et al., 2007). On the other hand, nicotine, a cholinergic agonist, has the opposite effect. Activation

of nicotinic receptors increases arousal and enhances memory capability in rats (Sambeth et al. 2007).

In the central nervous system, ACh plays an excitatory role in brain plasticity and short term memory (Gold, 1993). It is presumably involved in arousal control, detail orientation and novelty processing (Gold, 1993). The use of ACh by postsynaptic receptors can be used as a chemical marker for the activation of hippocampal neurons involved in learning and memory formation. On the contrary, a decrease in ACh and cholinergic cells impairs a number of cognitive functions, including encoding capabilities (Hasselmo, Wyble & Wallenstein, 1996), memory of recent events (Beninger et al., 1989), responsiveness to sensory stimuli (Vinogradova, Brazhnik, Kitchigina & Stafekhina, 1993), object recognition, spatial discrimination ability, and performance on working memory tasks (Givens & Olton, 1995).

GABA. GABAergic neuron receptors utilize gamma-aminobutyric acid as a neurotransmitter and have an inhibitory role in the phase-setting of the theta rhythm (Vinogradova et al., 1996). Septohippocampal GABAergic neurons regulate the theta rhythm in the hippocampus (Bassant et al., 2005; Borhegyi, Varga, Szilágyi, Fabo & Freund, 2004). Accordingly, administration of a GABAergic agonist, such as muscimol, mimics the GABAergic inhibition and causes disruption of medial septal output to the hippocampus (Givens & Olton, 1990). As a result, it reduces theta power and impairs working memory (Givens, 1995; Givens & Olton, 1990). In other words, enhancement of GABAergic transmission causes destruction of the theta rhythm and subsequent behavioral impairment (Givens & Olton, 1990).

The GABAergic agonist ethanol also causes impairments on spatial memory tasks (Givens, 1996; Givens, 1995), sustained attention (Givens, 1997) and choice accuracy (Givens & McMahon, 1997; Givens, 1997), One study found that the infusion of ethanol causes

suppression of hippocampal theta activity via the N-methyl-D-aspartate receptor mechanism (Givens, 1995). Specifically, a phase shift caused a decrease in theta frequency (Givens, 1995). It is important to understand the effects of various drugs on the theta rhythm and how they can affect behavioral and cognitive functioning.

Current Study

A large amount of research has been done that has assessed the theta rhythm of the hippocampus and its relation to the medial septal area. It is evident that physical medial septal projections to the hippocampus are crucial for the modulation of hippocampal theta, which is involved in motor capabilities, attention levels, and memory formation. In addition, it is clear that the medial septal cholinergic and GABAergic neurotransmitter systems play an important role in modulating hippocampal theta. However, there is relatively little work done on the physical and chemical relation between the medial septum and the amygdala and how this affects the amygdala theta rhythm. The purpose of the current research project is to evaluate the theta rhythm of the amygdala and determine if it is maintained by oscillatory bursts of neurons of the medial septal area. To do this, various ACh-antagonistic and GABA-agonistic substances will be used to examine effects on amygdala theta. It is proposed that each of these drug treatments will result in significant decreases in amygdala theta power. With more information on amygdala theta and the medial septal area, it may be possible to establish a more coherent understanding of how learning and memory consolidation are controlled.

Method

Subjects

Ten adult male Long-Evans rats weighing between 300 and 500 grams were used for the experimental surgeries. They were housed in separate 8 x 8 x 16.5 inch cages. The rats were kept

on a 12 hour light/dark cycle and were given ad libitum food and water. Rats were handled according to protocol provided by the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, D.C., 1996) and according to the protocols approved by Illinois Wesleyan's Institutional Animal Care and Use Committee (IACUC).

