

SEROTONIN AND STRESS RESPONDING IN ANIMALS: ROLE OF 5-HT_{2A/C}
RECEPTORS IN THE CENTRAL AND PERIPHERAL NERVOUS SYSTEMS

A Dissertation

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DEDICATION

For Sam

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ABSTRACT

Behavioral responses to stressors can be influenced in different ways by both serotonin (5-HT) agonists and antagonists. Further study, of both different stressors as well as different 5-HT agents, is needed to clarify the place of 5-HT in stress responding. To date, no published report has investigated the influence of centrally and/or peripherally administered 5-HT_{2A/C} agonist DOI or the 5-HT_{2A/C} antagonist ketanserin on behaviors evoked by tail pinch or open field stressors. Five separate, related experiments were conducted to investigate this influence. It was hypothesized that that peripherally (Experiment 1), centrally (Experiment 2), and centrally + peripherally (Experiment 3) injected DOI would reduce stress responding to tail pinch and open field stressors, and that peripheral injection of ketanserin (Experiment 4) would increase behavioral responding to stress when injected alone, as well as reverse the reduction in behavioral responding from injection of DOI (Experiment 5). The results strongly supported the hypotheses. Administration of DOI resulted in significantly decreased behavioral responding to tail pinch stress in all five experiments, regardless of route of administration. Concomitant peripheral administration of KET and DOI resulted in a reversal of the decrease in stress-evoked behaviors seen with administration of DOI alone. This is the first report of the influence of centrally and peripherally administered DOI on behaviors evoked by tail pinch or open field stress, and the reversal of that influence by the 5-HT_{2A/C} antagonist ketanserin. Future investigations should be designed to study whether the effects observed in the current report are centrally or peripherally mediated.

INTRODUCTION

Serotonin

Serotonin is a substance present in both animals and plants, the properties of which have been observed and researched for over 100 years. Sixty years before it was isolated and identified, the vasoconstricting properties of serotonin in mammals were observed. By the 1930s and 40s, serotonin had been identified in blood serum in the United States and (called enteramine) in the gut of vertebrate animals in Europe, though it was not until the early 1950s that these two were recognized as identical substances (Villalon, Terron, Ramirez-San Juan, & Saxena, 1995). Serotonin has since been identified as a transmitter substance, known to be present in the periphery and the central nervous system.

The neurotransmitter 3-(β -aminoethyl)-5-hydroxyindole, commonly known today as 5-hydroxytryptamine, or serotonin (5-HT), has become the most thoroughly studied of the biogenic amines due to its established influence on several physiological and psychological phenomena (Graeff, 1994) (Figure 1). 5-HT is derived from the amino acid L-tryptophan, which is taken into cells and converted into 5-hydroxytryptophan by the enzyme tryptophan-5-hydroxylase. Another enzyme, α -aromatic amino acid decarboxylase, converts the 5-hydroxytryptophan into 5-hydroxytryptamine. After 5-HT is released from the cell into the synapse, reuptake of the transmitter (active transport from the synaptic cleft back into the cell) occurs. Once returned to the interior of the cell, 5-HT is broken down by monoamine oxidase A, forming 5-hydroxyindoleacetaldehyde. This metabolite is in turn made into 5-hydroxyindoleacetic acid (5-HIAA) by the enzyme aldehyde dehydrogenase (Watling, 2001).

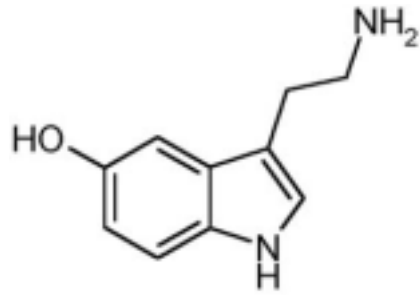


Figure1: 5-HT chemical structure.
From <http://www.biopsychiatry.com/serotonin/>

5-HT can be found in three cell types in the mammalian body: neurons in the CNS, blood platelets, and enterochromaffin cells of the GI tract (Steckler, Ruggenberg-Schmidt, & Muller-Oerlinghausen, 1993). Whereas the cells in the GI tract and the neurons in the CNS are able to synthesize 5-HT from the precursor L-tryptophan and application of the enzymatic cascade outlined above, platelets must rely on uptake of the transmitter from the blood (Lingjaerde, 1969). The 5-HT in platelets is stored as a 5-HT/ATP complex, which is released during vascular damage, helping to reduce blood loss by exerting vasoconstrictive properties on the surrounding vessels (Martin, 1994). Stimulation of the vagal nerve releases 5-HT from the enterochromaffin cells in the gut causing nausea and vomiting via stimulation of the area postrema (Minami et al., 2003). Release of 5-HT from neurons in the CNS can result in many potential physiological and behavioral changes, depending upon the receptor subtypes involved.

Review of 5-HT Receptor Function

The primordial 5-HT receptor is, based on molecular evolution analysis, reportedly seven to eight hundred million years old, and may have been the first evolutionary change in receptor structure from the G-protein receptors known to be present in yeasts and molds one billion years

ago. In fact, some theorize that all the biogenic amine receptors are mutants (i.e., descendants) of the original 5-HT receptor (Peroutka, 1994). The current classification and understanding of the 5-HT receptor system is based on discoveries in the middle of the last century.

While others had suggested that the action of 5-HT in vasoconstriction and smooth muscle contraction in the gut could be due to the presence of different receptors (Villalon et al., 1995), Zuleika Picarelli and Sir John Gaddum were the first to recognize and classify two distinct 5-HT receptors in guinea pig intestine, which they called 'M' and 'D' after the two substances (morphine and dibenzylamine) that prevented the contraction of this smooth muscle (Gaddum & Picarelli, 1957). Ten years following this discovery and original classification system, the 'M' and 'D' receptor distinction was thought to be insufficient in that it did not account for the failure of some potent 5-HT 'D' antagonists to prevent the vasoconstrictive properties of 5-HT in the canine carotid while successfully blocking vasoconstriction at other vascular sites. These failures led researchers to believe that more than two 5-HT receptors existed in the mammalian circulatory system (Villalon et al., 1995). During the 1970's technological advances in radioligand labeling of 5-HT agonists and antagonists allowed researchers to begin classifying receptor subtypes with more precision, and by the mid-1980's the need for a new classification system was apparent (Villalon et al., 1995).

Today there are at least seven 5-HT receptor families known to be present in human and/or animal tissue. Within three of the seven receptor families, there exist several receptor subtypes. For example, in the 5-HT₂ receptor family there are three subtypes, designated 5-HT_{2A}, 2B, 2C. With the advent of more advanced ligand binding techniques, improved molecular genetic studies of the 5-HT receptors, and insight into the second messenger systems involved in postsynaptic receptor activation, the identification of new receptor subtypes has increased

dramatically in the recent past – bringing the total number of known 5-HT receptors to 14 (Barnes & Sharp, 1999). This large number of receptors is thought to be a function of the 5-HT system's evolutionary age (Peroutka, 1994). The function and activity of several of these receptors has not yet been discovered. The distribution, location, and function of the 5-HT₁, 5-HT₂, and 5-HT₃ families are the most well-profiled of the receptor families.

The 5-HT_{1A} receptor is located both pre- and postsynaptically (Barnes et al., 1999). This autoreceptor controls reuptake of serotonin after it is released into the synaptic cleft. The presynaptic receptors are located primarily in the dorsal raphe nucleus (DRN). The postsynaptic 5-HT_{1A} receptors are located predominately in the hippocampus, but are also located in the septum, neocortex, hypothalamus, and parts of the amygdala (Graeff, 1993). Both the pre- and postsynaptic 5-HT_{1A} receptors work through the cyclic adenosine monophosphate (cAMP) second messenger system, decreasing cAMP activity when stimulated (Humphrey, Hartig, & Hoyer, 1993). Despite the fact that the pre- and postsynaptic receptors use the same second messenger system and are not morphologically distinct, 5-HT_{1A} agonists and antagonists do not act the same at both receptors. For example, the 5-HT_{1A} agonist ipsapirone is a full agonist of the presynaptic autoreceptors in the DRN, but is an incomplete agonist at the postsynaptic receptor located outside of the DRN (Graeff, Viana, & Mora, 1997). Also, the pre- and postsynaptic 5-HT_{1A} receptors show different patterns of reactivity with repeated exposure to agonists or selective serotonin reuptake inhibitors (SSRIs). While the presynaptic receptor tends to show desensitization with chronic administration of these agents, the postsynaptic receptor does not (Blier, de Montigny, & Chaput, 1987).

When stimulated, the 5-HT_{1B} receptors (prevalent in rodents) and the 5-HT_{1D} receptors (prevalent in human tissue) cause a decrease in 5-HT release (Saxena, 1995). The 5-HT_{1B/D}

receptors are found centrally in the substantia nigra, hippocampus, hypothalamus, and basal ganglia as well as in the cerebral arteries (Curzon, 1990; Simansky, 1996; Saxena, 1995). Like the 5-HT_{1A} receptor, the 5-HT_{1B/D} are found both pre- and postsynaptically. They are also considered autoreceptors and use the same second messenger (cAMP) as the 5-HT_{1A} receptor. However, the 5-HT_{1B/D} receptors are located on the neuron terminal, unlike the 5-HT_{1A} autoreceptor, which is located on the neuron soma (Graeff, 1993).

Despite the fact that the 5-HT_{1D} receptor in humans performs the same function in the circulatory system as the 5-HT_{1B} receptor in rats (e.g., arterial contraction), these two receptors do not share pharmacological properties, having different agonists and antagonists (Saxena, 1995). The 5-HT_{1B} receptor has also been reported to play a role in ingestive behavior in animals (Vickers & Dourish, 2004), whereby stimulation of this receptor has been reported to lead to satiety and decreased food intake (Lee, Kennett, Dourish, & Clifton, 2002; Curzon, 1990) and has been discussed as a possible mechanism of eating disorders such as anorexia nervosa (Curzon, 1990). However, results of feeding experiments involving the 5-HT_{1B} receptor are ambiguous (e.g., Grignaschi, Mantelli, & Samanin, 1993; Grignaschi, Sironi, & Samanin, 1996; Grignaschi, Fanelli, Scagnol, & Samanin, 1999; Hewitt, Lee, Dourish, & Clifton, 2002) and some propose it is the activity of the 5-HT_{2C} and _{1B} receptors together that have the strongest influence on feeding (Schreiber & De Vry, 2002).

The 5-HT_{1E} and 5-HT_{1F} receptors are not well researched, having no known specific antagonists (Barnes et al., 1999; Watling, 2001). They are known to be present in human tissue and are thought to use the cAMP second messenger system as do the other members of the 5-HT₁ family (Humphrey et al., 1993). When specific antagonists are identified for these receptors, researchers will be better able to elucidate their function.

The 5-HT_{2C} receptor was originally classified as 5-HT_{1C} (Pazos, Cortes, & Palacios, 1985). However, molecular cloning research and investigation into the second messenger system of the 5-HT_{1C} receptor have shown it to be more similar in structure, function, and pharmacological properties to the 5-HT₂ receptor family, and it has therefore been reclassified. The 5-HT_{2C} receptor is present exclusively postsynaptically and is located in many areas of the cortex, limbic system, and basal ganglia (Barnes et al., 1999). When activated, 5-HT_{2C} receptors increase the activity of the postsynaptic neuron via the inositol phosphate second messenger system (Graeff, 1993). The 5-HT_{2C} receptor is involved in a number of physiologic responses, including eating, movement, and temperature control (Koek, Jackson, & Colpaert, 1992).

The 5-HT_{2A} receptor was first found in the periphery on smooth muscle cells (Gaddum & Piccarelli, 1957), however today we know it to be present in the CNS as well. In the brain, the location of the 5-HT_{2A} receptor overlaps somewhat with the 5-HT_{2C} receptor, with both being present in the neocortex and limbic system. The 5-HT_{2A} receptor is also highly concentrated in the amygdala, the claustrum, the olfactory tubercle and the cingulate cortex (Pazos, Probst, & Palacios, 1987). Like the 5-HT_{2C} receptor, 5-HT_{2A} uses the inositol phosphate second messenger system to increase the firing rate of the postsynaptic neuron (Boess & Martin, 1994).

The 5-HT_{2B} receptor has been found in both human and rodent tissue (Hoyer et al., 1994). While it is known to be present in the human brain (Kursar, Nelson, Wainscott, & Baez, 1994) and involved in the regulation of processes like embryonic cardiac development and hypertension (Nebigil et al., 2001), this receptor has just recently been located in nervous tissue in the rat (Nicholson, Small, Dixon, Spanswick, & Lee, 2003). Probably for this reason, this receptor is not well covered in reviews of 5-HT receptor function, despite the fact that specific agonists and antagonists and the second messenger system (inositol phosphate) are known.

An important and perplexing finding about the 5-HT₂ receptor family is that receptors down-regulate as a result of chronic administration of antagonists. In most receptor systems, and indeed in the other serotonin receptor families, chronic administration of a pharmacological antagonist results in receptor up-regulation (i.e., a homeostatic mechanism where decreased transmitter activity leads to an increase in the number of receptors in the cell membrane). In the 5-HT₂ family however, specifically with the 5-HT_{2A&2C} receptors, the opposite effect has been shown to occur – that is, repeated administration of receptor antagonists, like ketanserin, have been shown to cause receptor down-regulation (Graeff, 1997). This may be due to these antagonists having inverse agonist-like properties, whereby the activity of a cell's second messenger system is decreased (as would be predicted with the application of an antagonist), but receptor down-regulation still occurs with chronic application of the antagonist (a phenomenon that typically occurs with the repeated application of an agonist, not an antagonist) (e.g., Labrecque, Fargin, Bouvier, Chidiac, & Dennis, 1995).

The 5-HT₃ receptor was also first discovered in the periphery, being the main 5-HT receptor present in the gut (Watling, 2001). It has been located in areas of the brainstem as well as the hippocampus, amygdala, nucleus accumbens, entorhinal cortex, and olfactory tubercle (Graeff, 1993). This is the only known 5-HT receptor where activation directly opens cation channels without the use of a second messenger system (Derkach, Surprenant, & North, 1989).

The pharmacology and function of the remaining classes of serotonin receptors (5-HT_{4, 5, 6, & 7}) are poorly characterized at present. The gene that transcribes the 5-HT₄ receptor has not yet been located, but researchers do know that it uses the cAMP second messenger system in the opposite way that the 5-HT₁ family uses it – i.e., activation of 5-HT₄ leads to increased cAMP activity and increased neuronal activity (Chaput, Araneda, & Andrade, 1990). The 5-HT₅ family

(consisting of 5-HT_{5A} and 5-HT_{5B}) is the only class of the seven known receptor types for which the method of signal transduction (directly through ion channels or a second messenger system) is unknown (Watling, 2001). As with the 5-HT₄ receptor, the genes responsible for transcribing the 5-HT_{6 & 7} receptors are unknown. It is known that the 5-HT_{6 & 7} receptors both use the cAMP system for signal transduction, increasing cAMP activity when stimulated (Barnes et al., 1999).

Influence of 5-HT in the Periphery

5-HT does have several other physiologic actions in the body outside of the CNS, via the aforementioned gut and platelet stores of the transmitter. 5-HT can impact the respiratory system by increasing respiration volume and causing bronchial constriction (Saxena & Villalon, 1990). Systemic administration of 5-HT can cause vasoconstriction or dilation in uninjured vascular tissue. 5-HT also influences cardiac output and blood pressure by increasing the force of the contraction of the heart muscle (Gershon, 1991). Stimulation of the smooth muscle of the lower intestine occurs with 5-HT, but the opposite effect occurs in the large intestine, where 5-HT inhibits its activity (Dhasmana, Zhu, Cruz, & Villalon, 1993).

5-HT Pathways in the CNS

Several neural pathways originating in the brainstem release 5-HT. The 5-HT neurons in the pons and medulla send their projections to the spinal cord, where they are involved in the mediation of nociception and movement (Graeff, 1997). A large population of 5-HT neurons in the brain is located in the dorsal and median nuclei of the raphe in the brainstem. These nuclei have diffuse projections to the rest of the brain. The DRN has three primary 5-HT projections that innervate several brain structures. The first is called the dorsal raphe-cortical tract and projects from the DRN to the cortex and basal ganglia. A second projection from the DRN, the dorsal raphe-periventricular tract, innervates the thalamus, the periaqueductal gray, and the

periventricular nucleus of the hypothalamus. The third major projection from the DRN (and most diffuse of the three), the dorsal raphe-forebrain tract, goes to the basal ganglia, the thalamus, the septum, the hippocampus, and the amygdala. Like the DRN, the median raphe nucleus (MRN) projects (via the median raphe-forebrain tract) to the thalamus, septum, and hippocampus, but also sends 5-HT fibers to the olfactory bulb (Deakin, 1991; Graeff, 1997). Together, the median raphe-forebrain tract and the dorsal raphe-forebrain tract make up part of what is known as the medial forebrain bundle. It is through the projections from the DRN and MRN that 5-HT exerts its influence on thinking, emotion, and functioning of the neuroendocrine system (Graeff, 1997).

The neurons of the DRN and MRN appear to be morphologically as well as functionally distinct. Neurons originating in the DRN are small in diameter with little bulging along the axon, while MRN fibers are thicker and have more large bulges along the axons. Functionally, even within structures that both the MRN and DRN project to, such as the thalamus and hippocampus, the DRN fibers synapse with cells that have 5-HT_{2A} and 5-HT_{2C} receptors, and the MRN fibers synapse with cells that contain 5-HT_{1A} receptors in their membranes (Deakin, 1991). Despite these distinctions between the MRN and the DRN, the two nuclei are linked directly and indirectly (via numerous other brain areas).