Procedure

Surgery. Rats were handled regularly for several weeks before surgery. To increase effectiveness of the anesthesia, food and water was withheld the night before surgery if it was planned for the morning. For afternoon surgeries, food and water was withheld during the morning. Rats were initially anesthetized with a ketamine/xylazine solution (8 mg ketamine; 1.2 mg xylazine). Dosage amount was determined based on the weight of the rat (1.0 ml/kg). Additional injections of lower doses (0.8 ml/kg, 0.6 ml/kg) were administered when whisker movement or foot reflexes occurred. White Petroleum Mineral Oil and Lanolin Oil lubricant was administered to the eyes to prevent drying. The rat's head was then shaved from between the ears to the back of the neck and was placed in a stereotaxic apparatus to hold it in place.

An incision was made with a scalpel to expose the skull. After the incision was made, four mouse clips were attached to the faschia to hold back the scalp. Following the incision, measurements were taken to determine where to drill the holes. Six holes were drilled: one into the amygdala (-2.6mm posterior to bregma; -5.0mm lateral to midline; -7.8mm inferior to dural surface), one into the medial septal area (0.7mm anterior to bregma; 1.5mm lateral to midline; -4.4mm inferior to dural surface), one for a ground wire, and three additional holes into each quadrant. A stainless steel Teflon coated recording electrode with a gold pin at one end was inserted into the amygdala at a 15 degree angle. A Plastics One (Roanoke, VA) C315GA 26GA guide cannula was placed into the medial septal area. Finally, an A-M Systems, Inc. (Sequim,

WA) ground wire with a gold ITT/Cannon Centi-Lok pin attached to one end was placed into the left posterior parietal cortex. Screws (Small Parts, Inc., Miami Lakes, FL) were inserted into the three additional holes to anchor the insulator strip. Finally, Plastics One dental cement was used on the exposed area to cover the screws and fixate the wires to the skull. The wires were inserted into an ITT/Cannon insulator strip to prevent movement of the surgical apparatus and to prohibit the rat from gnawing at the wires.

After the cement dried, Neosporin was administered to the edges of the wound to prevent infection and irritation and pain medicine was administered subcutaneously. The rat was then removed from the stereotaxic device and given a 0.1 ml/kg dose of Rimadyl, a pain medication, in a subcutaneous injection. Additional doses were given every 24 hours for three days, depending on the behavior of the rat. A 0.1 ml/kg dose of Buprenorphine was also administered immediately when the rat came out from the anesthesia. The rat was then returned to its cage where it resumed its normal food and water schedule. Post-surgery, the rat was handled frequently and its weight was monitored regularly to ensure proper health. It was given at least one week for recovery before post-surgery testing began.

Data Recording

Rats were anesthetized with a dose of 1.0 ml/kg ketamine/xylazine. Additional supplements of 0.8 ml/kg were administered if necessary. The wires from surgery were connected to EEG gold recording pins through the ITT Canon Insulator. The pins were connected to amplifiers (Biopac, Goleta, CA) and the signals were transmitted to a nearby computer. Recordings were taken by AcqKnowledge Version 3.9.0 software (Copyright Biopac Systems, Inc., 1992-2006). Initial baseline recordings of EEG theta activity were taken for 5 minutes. Rats were then administered with 0.5 µL of one of the following drug treatments:

saline, scopolamine (10 μg or 20 μg) scopolamine, muscimol (30 ng or 60 ng), 0.1% ethanol or 1.0% ethanol. Two minutes were given before post-infusion recording to ensure the drug took effect. One post-infusion ten-minute EEG recording session was taken.

Rats were disconnected from the EEG amplifiers and placed back in their cages with ad libitum food and water. Every rat received every drug and combination once; and two to three days were given between recordings to eliminate carryover effects of the anesthesia or the drug. The order of drug distribution across rats differed to remove possible order effects.

Data Analysis

All electrophysiological data was recorded using Biopac Systems, Inc., software. All frequencies were filtered out except those from 4 to 8 Hz, which characterize the theta rhythm. Power spectral density analysis was used to locate the maximum theta power (volts) and the frequency (Hz) at which maximum power occurred in that range. The maximum frequency was recorded in Hz and the power of the highest amplitude was recorded in volts. A within-subjects ANOVA was used to analyze the results, using drug infusion as the independent variable and theta power and frequency as the dependent variables.

Results

Data analysis was conducted on nine rats in all. Five rats completed all the recording sessions, while four rats had incomplete recording sessions. Specifically, two rats did not complete the 0.1% ethanol recording, another did not complete the saline and the 60 ng dose of muscimol recordings, and the last did not complete the 10 mg/ml concentration of scopolamine and 0.1% ethanol recordings. A quantitative analysis was performed using AcqKnowledge Software to obtain the power spectral densities for power and frequency of all post-infusion recordings.

Theta Power

A repeated measures ANOVA was conducted using drug as the independent variable and theta power as the dependent variable, revealing a significant main effect for drug, $F(7, 28) = 4.100, p < .05$. Subsequent analyses revealed significant main effects for muscimol, $F(2, 14) = 8.096, p < .05$, and for ethanol, $F(2, 8) = 4.981, p < .05$, but no main effect for scopolamine, $F(2, 12) = 1.046, p > .05$ (See Figure 1). Based on the results of the ANOVAs, subsequent T-tests were conducted to compare individual doses and concentrations of muscimol and ethanol treatments with the saline treatment. There was a significant difference in theta power between the saline treatment and both the high, $t(7) = 4.015, p < .05$, and low $t(7) = 2.934, p < .05$, doses of muscimol (See Figure 1). There was also a significant difference in theta power between the saline and the high, $t(6) = 2.602, p < .05$, and low, $t(4) = 3.240, p < .05$, doses of ethanol (See Figure 1). There was no significant difference between the 30 ng dose and the 60 ng dose of muscimol. There was also no significant difference between the 0.1% and the 1.0% doses of ethanol. These results suggest that the GABAergic pathway plays a more important role in amygdala theta modulation than the cholinergic pathway.

Theta Frequency

A repeated measures ANOVA was also performed to determine if there was a main effect of any drug on theta frequency, and it revealed no significant main effect, $F(6, 24) = .800, p > .05$ (See Figure 2). These results demonstrate that neither the cholinergic nor the GABAergic pathway affect the frequency of the amygdala theta rhythm.

Discussion

The present study examined whether theta EEG activity throughout the brain is controlled by a single modulator or if each separate area has its own modulator. The medial septal area is

physically and chemically connected to the hippocampus (Alonso et al., 1986; Vinogradova, Kitchigina & Zenchenko, 1998; Alonso et al., 1986; Partlo & Sainsbury, 1995). In other words, the control of the hippocampal theta rhythm depends on the integrity of the rhythmically bursting neurons of the medial septal area and the septal connection to the hippocampus (Bennett, 1973). The medial septal area also sends physical projections to the amygdala (Paré, Collins & Pelletier, 2002). However, it is unknown whether the medial septal area controls the neuronal firing in this area as well. The purpose of this experiment was to analyze the effects on amygdala theta power after infusion of various drugs into the medial septal area.

The current hypothesis was that medial septal modification with scopolamine, muscimol and ethanol would cause a decrease in theta power. The results of the experiment partially support this hypothesis. As previous studies on hippocampal theta have found, infusion of muscimol and ethanol into the medial septal area also caused a decrease in amygdala theta power. However, infusion of scopolamine did not cause a significant decrease in amygdala theta power. This has strong implications on the respective roles of each type of neurotransmitter receptor on the modulation of the theta rhythm in the amygdala. Because the current results only partially support the hypothesis, other considerations must be made regarding the true chemical pacemaker of the amygdala theta rhythm.

Other Neuronal Pathways

Due to the insignificant effects of the scopolamine on amygdala theta power, it is possible that brain areas apart from the medial septal area could influence theta power in the amygdala. A few of these pathways include the perirhinal cortex pathway, the lateral entorhinal cortex pathway (Liu & Bilkey, 1996), and the median raphe nucleus pathway (Partlo &

Sainsbury, 1995). Also, an alternate hypothesis is that an indirect pathway from the medial septum to the amygdala is most important in amygdala theta modulation.