Role of 5-HT in Psychological Pathologies

5-HT has been implicated in several psychological disorders, such as impulsivity, psychosis, and depression. Research in this area is extremely profuse and wide-ranging in both laboratory and clinical settings. While a complete review of this literature is beyond the scope of this dissertation, a few examples are noted below, followed by a more comprehensive review of 5-HT involvement in stress responding, the focus of this research.

Increased impulsive behavior, including aggression, overeating, alcoholism, as well as compulsive gambling and sexual activity have been linked to decreased levels of the 5-HT metabolite, 5-HIAA (Graeff, 1997) and decreased 5-HT uptake sites on platelets (Patkar et al., 2003). Several atypical antipsychotics prescribed today (e.g., quetiapine fumarate (brand name Seroquel) and aripiprazole (brand name Abilify)) have a higher affinity for serotonin receptors than dopamine receptors than do typical antipsychotics and act as 5-HT antagonists (Goldstein, 1999). Many researchers and clinicians now believe that depression is caused, at least in part, by a net decrease in 5-HT activity in the brain. However, there is disagreement in the field about the receptor subtypes involved - some feel it is an over-activity of the 5-HT_{1A} autoreceptor (Deakin, 1991), while others (Mikuni, Kagaya, Takahashi, & Meltzer, 1992) believe it is the result of supersensitivity of the 5-HT₂ receptor.

Stress

Stress has been defined in a number of ways, but most definitions include a core understanding that stress is a physiological and behavioral response to a noxious stimulus that threatens homeostasis (Fricchione & Stefano, 1994; Koob, 1999). The term *anxiety* is traditionally used to describe a subjective feeling reported by humans, often in response to actual or perceived stressors. In fact the dictionary definition of anxiety is: “an abnormal and overwhelming sense of apprehension and fear often marked by physiological signs (as sweating, tension, and increased pulse), by doubt concerning the reality and nature of the threat, and by self-doubt about one's capacity to cope with it.” (Merriam-Webster, 1987, p93). However, in scientific literature, the two terms are increasingly being used interchangeably. For example, drugs that reduce stress responding in animals are often referred to as ‘anxiolytic’ and those that increase stress responding are frequently called ‘anxiogenic’. As the subjective ‘feeling’ of anxiety

cannot be measured in animals, these terms, as they are used throughout this dissertation, are meant to refer to a measurable decrease or increase (respectively) in the physiological and/or behavioral response to a stressor.

Stressors

Several stimuli are stressors in the rat. These include foot shock, (Amat, Matus-Amat, Watkins, & Maier, 1998; Saphier, Farrar, & Welch, 1995), restraint (Kirby, Chou-Green, Davis, & Lucki, 1997; Nonaka, 1999; Singewald, Kaehler, Hemeida, & Philippu, 1997), noise stress (Saphier et al., 1995), injections of various substances (Adell, Casanovas, & Artigas, 1997; Rodriguez Echandia, Broitman, & Foscolo, 1983), forced swimming, heat or cold stress, handling (Adell et al., 1997; Kirby et al., 1997), tail pinch (Boutelle, Zetterstrom, Pei, Svensson, & Fillenz, 1990; Hawkins et al., 1999; Kirby et al., 1997; Pei, Zetterstrom, & Fillenz, 1990), and placement in an open field (Onaivi, Bishop-Robinson, Darmani, & Sanders-Bush, 1995). While the nature of the stressor may vary, the physiological and behavioral reactions that animals display share many common features (Chrousos & Gold, 1992).

Physiological Responses to Stress

Sympathetic Nervous System. The fight-or-flight response, as it was called by physiologist Walter Cannon, refers to activation of the sympathetic nervous system in response to a stressor. This response includes a number of physiological changes that prepare the body for activity. Epinephrine and norepinephrine are released by the adrenal medulla (Chrousos et al., 1992). The release of these transmitters has several effects on the body, including mobilization of nutrients from bodily stores, increased cardiac output, and redirection of blood flow away from the dermis, gastrointestinal tract, other internal organs and the extremities toward the major skeletal muscle groups (Chrousos et al., 1992). Increased circulation and nutrient availability to

the skeletal muscles prepares the body for quick and vigorous action in response to threatening or stressful stimuli. Sympathetic activation also results in bronchial relaxation, epocrine gland stimulation, and salivary gland inhibition, all of which further prepare the body for activity (Chrousos et al., 1992).

Hypothalamic-Pituitary-Adrenal (HPA) Axis. When a stressor activates the hypothalamic-pituitary-adrenal axis, a chemical cascade occurs that helps prepare the organism to react to the stressor (Raghavendra & Kulkarni, 2000a). The response starts in the hypothalamus with the release of corticotropin releasing factor (CRF) (Dohms & Metz, 1991; Owens & Nemeroff, 1991). CRF, in turn, stimulates the anterior pituitary gland to release adrenocorticotrophic hormone (Jorgensen, Knigge, Kjaer, Vadsholt, & Warberg, 1998) into the blood stream (Dohms et al., 1991; Jezova, Ochedalski, Glickman, Kiss, & Aguilera, 1999; Owens et al., 1991). When the adrenocorticotrophic hormone reaches the adrenal cortex, glucocorticoids, such as cortisol, are released into the blood stream (Dohms et al., 1991). Cortisol, in turn, increases the amount of glucose in the blood, enhances cardiac output, and stimulates energy expenditure (Dohms et al., 1991; Laue, Loriaux, & Chrousos, 1988). HPA axis activation is necessary for appropriate responding to a stressor (Lopez, Akil, & Watson, 1999), and deregulation of HPA axis activity has been implicated in both depression (Curtis & Valentino, 1994; Deakin, 1991; Nemeroff, 1988) and anxiety (Nemeroff, 1992).

Neurotransmitter Activity. 3,4-dihydroxyphenylethylamine (dopamine) is the precursor to norepinephrine and exerts similar effects to norepinephrine in the periphery, such as increased blood pressure and cardiac output. Dopamine, another neurotransmitter in the CNS (Dishman, 1997; Inoue, Tsuchiya, & Koyama, 1994), is well known for its involvement in centrally controlled activities such as extra-pyramidal movement (i.e., fine motor skills), regulation of

eating and sexual behavior, reward, learning processes, and psychiatric disorders like schizophrenia (Hoebel, 1985).

Dopamine has been implicated in the stress response in several ways. Activation of dopamine pathways has been observed as a consequence of a number of the physical stressors discussed above (Kalivas, Duffy, & Eberhardt, 1990). Microdialysis studies have revealed that stress increases dopamine and its metabolites in several brain sites (Inoue et al., 1994). Increases and decreases in stress behavior occur with administration of dopamine agonists and antagonists, respectively.

Gamma-aminobutyric acid (GABA) is a neurotransmitter found in the mammalian nervous system that was originally recognized for its anticonvulsant activity in the CNS (Corda, Lecca, Piras, Di Chiara, & Giorgi, 1997). GABA has also been found to be of relevance in the stress response. Microdialysis studies show increases in GABA in the basal ganglia, cerebral cortex and striatum after application of acute physical stressors (Otero Losada, 1989).

5-HT involvement in the physiological response to stress is discussed at length below (see section 5-HT and Physiological Responses to Stress).

Behavioral Responses to Stress

Many behavioral reactions to stressful stimuli have been reported for rats and other mammals. In some cases, a stressor can elicit a uniform behavioral profile. For example, in response to the application of a tail pinch, rats typically perform oral stereotypies such as eating and gnawing food, drinking, and licking both themselves (grooming) and other objects in the environment (Levine & Morley, 1981; Hawkins et al., 1999). In other instances, a single stressor can elicit a range of behaviors from an animal. When placed in the open field, rats show significant increases in freezing, rearing on the hind legs, sniffing, performing quick rotations of the body,

urinating, defecating, and spending more time near the wall of the open field (Kalin & Takahashi, 1990; Koob et al., 1993).

HPA Axis Involvement. Much research has addressed the role that increased HPA axis activity plays in mediating behavioral reactions to stressful stimuli. The release of CRF during stress has been linked to increases in locomotion and grooming, suppressed sexual behavior, and abnormal sleeping patterns (De Wied & Croiset, 1991; Koob, 1999; Levine et al., 1981; Nemeroff, 1988). Britton, Koob, Rivier, and Vale (1982) reported increased grooming and avoidance behaviors after central administration of CRF and placement of the animal in an open field. CRF is also linked to decreased food intake during stress. The number of approaches to food and the amount of food ingested decrease in rats that receive CRF (De Wied et al., 1991), and CRF has been shown to reduce food intake in animals that have been injected with substances that normally induce eating (Gosnell, Morley, & Levine, 1983). There is strong evidence that this reduction of food intake is a direct effect of CRF, as this effect persists in animals with lesioned adrenal glands, indicating that CRF's influence on other peptides (like ACTH) is not involved in the suppression of eating (Levine & Levine, 1989). CRF has also been reported to directly influence freezing behavior by activating the locus coeruleus. Swiergiel, Takahashi, Rubin, and Kalin (1992) reported that injection of the CRF receptor antagonist alpha-helical CRF 9-41 reduced freezing behavior induced by foot shocks.

Neurotransmitter Involvement. Administration of different transmitter substances (agonists/antagonists) often leads to observable changes in behavioral stress responding. For example, central injections of the GABA agonist, muscimol, result in significantly increased stress behavior in rats (Hawkins et al., 1999). However, there is some evidence that enhanced activity at the same GABA receptor (subtype A) that muscimol stimulates can reduce stress responding

(Corda et al., 1997). Dopamine has also been shown to increase stress responding in animals. It appears that these two transmitter systems (dopamine and GABA) interact during stress, such that the increase in responding to stress observed after administration of a GABA agonist can be prevented with the co-administration of a dopamine antagonist (Hawkins et al., 2000).

5-HT is another neurotransmitter that is known to play a significant role in stress responding. The evidence for the influence of 5-HT in physiological and behavioral responses to stress is discussed at length in the following section.

5-HT Involvement in Stress Responding

5-HT and Physiological Responses to Stress

HPA Axis and Sympathetic Nervous System. 5-HT is reported to be a potent stimulator of CRF release via its effect on the periventricular nucleus of the hypothalamus (Jorgensen et al., 1998). Bell, Butz, and Alper (1999) found that the 5-HT_{2A/C} agonist DOI injected into the periventricular nucleus increased blood pressure and heart rate by stimulating the release of CRF. Lesions of serotonergic neurons in the CNS lead to decreased or abolished ACTH release (Chaouloff, 1993; Owens et al., 1991).

Stimulation or antagonism of 5-HT receptors has been shown to influence the release of hormones from the anterior pituitary, including ACTH, during stress (Jorgensen et al., 1998). Independent of CRF release, 5-HT is able to directly stimulate the anterior pituitary to release ACTH (Chaouloff, 1993). However, the influence of 5-HT on the HPA axis is not consistent. Exemplifying this ambiguity, Welch and Saphier (1994) reported that the 5-HT agonist DOI (administered both peripherally and centrally) increased plasma ACTH, as did high doses of the 5-HT antagonist ketanserin (administered ICV). Adding to this inconsistency, there is some evidence that the influence of 5-HT varies depending on the type of stressor with which an animal

is confronted. Chaouloff (1993) reported that animals with lesions of the 5-HT neurons in the hypothalamus exhibited lower than normal corticosterone levels after exposure to photic stress or conditioned fear stress (as would be predicted), but the corticosterone levels were normal following exposure to ether or acoustic stress. Thus, it appears that some stressors activate the HPA axis via central 5-HT mechanisms, while other kinds of stressors activate the axis without affecting 5-HT release.

Although the exact nature of 5-HT's role in the stimulation of the autonomic nervous system is unclear, 5-HT does seem to be involved in the sympathetic nervous system response to stress. For instance, 5-HT is taken up with norepinephrine by sympathetic nerves (Watling, 2001). Additionally, 5-HT is known to be involved in the inhibition of norepinephrine containing neurons in the locus coeruleus, a brain area that is active during stress responding (Sinner, Kaehler, Philippu, & Singewald, 2001).

CNS. Several studies have investigated the effects of different stressors on the amount of 5-HT or a metabolite of 5-HT, 5-HIAA, in various areas of the brain. Restraint stress has been shown to increase levels of both 5-HT and 5-HIAA in the locus coeruleus (Singewald et al., 1997), hippocampus (Bonnin, Grimaldi, Fillion, & Fillion, 1999; Boutelle et al., 1990; Vahabzadeh & Fillenz, 1994), cortex, hypothalamus, and substantia nigra (Bonnin et al., 1999). Similarly, tail pinch has been shown to increase 5-HT in the striatum (Kirby et al., 1997), hippocampus (Boutelle et al., 1990; Kirby et al., 1997; Pei et al., 1990; Vahabzadeh et al., 1994), and frontal cortex (Pei et al., 1990).

CNS Pathways. As indicated above, pathways from the DRN are thought to be activated when animals are stressed, however there is evidence for differential activation depending on whether the type of stress the animal is subjected to activates a panic or an anxiety reaction.

Deakin and Graeff distinguish between types of stressors by classifying them as either *anxiety provoking* (from distal threat) or *panic provoking* (from proximal physical stressors). There is evidence that 5-HT plays a dichotomous role in behaviors evoked by these two types of stressors.

The DRN pathway to the amygdala appears to be important in the response to anxiety. If 5-HT activity in the amygdala is *decreased*, anxiolytic effects are seen (Graeff, 1993), and research shows that severing the connections between the DRN and the amygdala abolishes fear responding in animals. These findings are supported by evidence that microinjections of 5-HT antagonists directly into the amygdala and hippocampus prevent the typical fear response in animals (Deakin, 1991; Graeff, 1994).

In panic reactions, the DRN connection to the periaqueductal gray is important. Serotonin activation of the DRN occurs with stimuli that are predictive of noxious events, and leads to escape and avoidance behavior in animals. In contrast to the anxiolytic effect described above with decreased 5-HT activity in the amygdala, *increasing* serotonin activity in the periaqueductal gray produces anti-panic effects (Graeff, 1993; Graeff et al., 1997).

These findings support a contradictory role for 5-HT in anxiety and panic and seem to be in agreement with at least some clinical research. For example, in humans the 5-HT antagonist ritanserin has been shown to *reduce* anxiety in generalized anxiety disorder, but to *increase* the symptomology of panic disorder (Graeff, 1993). Graeff, Zuardi, Giglio, Lima Filho, and Karniol (1985) reported that metergoline, another 5-HT antagonist, increased reports of anxiety in healthy human participants. Silva, Hetem, Guimaraes, and Graeff (2001) and Graeff et al. (2001) both reported that nefazadone, a 5-HT_{2A} antagonist, reduces conditioned anxiety, but increases unconditioned fear (i.e., panic). One group of participants was given nefazadone and tested after undergoing skin conductance conditioning to white noise (an aversive stimulus), while another

group was given nefazadone then tested after performing a public speaking task. Both groups were asked to rate their subjective feelings of anxiety during the testing. Compared to a placebo control group, the conditioned anxiety group experienced reduced changes in skin conductance as well as reduced subjective report of anxiety. The participants in the public speaking group who were given nefazadone, on the other hand, experienced increased feelings of anxiety compared to those participants who received placebo.

Graeff et al. (1997) describe this hypothesis of anxiety/panic reactions by considering anxiety a learned response, and panic an innate, fight-or-flight-like reaction. This definition of panic, reaction to a proximal threat causing activation of the fight-flight response, can be likened to the response to physical stressors seen in animals described in detail above. When considered in the context of this hypothesis, projections from the DRN to the periaqueductal gray would most likely become active with the application of an inescapable stressor like tail pinch or open field.

Neurotransmitters. In addition to influencing activity in specific brain structures such as the amygdala and periaqueductal gray discussed above, 5-HT also exerts complex effects on the release of other neurotransmitters (such as dopamine and GABA) in the CNS.

Activation of 5-HT_{2A} receptors in the nucleus accumbens is correlated with larger and longer-lasting increases of dopamine in that brain area (Yan, Reith, & Yan, 2000), and Pehek, McFarlane, Maguschak, Price and Pluto (2001) demonstrated the converse effect of 5-HT in the prefrontal cortex – application of a 5-HT₂ antagonist decreased dopamine release. However, Di Matteo, Di Giovanni, Di Mascio, and Esposito (2000) found that with systemic injection of the 5-HT_{2C} agonist RO 60-0175, dopamine release and firing rate of dopamine neurons in the ventral tegmental area were decreased.

Some researchers (Goudreau, Wagner, Lookingland, & Moore, 1994) have also observed a modulatory influence of 5-HT on dopamine release in the pituitary, indirectly through its influence on GABA transmission in the CNS. 5-HT₂ activation increases transmission and synaptic potentials of GABAergic neurons in the hippocampus (Lee, Dixon, & Pinnock, 1999). Abi-Saab, Bubser, Roth, and Deutch (1999) also found that the 5-HT_{2A/C} receptor agonist DOI activated GABA neurons, leading to increased extracellular GABA in the prefrontal cortex.

The influence of neurotransmitter activity on behavioral responding to stress in both humans and animals has been an increasingly popular area of study for the past 20 years. Systemic and central injections of various serotonin agonists and antagonists have resulted in measurable changes in stress-induced behaviors. Below, the direct role of 5-HT in stress responding is discussed at length.

5-HT and Behavioral Responding to Stress

While many studies of 5-HT and stress responding are in vivo dialysis studies describing 5-HT release in different regions of the brain during stress, such as the hippocampus (Kirby et al., 1997; Jorgensen et al., 1998; Rueter, Fornal, & Jacobs, 1997; Vahabzadeh et al., 1994; Rueter & Jacobs, 1996), the prefrontal (Mendlin, Martin, & Jacobs, 1999; Rueter et al., 1997) and frontal cortex, the corpus striatum (Kirby et al., 1997; Mendlin et al., 1999; Rueter et al., 1997), the locus coeruleus (Singewald et al., 1997; Sinner et al., 2001), and the amygdala (Rueter et al., 1996), many have described behavioral changes following manipulation of 5-HT systems as well.

The 5-HT_{1, 2, 3, & 4} receptor families have all been implicated in stress responding. 5-HT_{1A} receptor stimulation in the CNS has been shown to increase locomotion in a novel environment (Carli & Samanin, 1988) and to reduce the ACTH response to acoustic and conditioned fear stressors (Saphier et al., 1995). Peripheral stimulation of the same receptor reduced immobility to

forced swimming stress (Moser & Sanger, 1999). Increases in locomotion and gnawing have been seen with either central or peripheral administration of PBG, a 5-HT₃ receptor agonist. Antagonizing both 5-HT₃ and 5-HT₄ receptors with tropisetron has resulted in reduced ACTH secretion to both restraint and ether stress (Jorgensen et al., 1998).

Manipulating 5-HT activity at the 5-HT_{2A} and _{2C} receptors during stress has been shown to affect behavioral responses including feeding and ingestive behavior (De Vry & Schreiber, 1997), immobility (Redrobe & Bourin, 1997), sexual behavior (Gorzalka, Hanson, & Brotto, 1998), and grooming behavior (Scalzitti, Cervera, Smith, & Hensler, 1999).

Although evidence exists that serotonin is involved in the etiology of these and other stress induced behaviors, the exact nature of the influence is not completely clear – as administration of 5-HT agonists and antagonists has resulted in both increases and decreases in stress related behavior. That is, 5-HT can act as both an anxiolytic and an anxiogenic substance. These findings are presented in the following two sections.

5-HT as Anxiogenic. Two lines of evidence have been reported in support of 5-HT as an anxiogenic neurotransmitter: 1) administration of compounds that produce an increase in 5-HT release, resulting in increases in behavioral responding to stress, and 2) administration of compounds that produce a decrease in 5-HT release, resulting in decreases in stress responding. Reports of both types are presented below.

Several drugs that increase 5-HT release have been reported to produce anxiogenic effects. Intraperitoneal injection of serotonin agonists zimelidine, fluoxetine, quipazine, and MK212 increased avoidance behavior in rats (Kshama, Hrishikeshavan, Shanbhogue, & Munonyedi, 1990). Central and peripheral administration of the 5-HT_{2B/2C} agonist m-CPP has also been shown to increase avoidance behavior in the elevated T-maze (Zanoveli, Nogueira, &

Zangrossi, Jr., 2003) and to reduce open-arm entries (Gibson, Barnfield, & Curzon, 1994) and locomotion (Durand, Mormede, & Chaouloff, 2003) in the plus-maze. The SSRI paroxetine has also produced anxiogenic effects in the elevated plus-maze, resulting in a decrease in open-arm entries, time spent in open arms, and number of line crosses (Koks et al., 2001). Stimulation of the 5-HT_{1B} (Lin & Parsons, 2002), 5-HT_{2A}, and 5-HT_{2C} (Setem, Pinheiro, Motta, Morato, & Cruz, 1999) receptors have also produced anxiogenic effects in the plus-maze.

There are two ways of producing a net decrease in 5-HT release in the synapse: to administer a 5-HT antagonist, or to administer an autoreceptor agonist. The m-CPP findings reported above were attenuated by both administration of 5-HT antagonists (Gibson et al., 1994) and autoreceptor agonists (Zanoveli et al., 2003). Another study reports that mice display anxiolytic responses (increased entries and time spent in open arms) when given the autoreceptor agonist MKC-242 (Sakaue et al., 2003). Systemic injection of 5-HT antagonists chlorophenylalanine, zacopride, GR 38032F, and propranolol resulted in reduced avoidance behavior (Kshama et al., 1990), and other antagonists have been shown to increase grooming in response to various stressors (Rodriguez Echandia et al., 1983).

5-HT as Anxiolytic. Like the evidence presented above, there are two main types of reports that indicate an anxiolytic effect of 5-HT in stress responding: 1) administration of compounds that produce an increase in 5-HT release, resulting in decreases in behavioral responding to stress, and 2) administration of compounds that produce a decrease in 5-HT release, resulting in increases in stress responding. Both types of evidence are reviewed below.

Studies have found that increasing 5-HT activity in general results in decreased behavioral responding to stress. Amer, Breu, McDermott, Wurtman, and Maher (2004) report that peripheral administration of the 5-HT precursor, 5-hydroxy-L-tryptophan, results in decreased food intake

during tail pinch stress and Blokland, Lieben, and Deutz (2002) found that tryptophan depletion (which leads to decreased 5-HT availability) results in increased anxiety behavior in the open field. Grimaldi, Bonnin, Fillion, Prudhomme, and Fillion (1999) reported blocking an endogenous peptide that exerts an antagonistic effect at 5-HT receptors (i.e., resulting in a net increase in activity at 5-HT receptors) resulted in an anxiolytic effect on behavior in the open field.

Others have performed more receptor-specific manipulations of 5-HT activity during stress. Specifically, stimulating 5-HT₂ receptors in animals has been reported to have anxiolytic effects during exposure to various stressors. Aniracetam, a 5-HT_{2A} agonist, has anxiolytic effects in social interaction, elevated plus maze, and conditioned fear stress (Nakamura & Kurasawa, 2001). Similarly, dexfenfluramine and quipazine, both 5-HT₂ agonists, reduce tail pinch induced eating (Morley, Levine, Murray, Kneip, & Grace, 1982; Rowland & Souquet, 1989).

Alternatively, antagonizing 5-HT₂ receptors has resulted in increased ultrasonic stress vocalizations in rat pups (Olivier et al., 1998) and increased reports of anxious feelings in humans (Graeff et al., 2001; Silva, Hetem, Guimaraes, & Graeff, 2001). While not direct evidence using a specific antagonist, one study found that decreased sensitivity of 5-HT₂ receptors caused by social defeat resulted in increased anxiety behaviors in the elevated plus-maze (Benjamin et al., 1993).

Schreiber, Melon, and De Vry (1998) investigated which 5-HT receptors are involved in the anxiolytic effects of the SSRIs by injecting different 5-HT receptor agonists and antagonists (for activation or blockade of 5-HT_{1A}, _{1B/1D}, _{2A}, ₃, and ₄ receptors) peripherally in rats and testing for changes in ultrasonic vocalization to foot shocks. They found significant reductions in vocalization with the _{1A} agonist 8-OH-DPAT, the _{1B/1D} agonist TFMPP, the _{2A/C} agonist DOI, and the _{2C} agonist mCPP. Several of these effects were reversed with administration of receptor-specific antagonists. The anxiolytic effects of the SSRI paroxetine, however, were only reversed

by the 5-HT_{2A} antagonist MDL 100,907, leading the authors to conclude that the main pathway through which this SSRI works is the 5-HT_{2A} receptor. In support of this finding, Sanchez and Mork (1999) reported that peripherally injecting a precursor of 5-HT or the 5-HT_{2A/C} agonist DOI reduced the ultrasonic vocalization induced by foot shocks in rats, and that the antagonist ritanserin reversed this effect.

Onaivi et al. (1995) found that the 5-HT_{2A/C} agonist DOI acted as both an anxiolytic and an anxiogenic (depending on the dose administered) in Hooded rats and ICR mice in the elevated plus-maze. At doses of 2.5 mg/kg or below, a significant increase was observed in the amount of time animals spent in the open arms of the maze, while doses above 2.5 mg/kg resulted in significantly less time spent in the open arms. The anxiolytic effect of the lower doses of DOI was reversed by the 5-HT₂ antagonist ketanserin. Interestingly, in two other strains of mice, different results were found. In the DBA/2 strain, DOI (regardless of dose) decreased the amount of time spent in the open arms of the maze, but in the C57/BL6 strain, the opposite effect occurred and DOI only increased the amount of time spent in the open arms.

Both Mora, Netto, and Graeff (1997) and Zangrossi, Jr. et al. (2001) reported that administration of 5-HT₂ agonists decreases fear and escape behaviors in animals. Mora et al. gave rats peripheral injections of the 5-HT_{2C} agonists mCPP and TFMPP, finding that TFMPP significantly inhibited escape behavior in the elevated T-maze. Zangrossi et al., on the other hand, injected DOI centrally in the dorsal periaqueductal gray and also found that latency to escape was enhanced in the elevated T-maze. Additionally, Setem et al. (1999) reported that the 5-HT_{2A} antagonist SR 46349B injected intraperitoneally resulted in significant displays of anxiety behavior in rats placed in the elevated plus-maze. Animals given this drug spent less time in open arms, scanning, and exploring, all of which are behavioral indicators of anxiety.

Unlike many of those reported above, some studies have included central injections, allowing for some hypothesis on sites of anxiolytic action of 5-HT in the CNS. Using the elevated plus-maze, Audi, de Oliveira, and Graeff (1989) reported that increasing 5-HT activity by injecting propranolol, a 5-HT_{1A} (autoreceptor) antagonist, into the dorsal periaqueductal gray of rats resulted in increased open arm entries. The authors also injected ritanserin, a 5-HT₂ antagonist, into the same brain area and were able to block the propranolol effect. The combination of these two findings led Audi et al. (1989) to hypothesize that the increase in 5-HT in the dorsal central gray resulting from the blockade of the autoreceptor was subsequently acting on 5-HT₂ receptors to produce the anxiolytic effect. In a later study, Audi, de Oliveira, and Graeff (1991) injected the same 5-HT_{1A} antagonist into the dorsal periaqueductal gray and were able to elaborate upon the previously reported findings. Propranolol injected into the dorsal periaqueductal gray increased rats' open arm entries, not just total arm entries, indicating that this is a true anxiolytic effect, not just a byproduct of some nonspecific increase in activity. While this effect was not reversed by ritanserin as it was in the previous (1989) study, it was blocked by ketanserin, another 5-HT₂ antagonist that has an affinity for 5-HT₂ receptors that is approximately 70 times greater than that of ritanserin (Hoyer, 1988).

In addition to tests of anxiolytic effects of centrally administered drugs in the open-armed plus or T-maze, researchers have tested the anxiolytic profile of many centrally injected serotonergic drugs using another paradigm: aversive brain stimulation. In another investigation of the dorsal periaqueductal gray, Nogueira and Graeff (1995) reported that administration of the 5-HT_{2A/C} agonist DOI into this area in rats decreased the aversiveness of stimulation of that area, an effect that was reversed with pre-administration of a 5-HT_{2A} antagonist, spiperone. Schutz, de Aguiar, and Graeff (1985) reported that injecting 5-HT itself directly into the dorsal

periaqueductal gray, or peripherally, increases animals' threshold for aversive brain stimulation as indicated by significant decreases in escape behaviors with stimulation. This effect was increased by zimelidine, a 5-HT uptake inhibitor, and was reversed by both metergoline and ketanserin. Others have produced similar findings. Melo and Brandao (1995) found that injecting zimelidine or the 5-HT₂ agonist alpha-methyl-5-hydroxytryptamine into the inferior colliculus resulted in a longer latency and fewer attempts to stop aversive electrical stimulation of this area.

Ambiguity of 5-HT Influence on Stress Responding. In addition to the evidence outlined in the two previous sections supporting both an anxiolytic as well as an anxiogenic role for 5-HT in stress responding, a single study by Olivier et al. (1998) demonstrates the ambiguity of 5-HT's influence on anxiety and stress. In a 1994 review, Olivier et al. (1994) reported that several 5-HT_{1A} autoreceptor agonists (buspirone, ipsapirone, gepirone, flesinoxan) were found to have anxiolytic properties. In 1998, Oliver et al. tested several substances with 5-HT receptor affinity in two different stressful temperature conditions: the warm and the cold plate. While the anxiolytic properties of the autoreceptor agonists buspirone, ipsapirone, and flesinoxan were reproduced in both temperature conditions, causing reductions in ultrasonic vocalization, the authors reported significant anxiolytic effects for autoreceptor *antagonists* (e.g., NAN-190) as well. Further, the overall pattern of results was quite variable, with one 5-HT uptake inhibitor (fluvoxamine) reducing ultrasonic vocalization in both conditions, another (clomipramine) only reducing anxiety behavior in the warm stimulus condition, and the 5-HT₂ antagonist ketanserin increasing ultrasonic vocalization with the cold plate stimulus only.

In addition to type of stressor, the gender of the animal has also been reported to influence the way 5-HT impacts stress responding. One study showed that injecting ovariectomized female rats with estradiol benzoate alone or together with progesterone prevented the inhibition of

lordosis behavior induced by central injection of a 5-HT_{1A} receptor agonist, and progesterone (but not estradiol benzoate) prevented the restraint stress-induced reduction of lordosis behavior (Truitt et al., 2003). Dominguez et al. (2003), found more 5-HT activity as indicated by higher levels of the 5-HIAA metabolite in the female DRN, but not the MRN, when compared to male animals. The same group also reported that female rats exhibit more stress-induced behaviors than males when placed in the elevated plus maze, but only on the first day of diestrus, when animals are unresponsive to estrogen and progesterone levels are rising. Another report indicates that chronic restraint stress increases the amount of 5-HT in the hippocampus of female, but not male rats; this increase, along with changes in other neurotransmitter and hormonal systems, may be related to the protection against the detrimental effects of stress on certain memory tasks observed in female, but not male animals (Luine, 2002). Because of these sex-based differences, most research, including this dissertation, uses only male animals in studies of stress responding.

Summary

There are several reports that support 5-HT as an anxiogenic neurotransmitter as well as many reports that support the anxiolytic effects of 5-HT on behavioral responding to stress. The findings outlined in the “5-HT as Anxiolytic” section above tend to support Graeff and Deakin’s theory that with the application of actual, proximal stressors such as foot shocks, aversive brain stimulation, and placement in an open field, stress responding is decreased with administration of 5-HT agonists, and that this decrease is prevented with concomitant administration of 5-HT antagonists. However, as outlined in the section, “Ambiguity of 5-HT influence on Stress Responding,” behavioral responses to stressors of a similar nature (e.g., hot/cold plate) can be influenced in different ways by both agonists and antagonists. Further study, of different stressors as well as different 5-HT agents, is needed to clarify the place of 5-HT in stress responding.

Purpose of the Present Study

Based on the literature reviewed above, one can conclude that 5-HT influences stress responding in a number of ways depending on the strain of rat used, the type of agent administered (i.e., what receptor affinity it has, whether it is an agonist or antagonist, etc.), the dose of the agent administered, the route of administration, as well as the type of stressor employed. To date, no published report has investigated the influence of centrally and/or peripherally administered 5-HT_{2a/c} agonist DOI or the 5-HT_{2a/c} antagonist ketanserin on behaviors evoked by tail pinch or open field stress.

The purpose of the reported experiments was two-fold. First, to investigate the role of 5-HT_{2A/C} receptors in the behavioral response to two types of stressors in the rat: tail pinch and the open field. Second, to help clarify any differences in the central vs. peripheral influence of 5-HT on stress responding.

Hypotheses

1. DOI will reduce stress responding in both the tail pinch and open field conditions when injected either a) peripherally, b) centrally, or c) both simultaneously.
2. Ketanserin may a) increase behavioral responding to stress when injected alone, and will b) reverse the reduction in behavioral responding from injection of DOI.

METHODS

Five separate, but related experiments were conducted to test the above hypotheses. Each is described below in the section entitled *Experiments*. Refer to Table 1 for the basic tenets of each.

Drugs

5-HT_{2A} & _{2C} Agonist

(+/-)-1-(2,5-dimethoxy-4-iodophenyl)-2 aminopropane (DOI) acts as a potent agonist at the 5-HT_{2A} and _{2C} receptor subtypes (Figure 2). DOI is classified as a phenethylamine hallucinogen (Liu, Jolas, & Aghajanian, 2000) that, according to information supplied by the manufacturer (Sigma/RBI), readily crosses the blood brain barrier. While not used in research or treatment of human subjects (except ex vivo tissue studies e.g., Bax, Heuven-Nolsen, Bos, Simoons, & Saxena, 1992; Serres, Azorin, Valli, & Jeanningros, 1999), DOI has been used in many studies of 5-HT influence on physiological and behavioral stress responding in animals, as described in detail above.

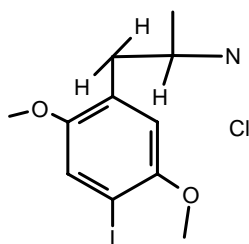


Figure 2: DOI Chemical Structure (from: <http://infonew.sigma-aldrich.com>)

5-HT_{2A} & 2C Antagonist

Ketanserin (KET) is an antagonist at the 5-HT_{2A} and 2C receptors (Figure 3). KET was originally used as an antihypertensive agent (Awouters, 1985) and has also been used to treat nociceptive disorders (Alhaider, 1991; Klimiuk et al., 1989) and Raynaud's syndrome (to increase circulation to the extremities) (Dormandy, Berent, & Downes, 1988).