Perirhinal cortex. The perirhinal cortex contains physical projections to the dentate gyrus of the hippocampus via the perforant path (Liu & Bilkey, 1996, Heynen & Bilkey, 1994). Subsequent blocking of the perforant path with procaine results in abolition of synaptic potentials in the hippocampus by perirhinal stimulation (Liu & Bilkey, 1996; Asaka, Griffin & Berry, 2002). Lesions to the perirhinal cortex also produced decreases in amplitude of hippocampal potentials, confirming the importance of this particular structure in hippocampal theta modulation (Liu & Bilkey, 1996). The perirhinal cortex is also interconnected with the amygdala (Koganezawa, Taguchi, Tominaga, Ohara, Tsutsui, Witter & Iijima, 2008). Due to the influence of the perirhinal cortex on hippocampal theta, it is possible that it also affects amygdala theta in the same manner. Future studies should assess the effects of the perirhinal cortex on amygdalar theta. To do this, a guide cannula could be inserted into the perirhinal cortex for drug infusion. Then, amygdala theta could be recorded after infusion of drugs to observe effects on theta power.

Entorhinal cortex. The entorhinal cortex is also physically connected to the hippocampus and serves as a mediator for information entering and exiting the hippocampus (Koganezawa et al., 2008). The entorhinal cortex contains physical projections to both the hippocampus and the amygdala (Höistad & Barbas, 2008). Lesions to the lateral entorhinal cortex had a large effect on the amplitude of hippocampal evoked potentials (Liu & Bilkey, 1996). Due to these findings, it is possible that the entorhinal cortex influences theta in the amygdala as well. The lack of significant results for scopolamine may be due to the fact that the cholinergic pathway to the

amygdala is not controlled by the medial septum but a different structure, such as the entorhinal cortex. Subsequent studies should analyze the entorhinal cortex's influence on amygdala theta.

Median raphe nucleus. A third pathway related to this study is that from the median raphe nucleus to the hippocampus. The medial septal area is not the only structure involved in regulation of hippocampal theta. The median raphe nucleus has also shown to be an important modulator of the rhythm. The median raphe nucleus is located near the brain stem, is rich in serotonergic neurons, and contains physical connections to the hippocampus (Partlo & Sainsbury, 1995). Stimulation of this area has been shown to result in scopolamine-sensitive Type II theta (Partlo & Sainsbury, 1995), which is recorded during periods of immobility. In the current experiment, had there been insertion of the guide cannula into the median raphe nucleus, there may have been a significant effect on the cholinergic receptors linked to the amygdala as well.

Indirect pathway. In addition to the several direct pathways that may influence theta power in the amygdala, there are also possible indirect pathways that may do the same. One possible indirect pathway is from the medial septal area to the hippocampus to the amygdala. It is evident that the medial septal area controls theta in the hippocampus (Cobb & Davies, 2005; Winters & Dunnett, 2004). It is also clear that the hippocampus is physically connected to the amygdala (Pape et al., 2005). Therefore, a possible mechanism for medial septal modulation of amygdalar theta is through the hippocampus. Future studies should assess whether the true pacemaker of theta in the amygdala is the hippocampus. One potential experiment would involve recording theta after lesioning the hippocampus. If the results show a decrease in theta power, it could be speculated that the direct influence on amygdala theta is not the medial septal area but actually the hippocampus.

Neurotransmitter systems

Each of the possible theta-modulating pathways involves a neurotransmitter system. Two in particular were examined in this experiment: the cholinergic and GABAergic systems. Neurons in the medial septal area contain two types of neurotransmitters—acetylcholine (ACh) and gamma-aminobutyric acid (GABA)—that bind to cholinergic and GABAergic receptors, respectively (Vinogradova et al., 1998). Cholinergic receptors, which utilize acetylcholine, produce excitatory impulses (Gold, 1993), whereas GABAergic receptors, which use gamma-aminobutyric acid (GABA), produce inhibitory impulses (Vinogradova, Brazhnik, Kichigina & Stafekina, 1996). The results of this study indicate that the GABAergic pathway has a more prominent role than the cholinergic pathway in the maintenance of theta in the amygdala.