As indicated by the research described above, KET is commonly used in stress research.

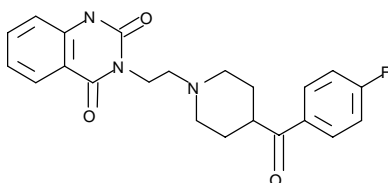


Figure 3: Ketanserin Chemical Structure (from: [http:// info new.sigma-aldrich.com](http://info.new.sigma-aldrich.com))

Pilot Work

The route of administration and specific doses of both drugs were determined using the literature as a guide (e.g., Jorgensen et al., 1998; Onaivi et al., 1995) as well as pilot work employing a range of doses of both drugs in our laboratory. The 30-minute latency between the injection of peripherally administered drugs and testing is based on our pilot work where animals were tested 0, 30, and 60 minutes after injection, as well as guidance from the literature (e.g., Diaz-Veliz et al., 1997; Nic Dhonnchadha et al., 2003).

Experiments

Experiment 1: Peripheral DOI

This experiment was designed to test part 'a' of hypothesis one, that peripherally injected DOI will reduce stress responding to tail pinch and open field stressors.

Animals. Forty-eight male Sprague-Dawley rats, approximately eight weeks of age, were used for this experiment. Animals were obtained from the Division of Laboratory Animal Medicine, Louisiana State University. Animals were housed in individual plastic cages with food and water available 24 hours/day. The room where the animals were housed was maintained at a temperature of 22°C and overhead lighting was on a 12-hour light/dark cycle, on at 0700 hr.

Drug. DOI was obtained from SIGMA/RBI (Sigma-Aldrich, Inc.). It was kept in its original container, in the dark, at room temperature. Each testing day, drug was weighed out and the appropriate volume of sterile normal saline was added to reach the desired concentration.

Experimental Group Assignment. Before the collection of data, animals were randomly assigned to a drug or control group. In this experiment, the groups consisted of saline control (n = 15), 0.1 mg/kg DOI (n = 9), 0.5 mg/kg DOI (n = 12), and 1.0 mg/kg DOI (n = 12).

Experiment 2: Central DOI

This experiment was designed to test part 'b' of hypothesis one, that centrally injected DOI will reduce stress responding during tail pinch and placement in the open field.

Animals. Fifty-five animals were obtained from the same colony and were the same sex and approximate age as previously described. Housing conditions were as described above.

Drug. DOI was obtained from Sigma/RBI (Sigma-Aldrich, Inc.). It was dissolved using sterile water and distributed into aliquots. Aliquots were lyophilized and stored at 0°C until just prior to testing. For injection, the drug was reconstituted with sterile 0.9% saline.

Experimental Group Assignment. Animals were randomly assigned to one of 4 groups: saline control (n = 17), 20 µg (n = 13), 100 µg (n = 18), or 200 µg (n = 14) of DOI.

Surgery. In order to receive ICV injections, animals underwent stereotaxic surgery for implantation of permanently indwelling bilateral cannulae. Animals were anesthetized with ketamine (90 mg/kg i.m.) and xylazine (5 mg/kg i.m.). Once the anesthetic had taken effect, the top of an animal's head was shaved and the head was mounted on the stereotaxic apparatus. The surface of the skull was exposed and irrigated with approximately 0.25 ml of a bupivacaine:lidocaine mixture to reduce postoperative pain. Stainless steel anchor screws were inserted into the skull and permanently indwelling, stainless steel guide cannulae were implanted 0.0 mm anterior to bregma, +/-1.6 mm lateral from midline, and -3.0 mm ventral from dura. Dental acrylic was used to hold the cannulae in place and prevent the recovered animal from disturbing the implant. Animals were closely monitored following surgery, until they emerged from anesthesia. Animals were given five days following the completion of the surgical procedure to recover before experimental testing began. This surgical protocol was reviewed and approved by the Louisiana State University Institutional Animal Care and Use Committee (protocol #99-115).

Experiment 3: Central + Peripheral DOI

This experiment was designed to test part 'c' of hypothesis one that simultaneous central and peripheral injections of DOI will reduce stress responding in both the tail pinch and open field conditions.

Animals. Twenty-three male animals were used in this experiment. They were obtained from the same colony, were within the same age/weight range, and were housed as described above.

Drug. DOI was used as described in Experiment 1 and Experiment 2, above.

Experimental Group Assignment. Two groups were employed in this experiment: a saline control that received both a central and a peripheral injection of saline (n = 13) and a drug group that received a 200 µg central injection of DOI and a 0.1 mg/kg peripheral injection of DOI (n = 15).

Surgery. Animals in this experiment underwent surgery as described above for Experiment 2.

Experiment 4: Peripheral KET

This experiment was designed to test part ‘a’ of hypothesis two, that administration of KET may increase stress responding during tail pinch and in the open field.

Animals. Thirty-one male rats were used for this portion of the study. Animals were obtained from the same colony, were within the same age/weight range, and were housed as described above.

Drug. KET was obtained from Sigma-Aldrich and stored according to the manufacturer’s direction, at 0°C. Drug was weighed prior to testing and diluted in sterile 0.9% saline.

Experimental Group Assignment. Animals were randomly assigned to one of three drug groups: 0.5 mg/kg KET (n = 11), 2.5 mg/ KET (n= 10), and 5.0 mg/kg KET (n = 10). The control group for this experiment was shared with Experiment 1, as the two experiments had identical injection and testing protocols.

Experiment 5: Peripheral DOI + Peripheral KET

This experiment was designed to test part ‘b’ of hypothesis two, that KET will reverse the effect of DOI, and prevent the reduction in behavioral responding to tail pinch and open field stressors.

Animals. Ninety-six male animals were used in this experiment. Animals were obtained from the same colony, used within the same age/weight range, and housed as described above.

Drug. Both DOI and KET were obtained, stored, and prepared for injection as previously described.

Experimental Group Assignment. Animals were randomly assigned to one of six groups: double saline control group (n = 16), 0.5 mg/kg DOI + saline (n = 16), 1.0 mg/kg DOI + saline (n = 16), 5.0 mg/kg KET + saline (n = 16), 0.5 mg/kg DOI + 5.0 mg/kg KET (n = 16), and 1.0 mg/kg DOI + 5.0 mg/kg KET (n = 16).

Table 1: Design for all 5 Experiments

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
Injection Type	Peripheral	Central	Peripheral + Central	Peripheral	Double Peripheral
N	48	55*	23*	31	96
Drug	DOI	DOI	DOI	KET	DOI + KET
Dose	Saline 0.1 mg/kg 0.5 mg/kg 1.0 mg/kg	Saline 20 µg 100 µg 200 µg	Saline 200 µg + 0.1 mg/kg	0.5 mg/kg 2.5 mg/kg 5.0 mg/kg	Saline DOI: 0.5 + saline 1.0 + saline KET: 5.0 + saline Both: 0.5 DOI + 5.0 KET 1.0 DOI + 5.0 KET

*Number represents post-histology *n*.

General Procedure

Rater Training Criterion

Data collectors were trained to 80% accuracy on the measurement of all dependent measures. Training was conducted prior to the collection of data using animals that were not

included as subjects. Data collectors were trained to properly handle the animals, apply the tail pinch stressor, and collect behavioral data. Each collector was required to measure every behavior in two consecutive trials to at least 80% concordance with another (previously trained) rater on both trials.

Injections

Single Peripheral Injection Procedure: Experiments 1 & 4. Animals in Experiments 1 and 4 were removed from their home cage, restrained by hand and administered a subcutaneous injection at the back of the neck. Animals were weighed a few minutes prior to injection and all injections were given at a volume of 1.0 ml per kg of body weight. Following injection, animals were returned to their home cages for a period of 30 minutes prior to being placed in the testing cage and subjected to the tail pinch stressor.

Single Central Injection Procedure: Experiment 2. Animals were removed from their home cages and restrained by hand. Stylets placed in the guide cannulae (to prevent blockage during the post-operative recovery period) were removed and sterile injectors were inserted into the guide cannulae. The injector tip extended 1.0 mm past the ventral end of the cannula. Injectate was administered simultaneously to the lateral ventricles at a rate of 5.0 μ l per minute via polyethylene tubing connected to 100 μ l syringes held in a Sage infusion pump. A total volume of 10 μ l was delivered to each ventricle over a two-minute period. The animals were held, with injectors in place, for an additional one minute to allow the injectate to diffuse from the site of injection. Animals were exposed to the tail pinch stressor immediately following the injection procedure.

Central + Peripheral Injection Procedure: Experiment 3. The injection procedure for the animals in experiment three had two phases. The first phase was identical to the peripheral

injection procedure previously described. However, approximately 5 minutes before animals were scheduled to go into the tail pinch testing cage (i.e., 25 minutes following peripheral injection), they were removed from their home cages and given the ICV injection as described above.

Testing immediately followed the completion of the ICV injection.

Double Peripheral Injection Procedure: Experiment 5. Each animal in Experiment 5 received two consecutive peripheral injections. The injections were administered subcutaneously at the back of the neck as described above. For the groups that received drug, animals received the drug injection first and the saline injection immediately following. Animals that received two injections of drug always received the DOI injection first and the KET second. As previously described, animals were returned to their cages for 30 minutes before tail pinch testing.

Testing

All animals (Experiments 1-5) were tested for behavioral responding to stress under two conditions. Testing was always conducted between 0900 and 1300, during the animals' light cycle. Animals were first subjected to a tail pinch stressor, where the animal was placed in a suspended wire cage, the length of its tail guided through the bottom of the cage, and a clamp (modified haemostatic forceps) was applied to the animal's tail outside of the cage at a diameter of 4.3 millimeters. The clamp was applied for a period of four minutes, during which animals were observed for behavioral responses. When the four-minute testing period was over, the clamp was removed and animals were returned to their home cage.

Forty-five minutes after injection (approximately ten minutes after the conclusion of the tail pinch test), animals were again removed from their home cage and placed in the open field apparatus for a four-minute observation period. This apparatus is an open topped box constructed of clear Plexiglas with dimensions of 2'x2'x2'. The bottom of the box is demarcated

into four 12” squares. A 75-watt light is positioned directly above the field. Following the testing period, animals were returned to their home cage.

Rotarod

Twenty-four hours following stress testing (tail pinch and open field) animals were trained on the rotarod apparatus. This instrument is a rotating stainless-steel drum (with a textured surface for traction) 7.2 cm in diameter used to test for motor impairment. The training procedure consisted of repeatedly placing the animal on the rotating drum (10 RPM) until it remained on the drum for 30 consecutive seconds without losing balance.

Testing for motor impairment due to drug administration occurred 24 hours after the animals were trained (that is 48 hours after testing in the stress conditions). Animals were removed from their home cages and underwent the same injection procedure described above. Thirty minutes following peripheral injection (Experiments 1, 4, & 5) or immediately following central injection (Experiment 2 & 3), they were placed on the rotarod and were observed for thirty seconds or until they fell from the rotarod, whichever occurred first.

Dependent Variables

Tail Pinch

The following seven variables were measured in the tail pinch condition:

1. **Oral stereotopy with food** was defined as the amount of time an animal engaged in any oral behavior (i.e., licking, chewing, etc.) directed at lab chow.
2. **Eating** was recorded by subtracting the post-test weight of lab chow placed in the tail pinch cage from the pre-test weight.

3. **Gnawing** was defined as the amount of chow that was shredded, but not ingested, by the animal during tail pinch. The shredded chow was collected beneath the testing cage during tail pinch.
4. **Grooming** was measured as the amount of time the animal licked or combed any part of its body or whiskers with its paws.
5. **Oral stereotopy without food** was recorded as the amount of time the animal engaged in oral movement that did not involve food or self. This included licking and biting of the cage and chattering of the teeth.
6. **Vocalization** was defined as the number of vocal emissions an animal made over the course of the testing period.
7. **Fecal boli** were recorded as the number produced over the course of the testing period.

An *increase* in any of the behaviors described above is indicative of increased stress responding during exposure to a tail pinch stressor.

Open Field

The following six variables were measured in the open field condition:

1. A **line cross** was tallied each time an animal crossed with its front paws from one quadrant to another in the open field box.
2. **Rearing** was defined as the number of times the rat simultaneously lifted both front paws from the cage floor.
3. **Freezing** was recorded as the amount of time an animal displayed cessation of all movement except breathing.
4. A **headshake** was recorded each time an animal vigorously shook its head.

5. **Wet dog shakes** were recorded as the number of times the animal shook its head and torso (in this case a headshake was *not* recorded).

6. **Flat body posture** was noted (presence or absence) and was defined as an elongated posture in combination with a creeping gait.

In the open field, an *increase* in the amount of time spent freezing and a *decrease* in line crosses or in rearing is indicative of increased stress responding. The remaining three variables (head/ wet dog shakes and flat body posture) are not measurements of stress responding, but rather of 5-HT_{2A/C} receptor activity - an increase in these behaviors is indicative of increased activity at the 5-HT_{2A/C} receptors.

Rotarod

The dependent measure for the rotarod test was latency to loss of balance. Each animal was allowed to walk on the apparatus until it fell or the 30-second criterion was reached. Scores were recorded to the nearest tenth of a second.

Note on Data Collection

In order to ensure accurate measurement, two trained observers collected data during each trial of tail pinch and open field testing, with the dependent variables divided between them. During a tail pinch trial for example, one observer recorded oral stereotopy with food and grooming, while the other recorded oral stereotopy without food and vocalizations. A similar division of dependent variables was adopted for the open field condition. Unless otherwise noted above, duration measurements (e.g., oral stereotopy with food) were recorded to the nearest hundredth of a second. All measurements of weight (e.g., eating) were recorded to the nearest hundredth of a gram.

Histology

Animals that received stereotaxic surgery were euthanized following the rotarod procedure and a histological analysis was performed to insure proper placement of cannulae for ICV injections. Animals were euthanized with an overdose of ether and 5 μ l of ink was injected bilaterally using the guide cannulae. Following ink injection, animals were perfused transcardially with 30 cc of physiologic saline followed by 30 cc of phosphate-buffered formalin. The brains were extracted and stored in formalin for at least 24 hours. Eighty μ m sections were taken through the site of implantation using a freezing microtome. Sections were examined under the microscope and visual confirmation of bilateral implantation was made. Only data for those animals with correct implantation were considered in analysis.

RESULTS

Statistical Procedures

Tail pinch and open field data were analyzed using Multivariate Analysis of Variance (MANOVA), with two exceptions: flat body posture and rotarod. Due to the nominal nature (i.e., presence or absence) of the flat body posture data, they could not be included in multivariate analysis and were analyzed separately using χ^2 analysis. Because all animals did not complete rotarod testing due to attrition or failure to satisfy the 30-second rotarod training criterion, rotarod data were analyzed separately using univariate analysis of variance. Significant MANOVA result for each experiment was followed up with univariate analysis of variance for each dependent variable included in the design, and tests of Least Significant Differences were conducted on each significant univariate test in order to describe the between-group differences observed. Alpha was set a priori at .05 for all tests. Significant findings are reported by experiment below (refer to the Appendix for non-significant data).

General Notes

For ease of reading, drug doses from this point forward are referred to as low, middle and high where appropriate. Doses are labeled in each figure, however Table 2 below is provided as an additional reference.

Table 2: Doses designated as ‘low,’ ‘middle,’ or ‘high’ for each experiment.

Experiment 1	Experiment 4	Experiment 2	Experiment 5	
DOI (mg/kg)	KET (mg/kg)	DOI (μ g)	DOI (mg/kg)	KET (mg/kg)
Low = 0.1	Low = 0.5	Low = 20	Low = 0.5	5.0
Middle = 0.5	Middle = 2.5	Middle = 100		
High = 1.0	High = 5.0	High = 200	High = 1.0	

In no experiment did administration of either drug result in significant motor impairment as measured by rotarod performance (see the Appendix).

Experiments 1 & 4: Peripheral DOI and KET

Data for Experiments 1 and 4 were combined into one multivariate analysis for conservation of alpha error. Overall MANOVA was significant [$F(78, 390) = 2.705, p < .001$]. Significant differences were found between drug groups for eight of the thirteen variables included in the analysis: oral stereotopy with food [$F(6, 72) = 4.169, p = .001$] and without food [$F(6, 72) = 5.479, p < .001$], vocalizations [$F(6, 72) = 2.569, p = .026$], gnawing [$F(6, 72) = 2.415, p = .035$], rearing [$F(6, 72) = 13.59, p < .001$], headshakes [$F(6, 72) = 10.177, p < .001$], body shakes [$F(6, 72) = 6.949, p < .001$], freezing [$F(6, 72) = 2.587, p < .025$]. The groups differed on flat body posture as well [$\chi^2(3) = 34.29, p < .001$].

Oral stereotopy with food is depicted below in Figure 4. The group that received the high dose of DOI exhibited significantly less oral stereotopy with food than the group that received saline ($p = .037$). While neither group that received the low or middle dose of DOI behaved differently from the saline control group, a dose-related trend towards behavioral reduction is evident.

Unlike the dose-related stepwise reduction in behavior observed in Experiment 1 with DOI administration, a U-shaped increase in oral behavior directed at food emerged in animals administered KET in Experiment 4. Specifically, the group that received the low dose of KET performed significantly more oral stereotopy with food than the saline control group ($p = .023$), the groups that received the low ($p = .037$), the middle ($p < .001$), or the high ($p < .001$) doses of DOI, as well as the middle dose ($p = .028$) of KET. The group that was administered the high dose of KET also spent significantly more time engaged in oral behavior directed at food than the groups administered the middle ($p = .008$) or high ($p = .002$) doses of DOI.

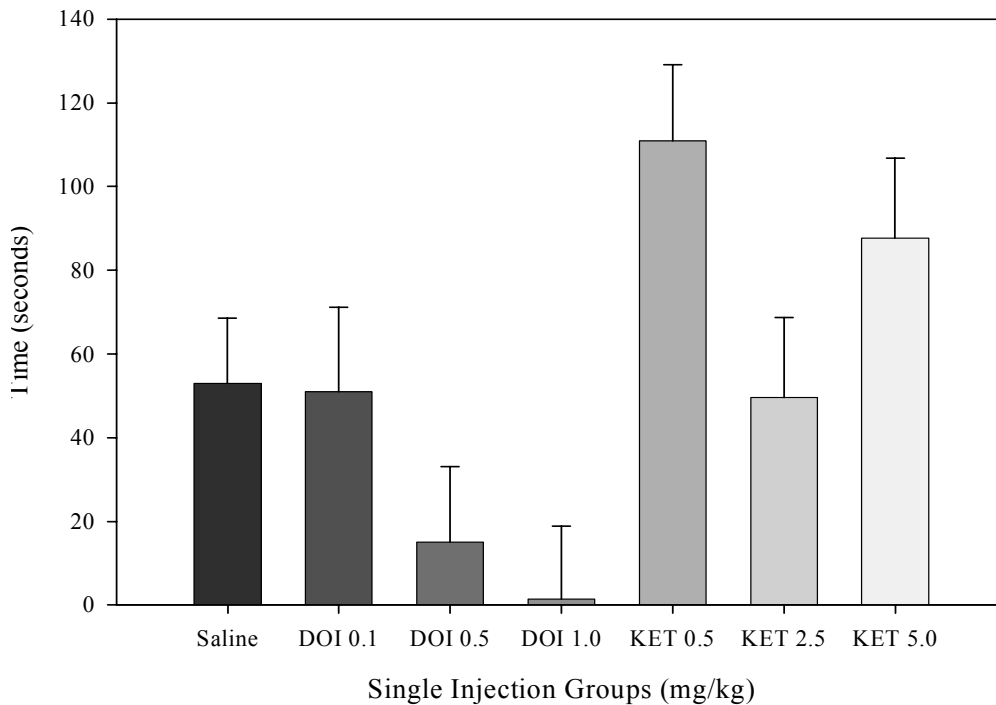


Figure 4: Oral Stereotopy with Food after Peripheral administration of Saline, DOI, or KET

Oral stereotopy without food is shown in Figure 5 below. The groups that received either the middle or high doses of DOI performed significantly less oral stereotopy directed at the testing cage than the saline control group with probability values of ($p = .004$) and ($p = .006$) respectively.

The group that was administered the low dose of DOI behaved no differently than the saline control group, and spent a significantly longer amount of time engaged in oral stereotopy without food than the groups receiving any of the three doses of KET (low ($p = .037$), middle ($p = .001$) and high ($p < .001$)), and the other two doses of DOI as well (middle ($p < .001$) and high ($p = .001$)). All three groups that received KET (low ($p = .001$), middle ($p = .007$) and high ($p = .004$) doses) performed significantly less oral stereotopy without food than did the saline control group.

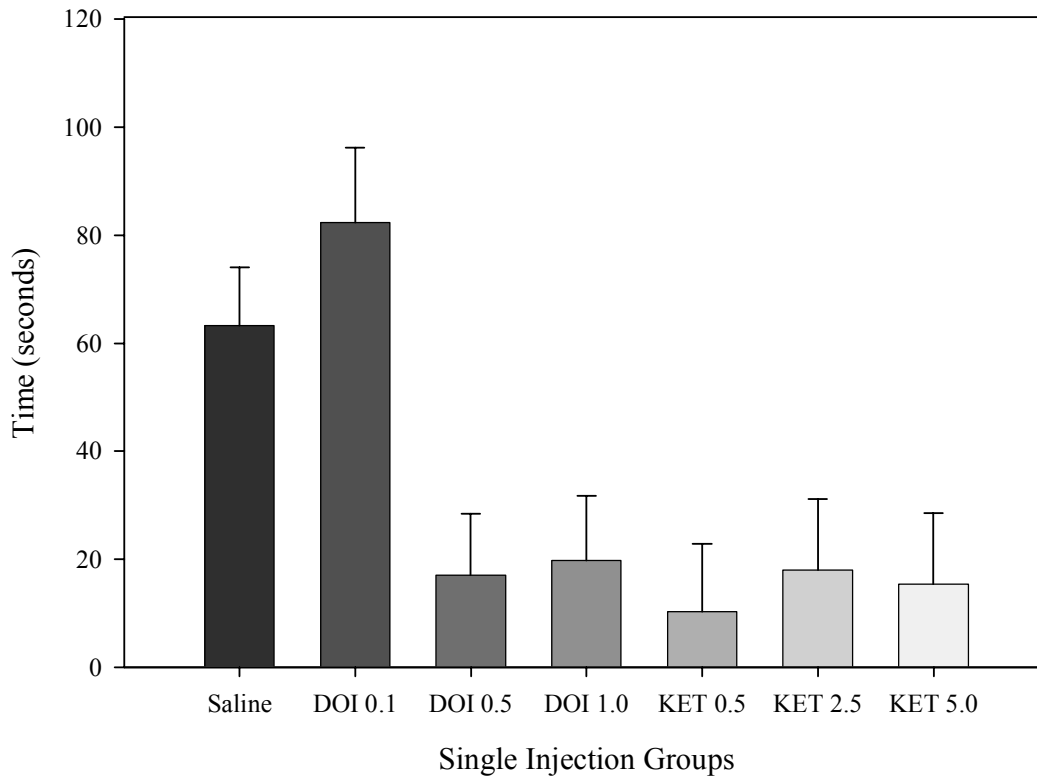


Figure 5: Oral Stereotopy without Food after Administration of Saline, DOI, or KET

Figure 6 below shows the data for Vocalizations. Despite a dose-related trend towards a reduction in the number of vocalizations emitted by animals that were administered DOI, none of the groups receiving DOI differed significantly from the saline control group on vocalizations. The group that was administered the low dose of DOI vocalized significantly more than the middle ($p = .032$) and high ($p = .023$) doses of DOI, as well as the low dose of KET ($p = .037$). The group receiving the middle dose of KET emitted significantly more vocalizations than the groups receiving saline ($p = .045$), the middle ($p = .005$) and high ($p = .003$) doses of DOI, as well as the low dose of KET ($p = .012$).

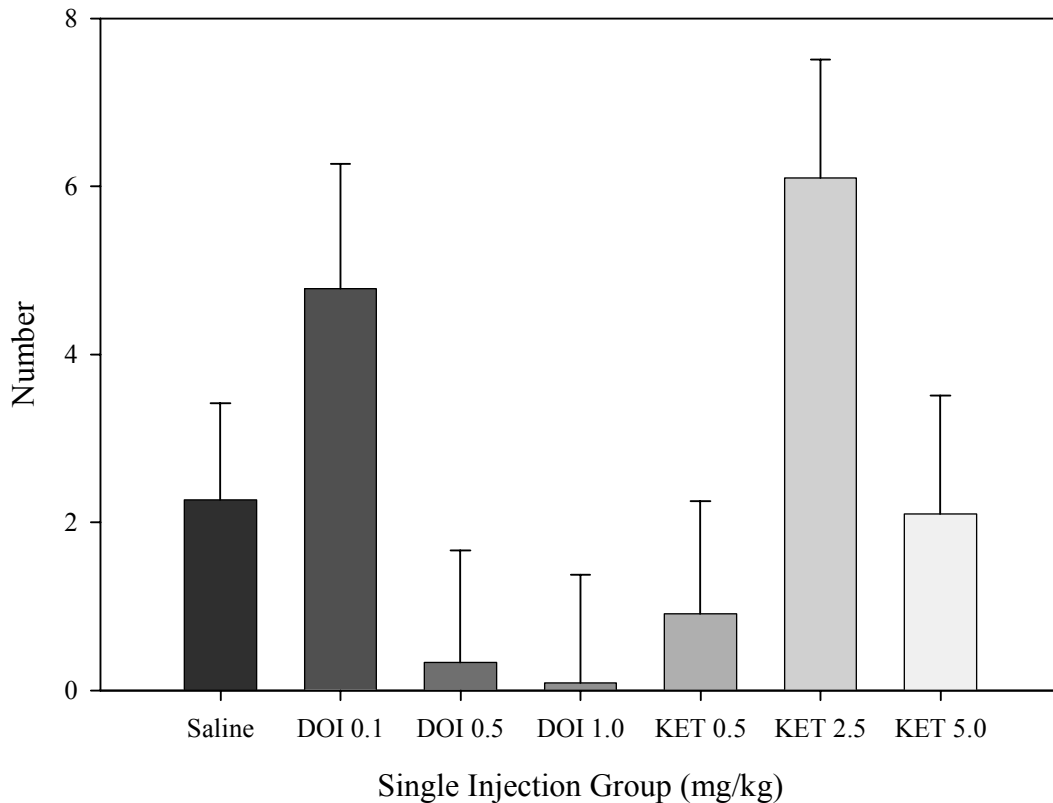


Figure 6: Vocalizations after Administration of Saline, DOI, or KET

Gnawing data is depicted in Figure 7 below. None of the groups that were administered drug (DOI or KET) behaved significantly differently from the saline control group on the amount of food gnawed during tail pinch. There was however, a non-significant trend towards behavioral reduction in the amount of food gnawed for animals that were injected with DOI.

The group that received the low dose of DOI gnawed significantly more food than those that received the middle ($p = .029$) or high ($p = .026$) doses of DOI. The animals that were administered the low dose of KET performed significantly more gnawing than the groups that were injected with either the middle ($p = .006$) or high ($p = .005$) doses of DOI, and the middle ($p = .034$) dose of KET as well.

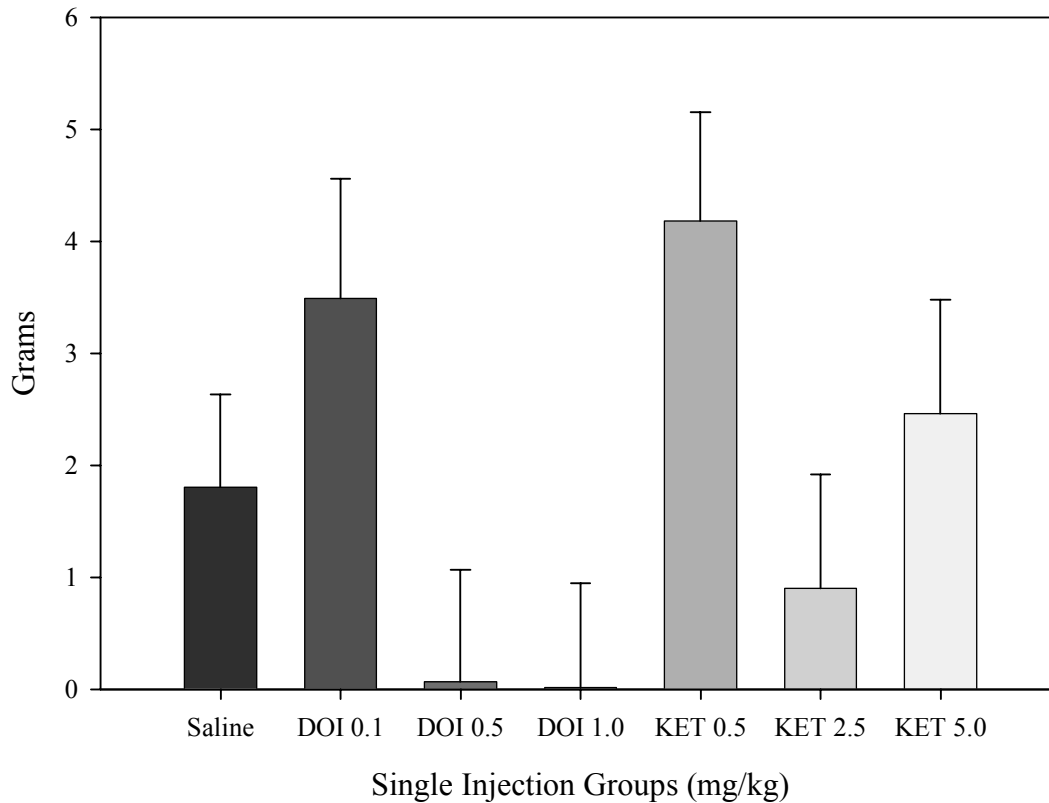


Figure 7: Grawing after Administration of Saline, DOI, or KET

The data for Rearing is presented below in Figure 8. For this variable, all groups that were administered drug (DOI or KET) performed significantly fewer instances of rearing behavior in the open field condition than did the saline control group. Specifically, the groups receiving the low ($p < .001$), middle ($p < .001$), and high ($p < .001$) doses of DOI exhibited a dose-dependent reduction in rearing compared to the control group, and the group that received the high dose of DOI performed significantly less rearing behavior than the low dose of DOI ($p = .042$).

While the animals that were administered the low ($p = .002$), middle ($p = .009$) and high ($p = .004$) doses of KET all performed significantly less rearing behavior than the control group, they performed significantly more rearing than the middle and high doses of DOI, with all probability values at or below $p = .001$. The group that received the middle dose of KET also

performed significantly more rearing compared to the group that received the low dose of DOI ($p = .050$).

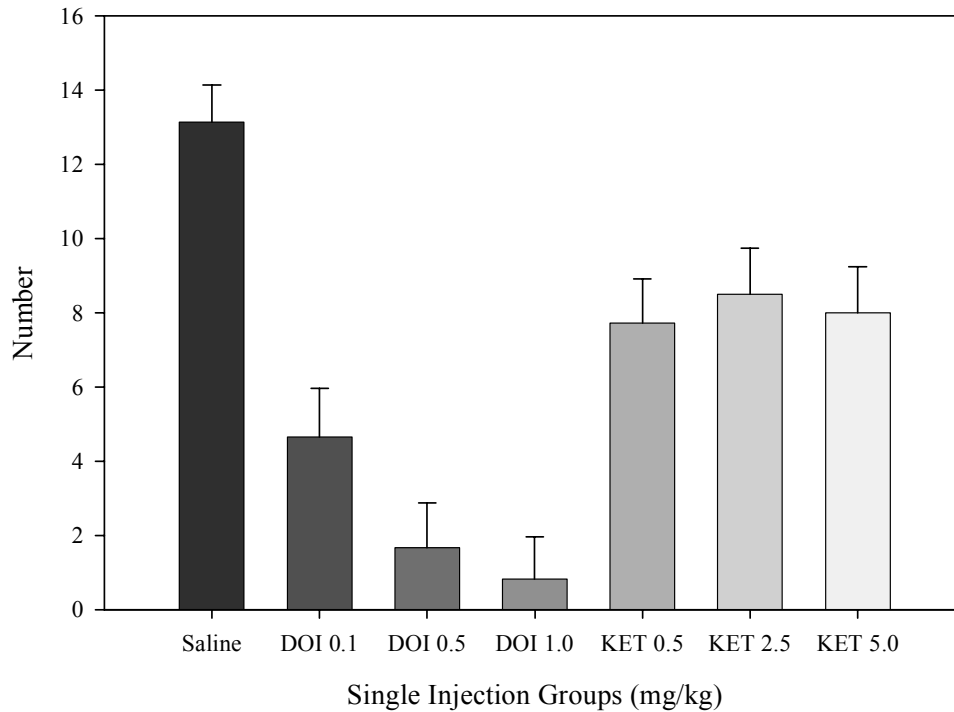


Figure 8: Rearing after Administration of Saline, DOI, or KET

The following two graphs depict Headshakes (Figure 9) and Wet dog shakes (Figure 10).

The groups receiving either the middle or high dose of DOI displayed a significantly higher number of headshakes in the open field than the saline control, all three groups that received KET, and the group that was administered the low dose of DOI (all comparisons $p < .001$).

Similar to the headshake variable, the group that received the middle dose of DOI displayed significantly more wet dog shakes in the open field than all other groups, with $p < .001$ for all comparisons.