GABAergic. The results of the present study demonstrated that manipulation of the medial septal area with muscimol, a GABAergic agonist, caused a significant decrease in theta power. This occurred after infusion of both the 30 ng dose and the 60 ng dose of muscimol. A decrease in theta power indicates that fewer neurons are firing in a rhythmic, synchronized manner. Due to this decrease, it can be proposed that the GABAergic system in the medial septal area plays a significant role in modulating amygdalar theta. The use of muscimol as a GABAergic agonist in the current experiment was based on the results of previous work in which muscimol was used. Results of these studies have revealed significant decreases in hippocampal theta power after infusion of muscimol (Givens & Breese, 1990; Givens & Olton, 1990). Therefore, the current results were expected based on previous work using muscimol infusions.

The role of medial septal GABAergic receptors in modulating amygdala theta is also supported by the results following the ethanol infusions. When ethanol affects the central

nervous system, it causes loss of concentration, impaired response time, and memory impairment, among other detriments (Givens, 1995). The GABAergic receptors in the medial septal area are sensitive to the effects of ethanol (Grobin, Matthews, Devaud & Morrow, 1998), which directly affects the area by allosterically modulating GABAergic receptors, disrupting synapse formation, and halting calcium functioning (Grobin et al., 1998; Givens, 1995). Ethanol inhibits medial septal neurons from firing, leading to a decrease in hippocampal theta power (Givens, 1995). The results of these previous studies validate the use of ethanol as a second GABAergic agonist for this experiment. The current results reveal that infusion of two different concentrations of ethanol resulted in a significant decrease in amygdala theta power. The decreases in theta power after infusion of ethanol confirm the importance of the GABAergic receptors on the modulation of amygdala theta.

Cholinergic. In contrast to the results of muscimol and ethanol infusion, the modification of the medial septal neurons with scopolamine, a cholinergic antagonist, did not cause a significant decrease in theta power. Scopolamine is a known ACh antagonist that has been used in a large number of hippocampal theta studies (Sambeth et al., 2007; Givens & Olton, 1990; Bennett, 1973). Past work on the effects of scopolamine revealed impairment of neuronal function along the medial septal/hippocampal pathway (Brazhnik & Vinogradova, 1988) and a significant decrease in hippocampal theta (Givens & Olton, 1990). Therefore, it was expected that the detrimental effects of scopolamine on the cholinergic receptors would cause a partial if not complete depletion of theta in the amygdala as well. However, the lack of a considerable decrease in theta power after infusion of scopolamine implies that cholinergic receptors may play a more prominent role in the medial septal/hippocampal pathway than they do in the medial septal/amygdala pathway.

However, there are alternate explanations for the lack of significant results after infusion of scopolamine, such as the number of doses and number of concentrations. In previous studies, similar doses of scopolamine (15 µg), were used in rats (Givens & Olton, 1990; Givens & Olton, 1995) and the results revealed significant decreases in hippocampal theta. Also, when 0.3-1.0 mg/kg scopolamine was used, the results revealed significant blocking of theta activity in the hippocampus (Vinogradova et al., 1993). Due to these results, it was expected that doses of 10 µg and 20 µg of scopolamine would have similar effects on amygdala theta power. If there had been a larger sample size, more potent concentrations of scopolamine, and different doses of the drug, there may have been a significant difference in amygdala theta power.

Theta frequency

There were no significant changes in theta frequency after infusion of any of the drug treatments. Analysis of theta power in addition to theta frequency was based on previous studies that observed increases in hippocampal theta frequency well beyond the normal theta range (Green & Rawlins, 1979). The lack of significant results in the current study indicates that the medial septal area is not as highly involved in amygdala theta frequency as it is in amygdala theta power. One limitation of the frequency analysis was the way in which the software program calculated theta frequency for each recording. Due to this limitation, the majority of theta research has focused on power (Ekstrom et al., 2005; Sederberg et al., 2003).