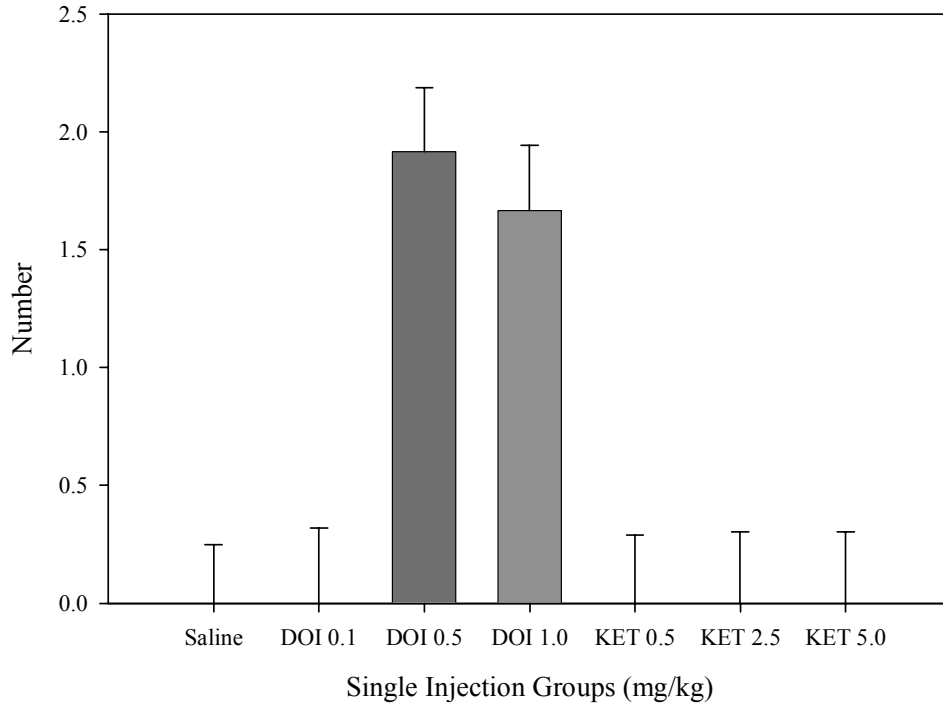


Figure 9: Headshakes after Administration of Saline, DOI, or KET

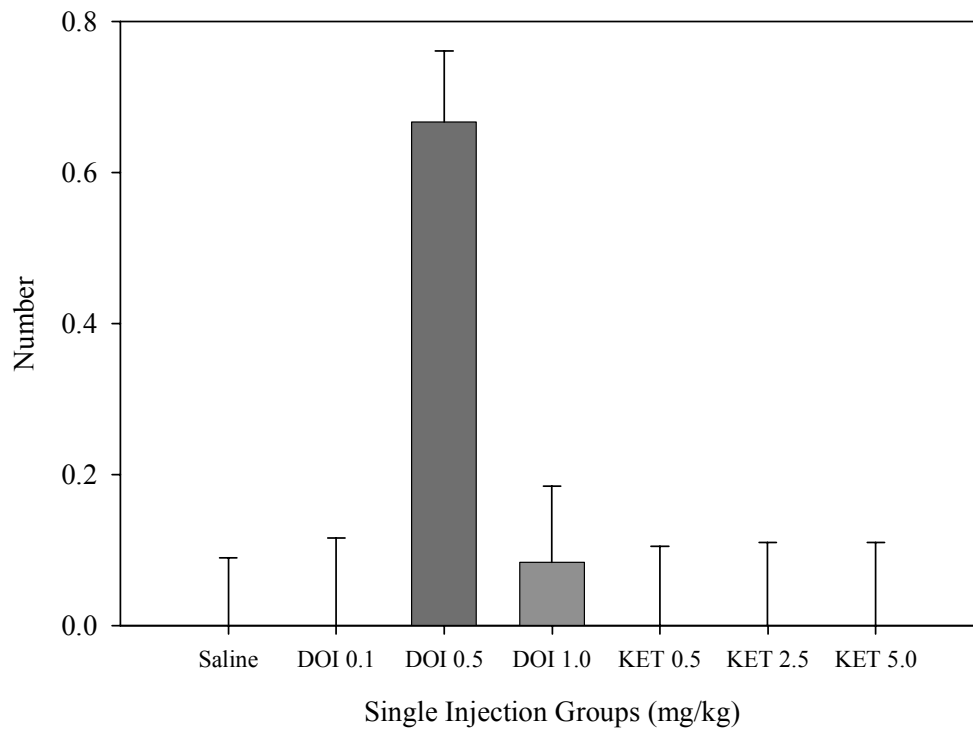


Figure 10: Wet Dog Shakes after Administration of Saline, DOI, or KET

Figure 11 below shows the data for Freezing. No group that was administered DOI behaved differently from the saline control group on the amount of time spent freezing in the open field. In fact, only one group of animals displayed a significant change in behavior on this variable. The group that was administered the high dose of KET spent a significantly longer amount of time freezing than the saline control group ($p = .004$), the group that received the low dose of DOI ($p = .005$), and the groups that received either the middle ($p = .004$) or low ($p = .009$) doses of KET.

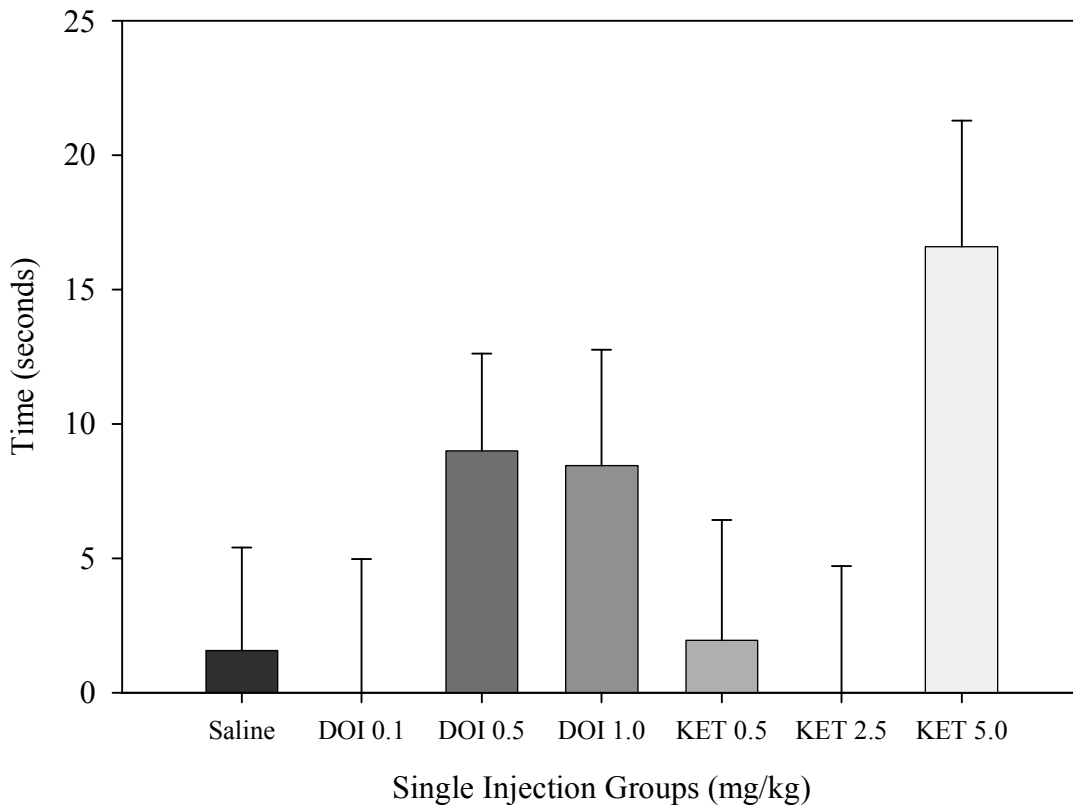


Figure 11: Freezing after Administration of Saline, DOI, or KET

Flat body posture data is depicted below in Figure 12. Flat body posture occurred in a significantly higher proportion of animals that received DOI than in those that received saline,

with 83.3% of animals in both the middle and high dose groups exhibiting flat body posture and 0% of animals receiving saline, the low dose of DOI, or any dose of KET exhibiting flat body posture.

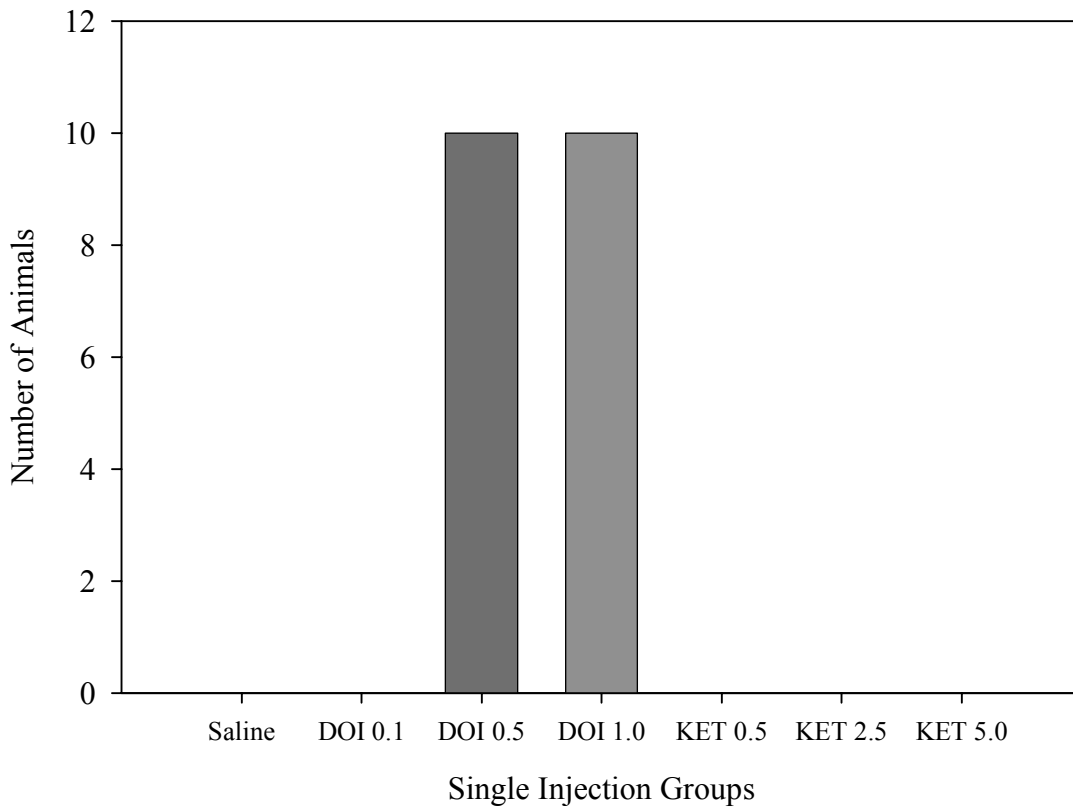


Figure 12: Flat Body Posture after Peripheral Administration of Saline, DOI or KET

Experiment 2: Central DOI

MANOVA revealed a significant effect of centrally administered DOI [$F(39,123) = 1.999$; $p = .003$]. In the tail pinch condition, only vocalizations were different between drug groups [$F(3,50) = 3.00$, $p = .04$], and in the open field condition, headshakes [$F(3,50) = 15.94$, $p < .001$] and wet dog shakes [$F(3,50) = 3.20$, $p = .03$] were different. Flat body posture was significantly increased by DOI [$\chi^2(3) = 13.35$, $p = .004$].

Figure 13 shows the average number of Vocalizations. Compared to the saline control, vocalizations were reduced by centrally administered DOI at the low ($p = .01$) and high ($p = .02$) doses.

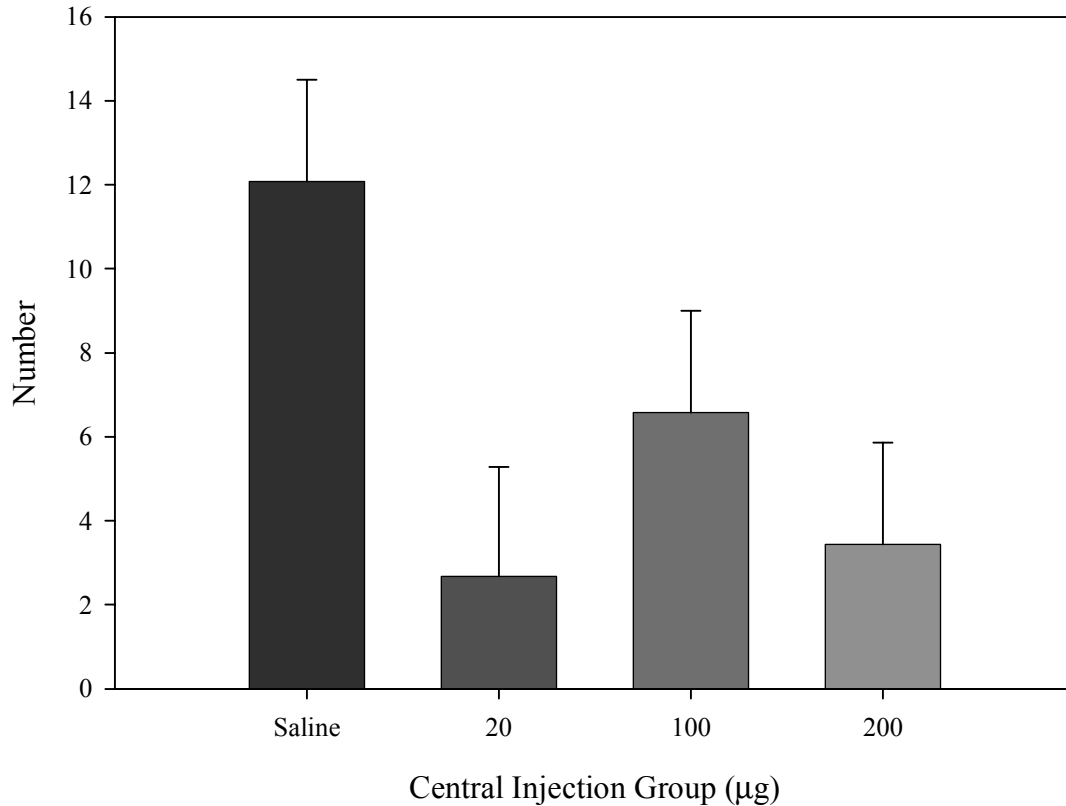


Figure 13: Vocalizations Following Centrally Administered DOI

Headshakes (Figure 14) and Wet dog shakes (Figure 15) for Experiment 2 are shown below.

Headshakes were significantly increased by both the middle ($p = .001$) and high doses ($p < .001$) of DOI in comparison to the control group. This effect was dose-dependent, with the high dose significantly increasing headshakes compared to the low ($p < .001$) and middle ($p = .004$) doses of DOI. Wet dog shakes were increased significantly only by the high dose of DOI ($p = .008$) compared to the saline control group.

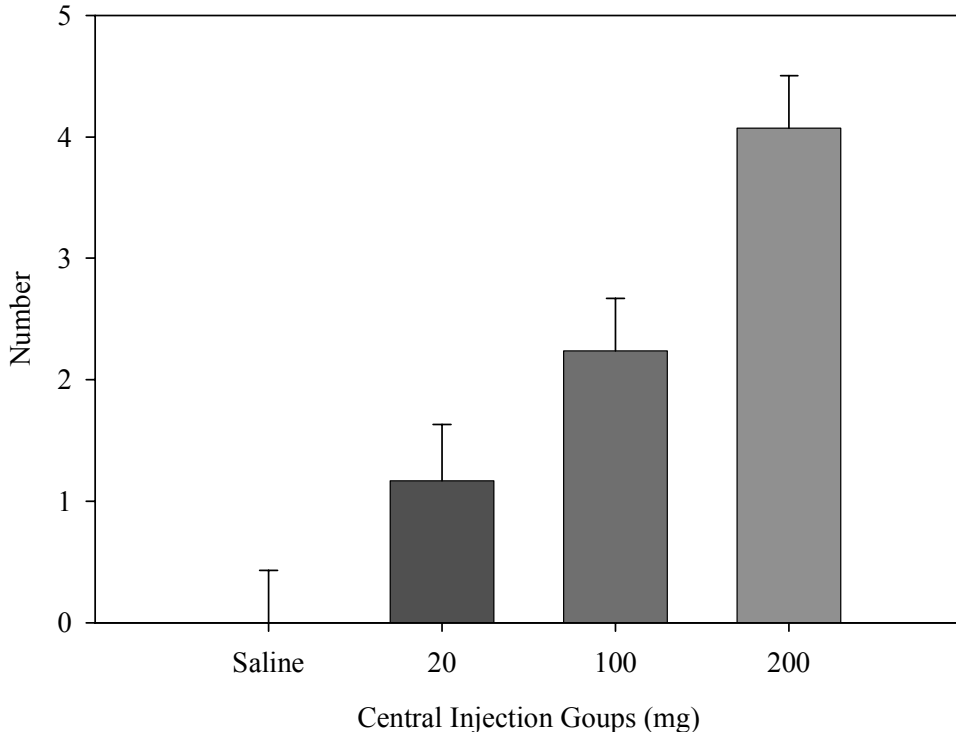


Figure 14: Headshakes Following Centrally Administered DOI

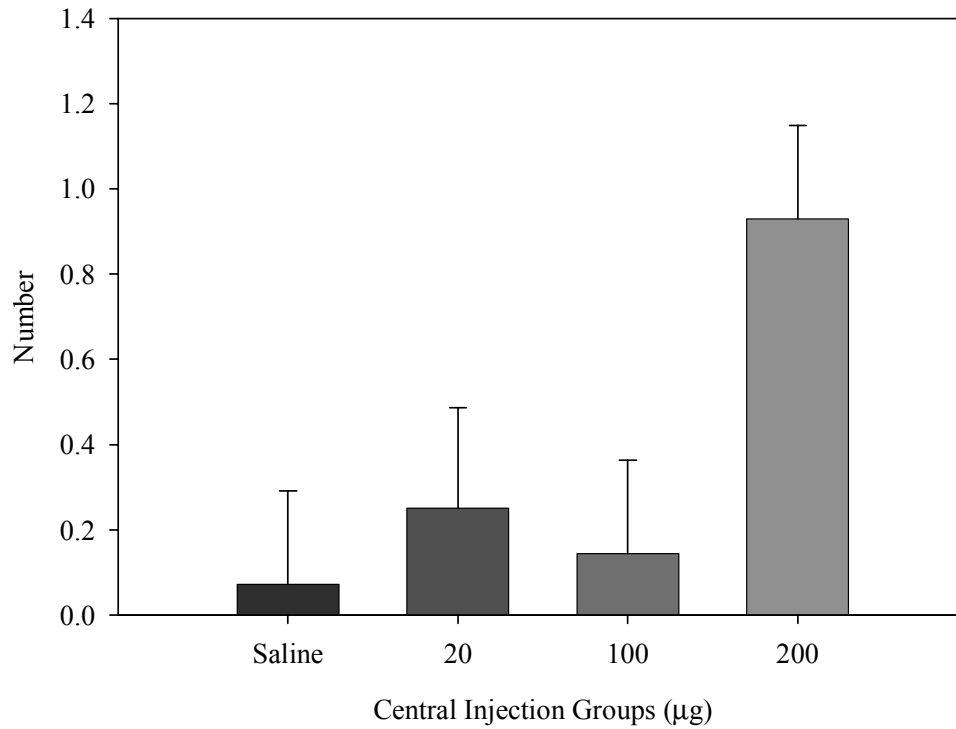


Figure 15: Wet Dog Shakes Following Central Administration of DOI

Flat body posture is shown below in Figure 16. No animal receiving saline injection displayed flattened body posture, but 8%, 50%, and 42% of animals in the groups receiving low, middle, and high (respectively) doses of DOI did display the posture.

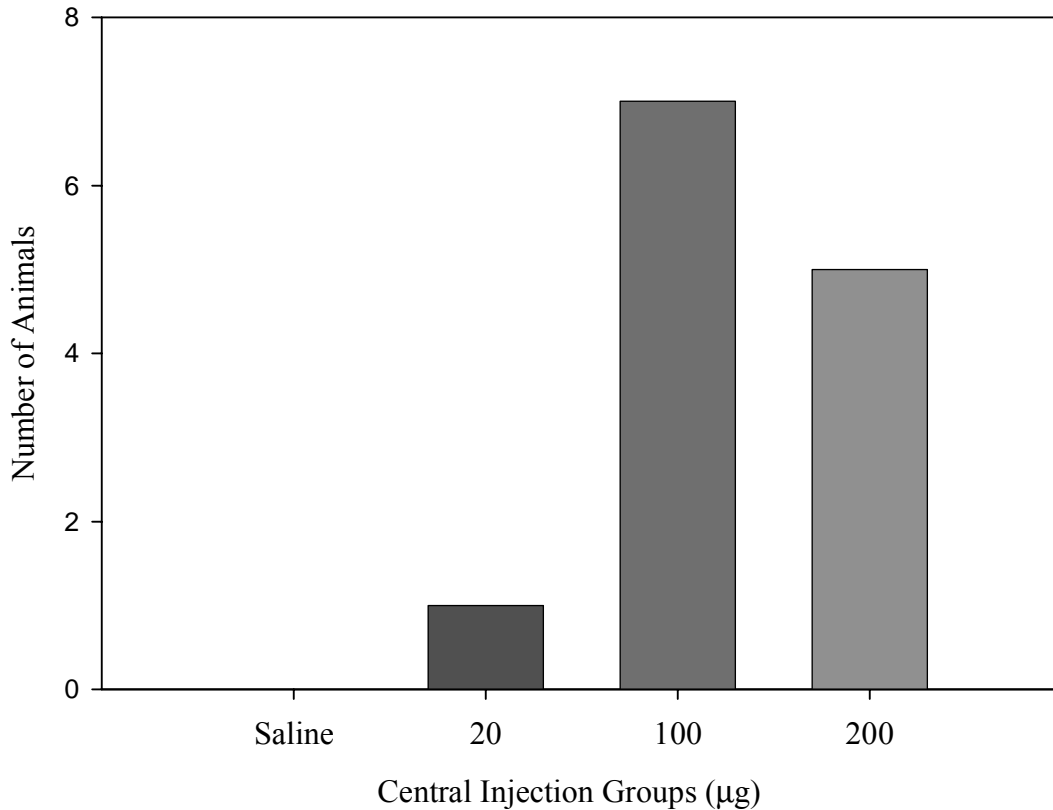


Figure 16: Flat Body Posture Following Central Administration of DOI

Experiment 3: Central + Peripheral DOI

MANOVA for this experiment was significant [$F(12,10) = 3.207, p = .037$]. In the tail pinch condition, behavioral reduction in three variables was observed following administration of DOI, and in the open field two behaviors were significantly increased by DOI.

Oral stereotypy with food is depicted in Figure 17, below. The amount of time animals engaged in oral stereotypy directed at food during the tail pinch test was significantly decreased in animals that were administered DOI [$F(1,21) = 10.47, p = .004$].

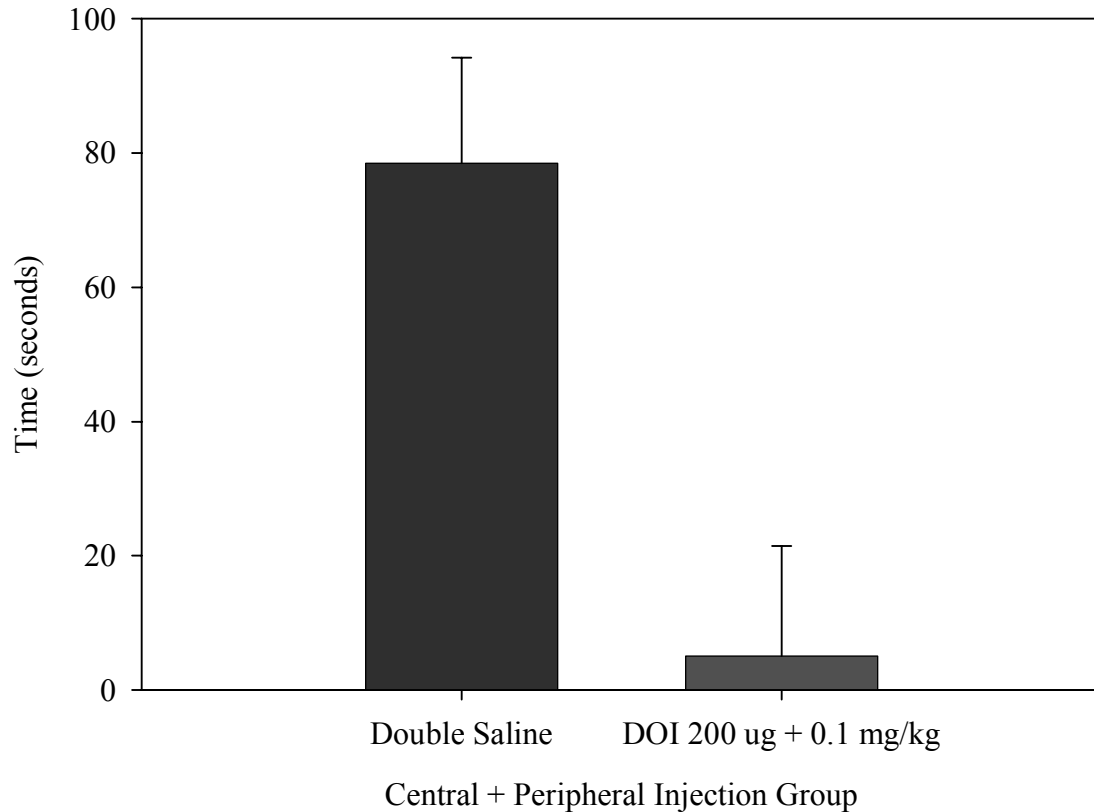


Figure 17: Oral Stereotypy with Food after Central + Peripheral Injections of Saline or DOI

Eating (Figure 18) and Gnawing (Figure 19) are shown below, followed by Headshakes (Figure 20) and Wet dog shakes (Figure 21).

Administration of DOI peripherally and centrally resulted in a significantly reduced amount of food material being eaten by animals [$F(1,21) = 6.53, p = .018$]. The amount of food gnawed was significantly reduced following central plus peripheral injection of DOI [$F(1,21) = 4.91, p = .038$].

The number of headshakes in the open field was significantly increased by DOI [$F(1,21) = 18.72, p < .001$]. Wet dog shakes were also significantly increased in animals injected with DOI compared to the saline control [$F(1,21) = 6.26, p = .021$].

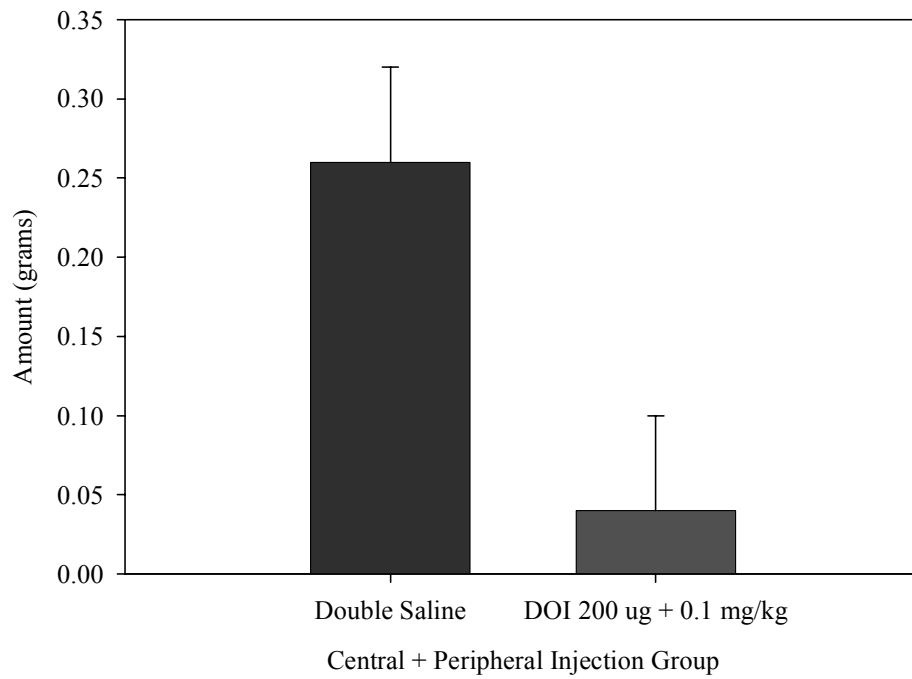


Figure 18: Eating after Central + Peripheral Injections of Saline or DOI

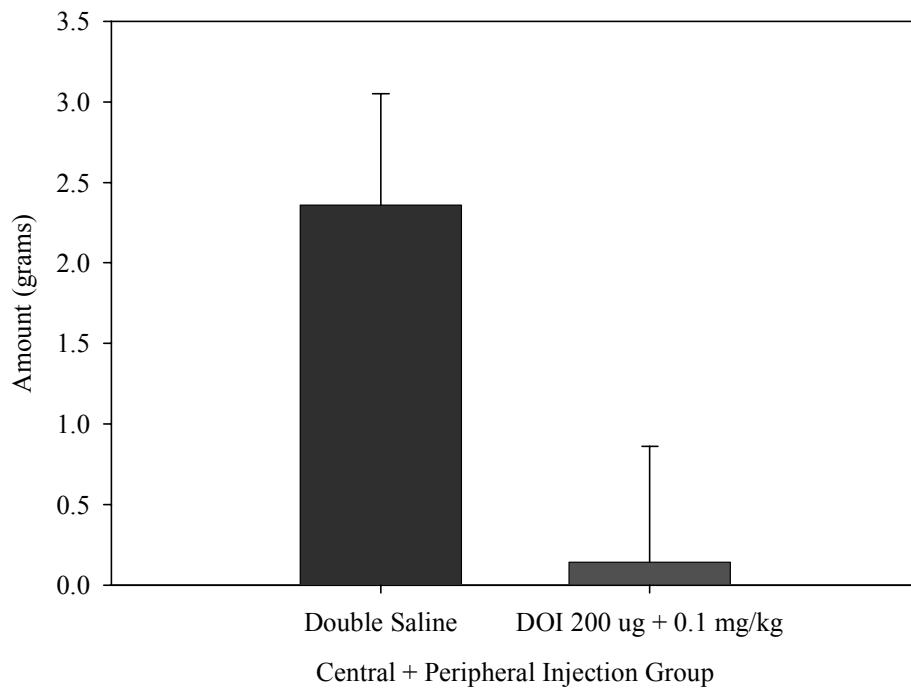


Figure 19: Gnawing after Central + Peripheral Injections of Saline or DOI

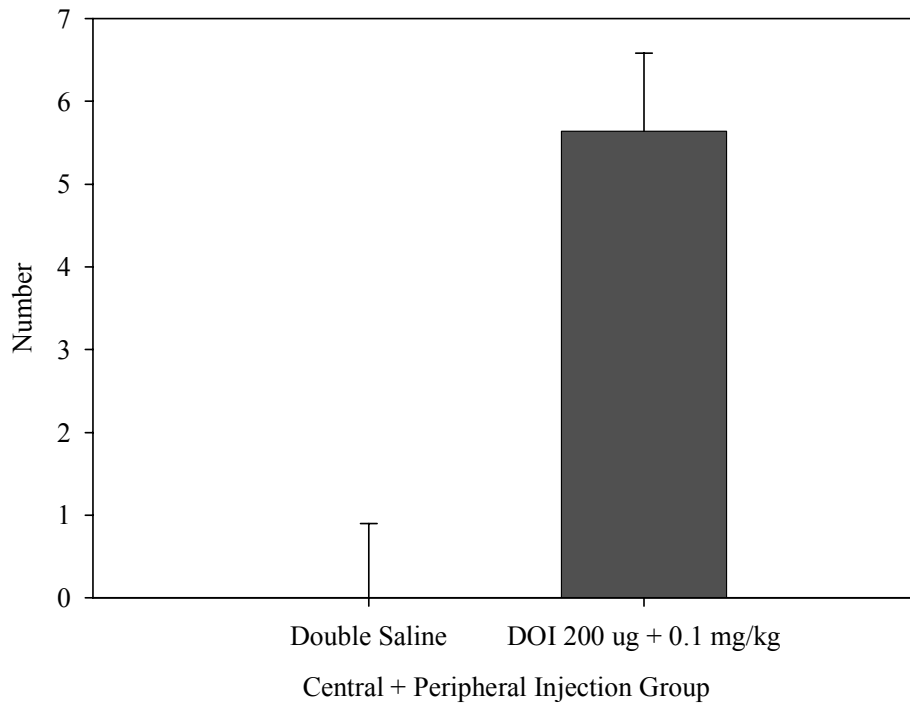


Figure 20: Headshakes after Central + Peripheral Injections of Saline or DOI

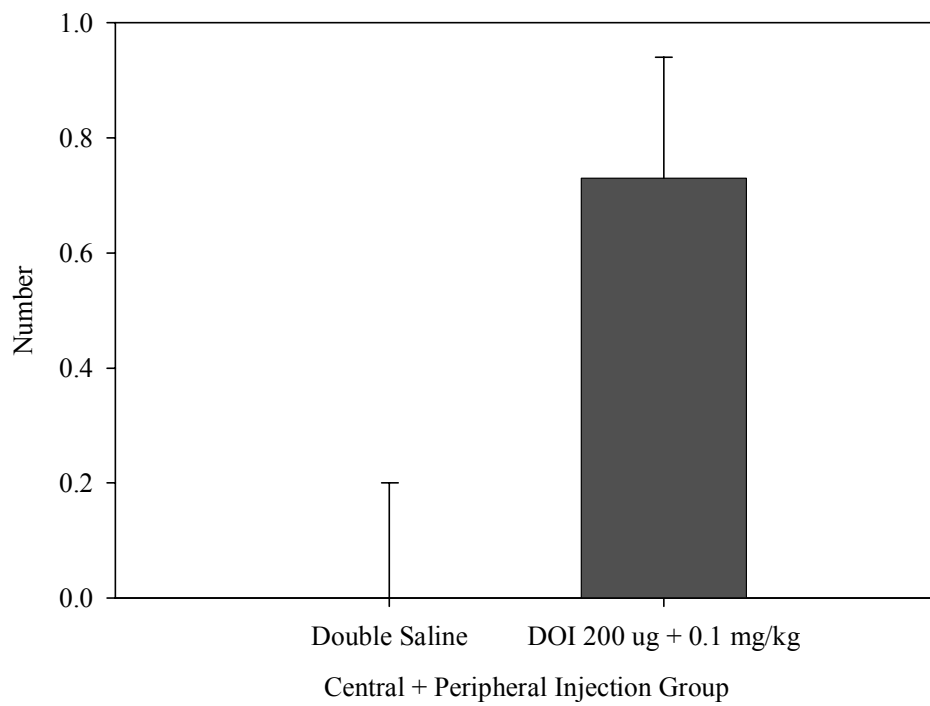


Figure 21: Wet dog shakes after Central + Peripheral Injections of Saline or DOI

Experiment 5: Peripheral DOI + Peripheral KET

Overall comparison of the behavior of animals that received one of six different combinations of double peripheral injections: saline, a low dose of DOI + saline, a high dose of DOI + saline, KET + saline, low dose of DOI + KET, or high dose of DOI + KET, resulted in a significant MANOVA [$F(65, 410) = 2.912, p < .001$]. Specifically, significant differences were observed between drug groups in three behavioral measures in the tail pinch condition: oral stereotypy with food [$F(5, 90) = 5.593, p < .001$], vocalizations [$F(5, 90) = 4.508, p = .001$], and eating [$F(5, 90) = 3.439, p = .007$]. The difference in the amount of time animals spent grooming also approached significance [$F(5, 90) = 2.213, p = .06$] in the tail pinch condition. In the open field, the amount of rearing [$F(5, 90) = 12.37, p < .001$], headshakes [$F(5, 90) = 16.983, p < .001$], and flattened body posture [$\chi^2(5) = 62.3, p < .001$] displayed by animals in the different drug groups was significantly altered in the open field condition.