Limitations

In addition to limited number of doses, there are several other limitations worth mentioning. These include the limited number of drugs used, using only one method of infusion, and the lack of histological analysis necessary for thorough assessment of drug effects on amygdala theta power.

First, there were a limited number of drugs used for each rat. Studies in the past have used a wide variety of cholinergic antagonists, such as atropine (Monmaur & Breton, 1991) and GABAergic agonists, such as physostigmine (Vinogradova, Kitchigina & Zenchenko, 1998), and noradrenergic antagonists, such as detomidine hydrochloride (Partlo & Sainsbury, 1995). However, in the current study, only four different chemicals were used with a low number of concentration/dose manipulations. The number of drug treatments was limited to seven to prevent possible tissue damage.

There are other forms of lesioning that may have produced significant results across the board. These methods include electrolytic lesions (Thinschmidt, Kinney & Kocsis, 1995; Rawlins, Feldon & Gray, 1979) and lesions with 192 IgG-saporin (Gerashchenko, Salin-Pascual & Shiromani, 2001). When 192 IgG-saporin is injected into the medial septal area, the cholinergic neurons are selectively damaged and theta activity is reduced (Gerashchenko et al., 2001). Lesions with IgG-saporin lead to permanent neuronal cell loss. In the current study, the detrimental effects of muscimol, ethanol and scopolamine were reversible. Therefore, although very unlikely, it is possible that the effects of the drugs wore off before or during the 10 minute recording session. The use of a more potent drug such as IgG-saporin may have resulted in significant findings.

Another limitation is the lack of histological analysis, which will be necessary to confirm the exact locations of the guide cannula and recording electrode in the brain. Caution was taken to ensure the proper placement of each object. Yet, the end of the wire may have bent and the pencil marks on the brain may not have been completely accurate. Either could have caused improper placement of the cannula and electrode into the brain. Therefore, possible effects of the scopolamine treatments may not have been detected by the particular location of the recording

electrode in the amygdala. Future histological examination will confirm the locations of the guide cannula and the recording electrode.

A final possible limitation in this study is the effects of the anesthesia. It is possible that the anesthesia had an effect on amygdala theta power. In fact, it is likely that it had a slightly dampening effect compared to theta power in a freely-moving behavioral study. However, numerous studies have been done analyzing theta power under the effects of various anesthetics. These studies have found similar decreases in hippocampal theta power (Soltesz & Deschenes, 1993; Heynen & Bilkey, 1994) following cholinergic and GABAergic manipulations.

First, there may have been lingering anesthetic effects from one session to the next. In past experiments, behavior and electrophysiology characteristics returned to baseline levels ninety minutes after infusion. In the current experiment, a minimum of two to three days passed between each recording session to prevent possible carryover effects of the drug or anesthesia from the previous session. Therefore, it is very unlikely that there would be effects from one session to the next. However, if this occurred, it may have caused errors in the recordings.

Second, the choice of anesthetic drug and dosage was based on previous work. Some studies have used mixtures of ketamine and xylazine, specifically 85 mg ketamine/10 mg xylazine per kilogram solution (Soltesz & Deschenes, 1993). Other hippocampal research has used urethane as the anesthetic (Heynen & Bilkey, 1994; Green & Rawlins, 1979). Previous dosages have included a 1.5 g/kg intraperitoneal dose (Heynen & Bilkey, 1994) and a 1.1-1.4 g/kg dose (Holsheimer, Stok & Lopes da Silva, 1983), which are both similar to the dose used in the current study. However, urethane is a one-time use drug that prohibits the use of multiple recordings from a particular area. Therefore, for the current experiment, a 1.0 ml/kg dose of ketamine/xylazine was used to allow for multiple recordings.

Future directions

Based on the various limitations of the current project, there are a number of ways in which amygdala theta can be further analyzed. In addition to more drug doses, possible future studies involve analysis of the two types of theta and implementation of a behavior.

First, there are two types of theta—Type I and Type II—and this study did not record for Type I theta. Type I theta is recorded at frequencies ranging from 6 to 12 Hz and is present during voluntary movements. Type I theta power is decreased by agents such as urethane and sodium pentobarbital (Partlo & Sainsbury, 1995). On the other hand, Type II theta has frequencies from 4 to 9 Hz and occurs during alertness and sensory processing. It is mainly controlled by the cholinergic system (Partlo & Sainsbury, 1995). The current study did not assess Type I theta because it did not include a behavioral aspect. Therefore, if the experiment included a behavioral assessment, there may have been slightly different results.

Next, this study did not use a behavior to assess drug effects on amygdala theta. Other studies have used a working (Raghavachari et al., 2001; Givens, 1996) or spatial memory task (Jacobs et al., 2007) to assess performance and response time. Future work on amygdala theta should assess behavior to determine if decreases in amygdala theta power cause deficits on memory tasks.

Conclusion

The majority of research on the theta rhythm has focused on the hippocampus, whereas less exists on theta in areas such as the amygdala. Work on hippocampal theta has revealed that the theta rhythm is particularly important for motor movement, high attention levels, and memory formation. To function properly, the theta rhythm relies on the cholinergic and GABAergic neurotransmitter systems from the medial septal area to the hippocampus. Knowing

there is also a physical connection from the medial septal area to the amygdala, this study set out to determine if the medial septal area is the chemical pacemaker of the amygdala theta rhythm. The origin or pacemaker of this rhythm must be known to understand how it can be enhanced or depleted. Results revealed that the GABAergic pathway is strongly involved in the medial septal/amygdala connection, whereas the cholinergic system may be less involved. If further research is done to confirm this findings, researchers can then explore therapeutics that may be able to permanently or temporarily enhance amygdala theta or prevent its destruction.

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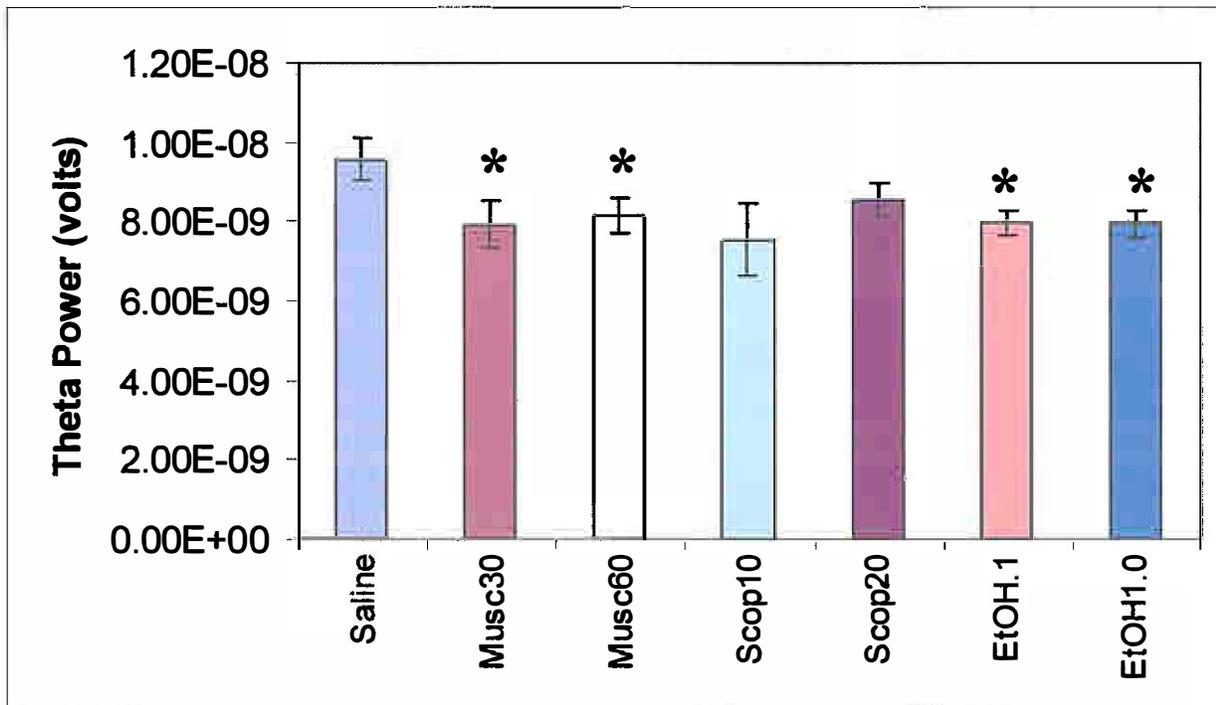
Figure 1. Mean measurements for amygdala theta power after infusion of various drug

treatments. Bars indicate standard error of the mean. Musc30 represents the low dose of

muscimol, Musc60 represents the high dose of muscimol, Scop10 represents the low dose of

scopolamine, Scop20 represents the high dose of scopolamine, EtOH.1 represents the low

concentration of ethanol, and EtOH1.0 represents the high concentration of ethanol.

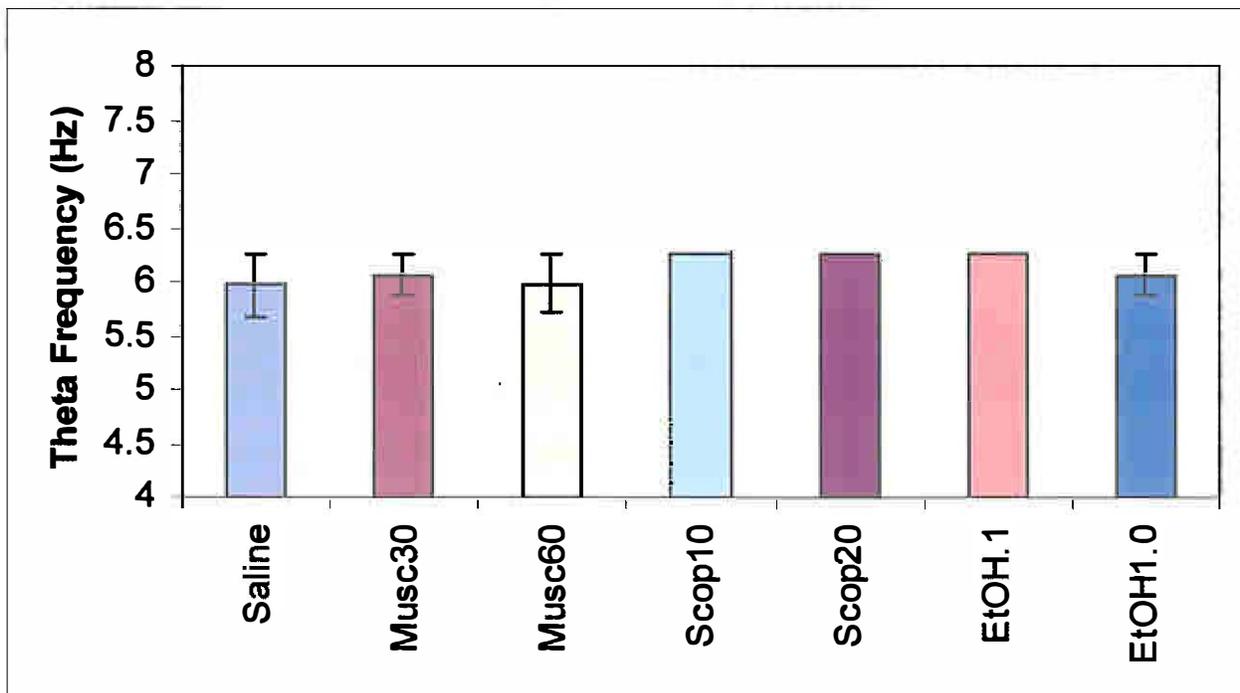* = significantly different from saline (p<.05)

Figure 2. Mean measurements for amygdala theta frequency after infusion of various drug treatments. Labels on the x-axis are the same as in Figure 1.