Oral stereotypy with food is depicted below in Figure 22. Post hoc findings were in the predicted direction, with behavior evoked by tail pinch stress being significantly decreased by DOI compared to the saline control group (the high dose ($p = .003$) or the low dose ($p = .021$) of DOI + saline). This DOI effect was reversed by KET administration. Animals that received either KET + saline or KET + DOI (either dose) did not differ from the saline control group in the amount of time engaged in oral behavior directed at food. The group that received the low dose of DOI + KET engaged in significantly more oral stereotypy with food than the group that received the low dose + saline ($p = .001$). Similarly, the group administered the high dose of DOI + KET spent significantly more time with food than the group that was given the high dose of DOI + saline ($p = .043$).

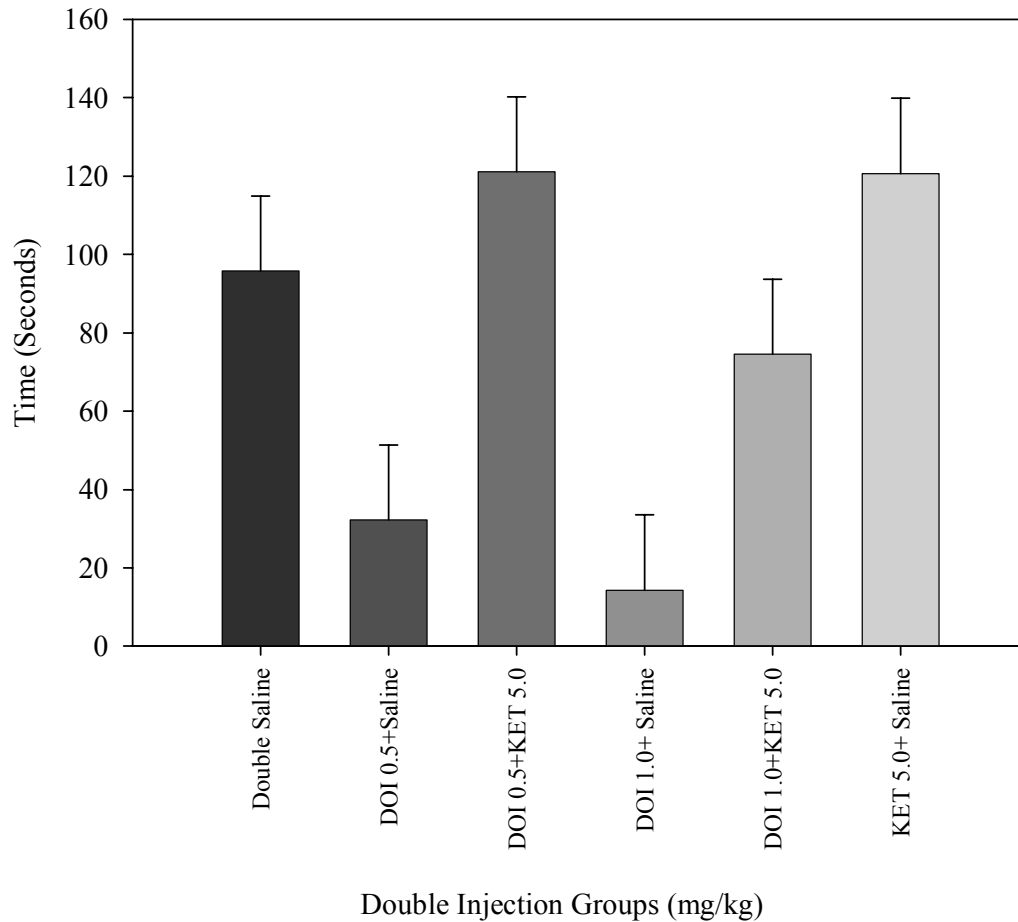


Figure 22: Oral Stereotopy with Food after Double Peripheral Injection.

The animals administered the low dose of DOI + saline spent less time engaged with food than those that received KET + saline ($p = .002$). The group that received the high dose of DOI + saline performed significantly less oral stereotopy with food than both the KET + saline group and the group administered the low dose of DOI + KET (both comparisons $p < .001$).

Eating is shown below in Figure 23. The groups that received DOI + saline ate significantly less during the tail pinch test than the saline control group (high ($p = .001$) and low ($p = .007$) doses). While the animals that were administered the low dose of DOI + KET did not eat significantly more than did the animals that received the low dose of DOI + saline, they (unlike the low dose of DOI + saline) also did not differ from the saline control, indirectly indicating a

reversal of the DOI effect. The group that was administered the high dose of DOI + KET ate less ($p = .027$) than the saline control group, suggesting that the ability of KET to reverse the effect of DOI on eating during tail pinch stress is dose-related.

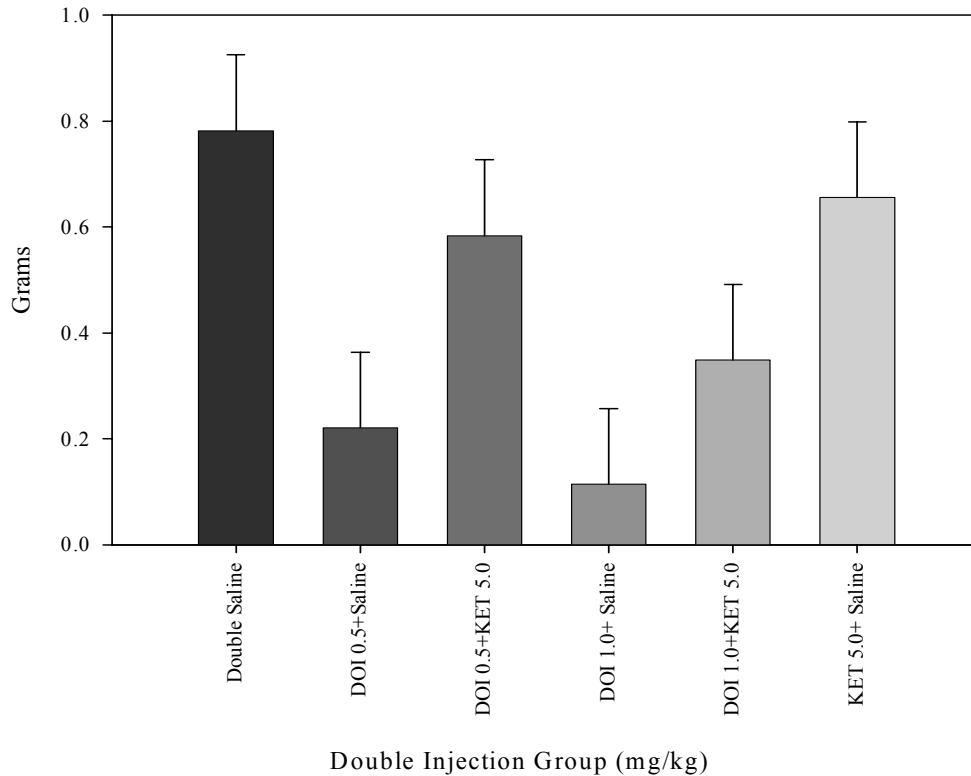


Figure 20: Eating after Double Peripheral Injection.

The group given the low dose of DOI + saline ate significantly less than the group given KET + saline ($p = .034$). The group administered the high dose of DOI + saline ate significantly less than both the low dose of DOI + KET ($p = .022$) and the KET + saline ($p = .009$) groups.

Figure 24 below shows data for Vocalizations. The two groups that received the low dose of DOI vocalized more than every other group with only one exception: low dose of DOI + KET compared to the high dose of DOI + KET. Specifically, the groups given the low dose of DOI + saline and low dose of DOI + KET vocalized more than the saline control ($p = .002$ and $p = .023$, respectively), the group administered the high dose of DOI + saline ($p < .001$ and $p =$

.003), and the group that was administered KET + saline ($p = .002$ and $p = .024$). The group that received the low dose of DOI + saline also vocalized significantly more than the group that received the high dose of DOI + KET ($p = .049$). A near significant increase in vocalizations was observed for the group that received the high dose of DOI + KET compared to the high dose of DOI + saline ($p = .065$)

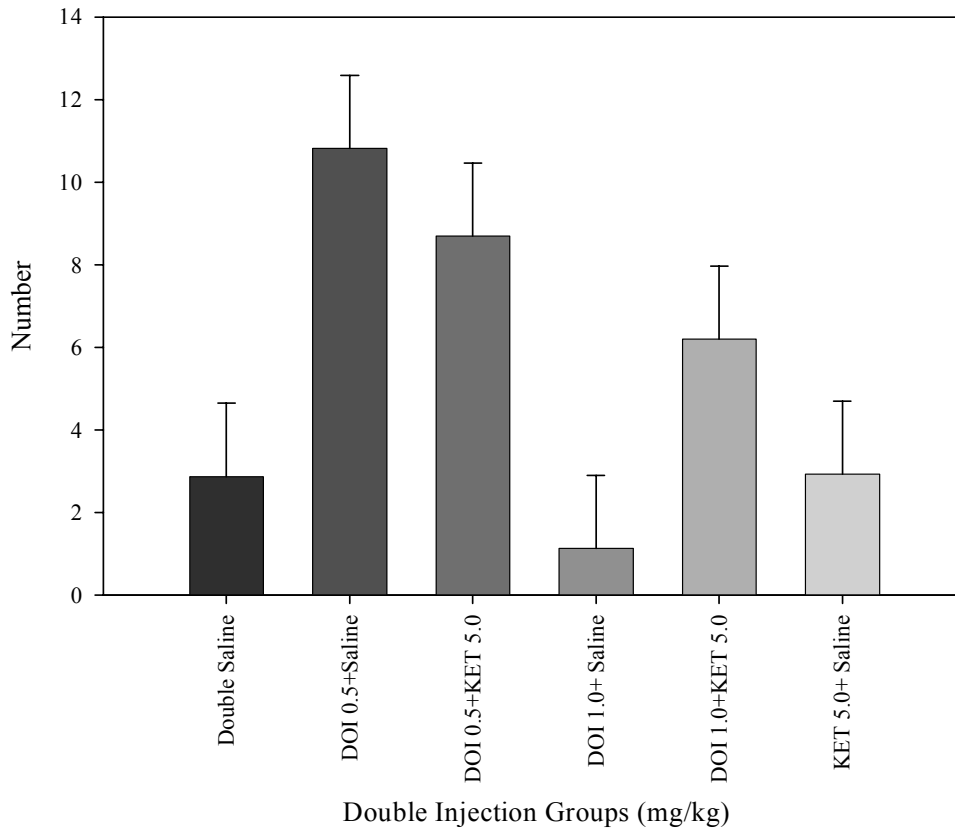


Figure 24: Vocalizations after Double Peripheral Injection.

Rearing is shown below in Figure 25. The groups that received either the high ($p < .001$) or the low ($p = .001$) dose of DOI + saline performed significantly less rearing than the saline control group in the open field. All three KET groups performed significantly more rearing than the groups that received the high or the low doses of DOI + saline (all comparisons at or below $p = .001$).

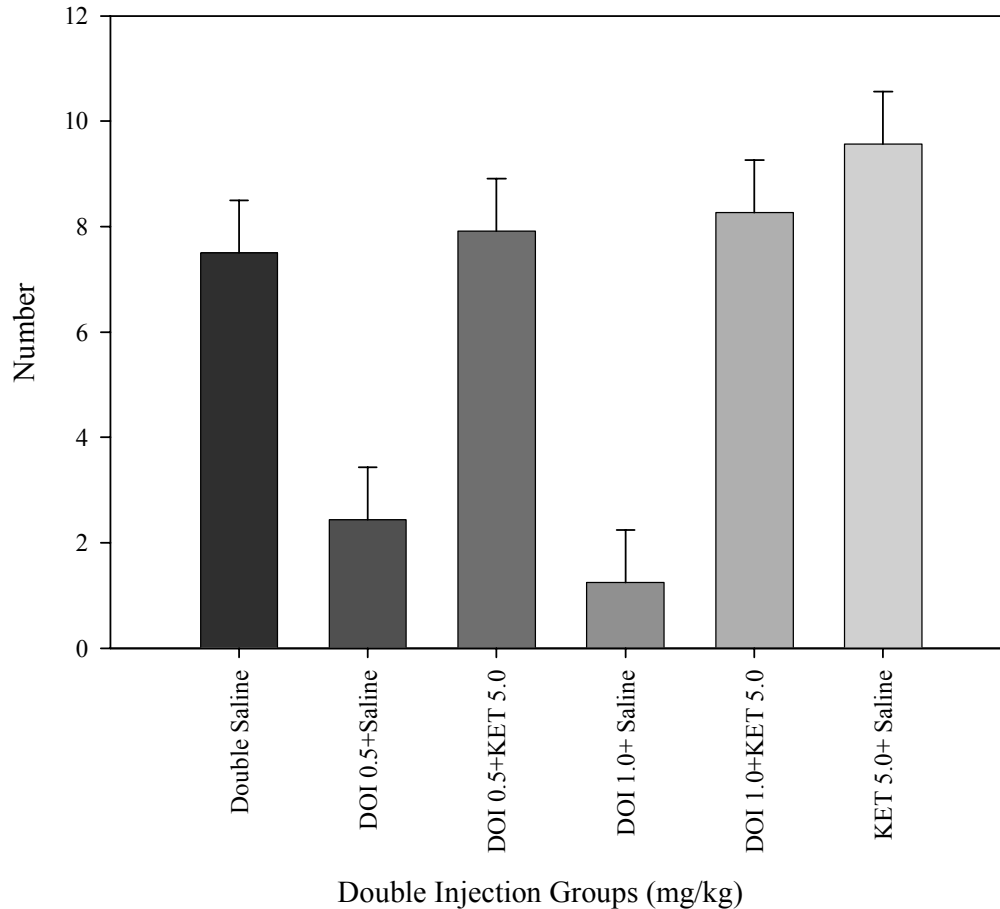


Figure 25: Rearing after Double Peripheral Injection.

Head Shakes (Figure 26) and Flat body posture (Figure 27) are shown below. The groups that received either the high or the low dose of DOI + saline performed significantly more headshakes than the saline control and all three groups that were administered KET, with all differences at or below $p = .001$. Flat body posture was observed in a significantly higher proportion of animals that received DOI than those that received saline or KET, with 100% of animals in the group that received the high dose of DOI + saline and 50% in the group that received the low dose of DOI + saline exhibiting flattened body posture. Only 6.25% of animals that received either dose of DOI + KET or saline, and 0% of animals that received KET + saline exhibited the flattened posture.

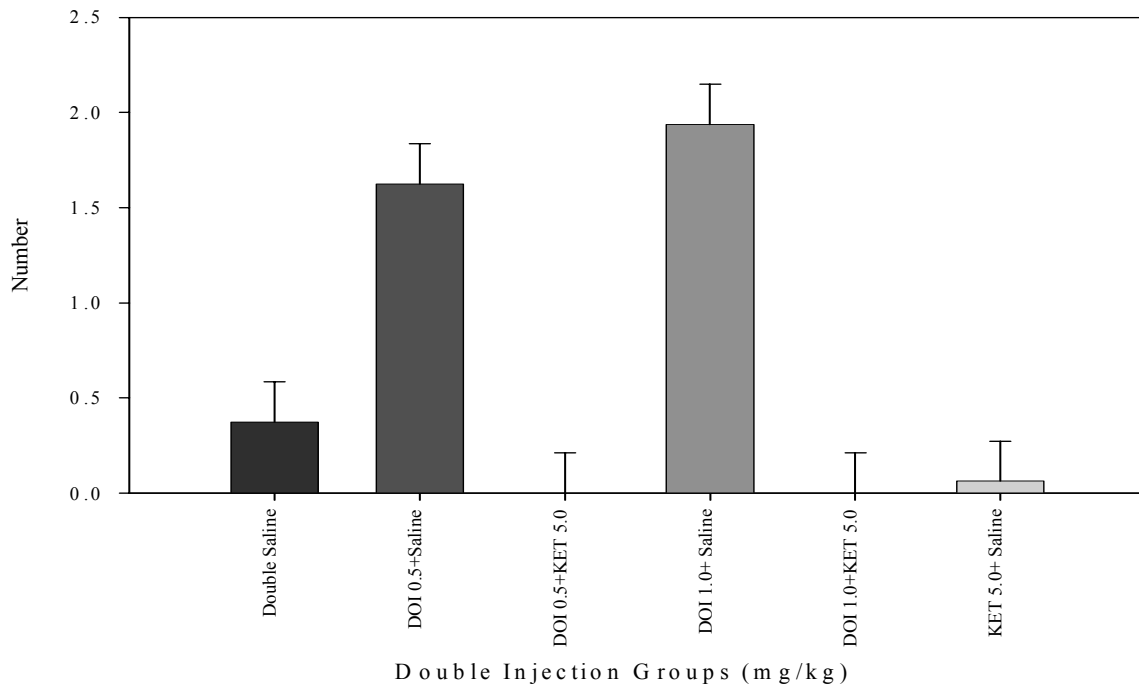


Figure 26: Headshakes after Double Peripheral Injection.

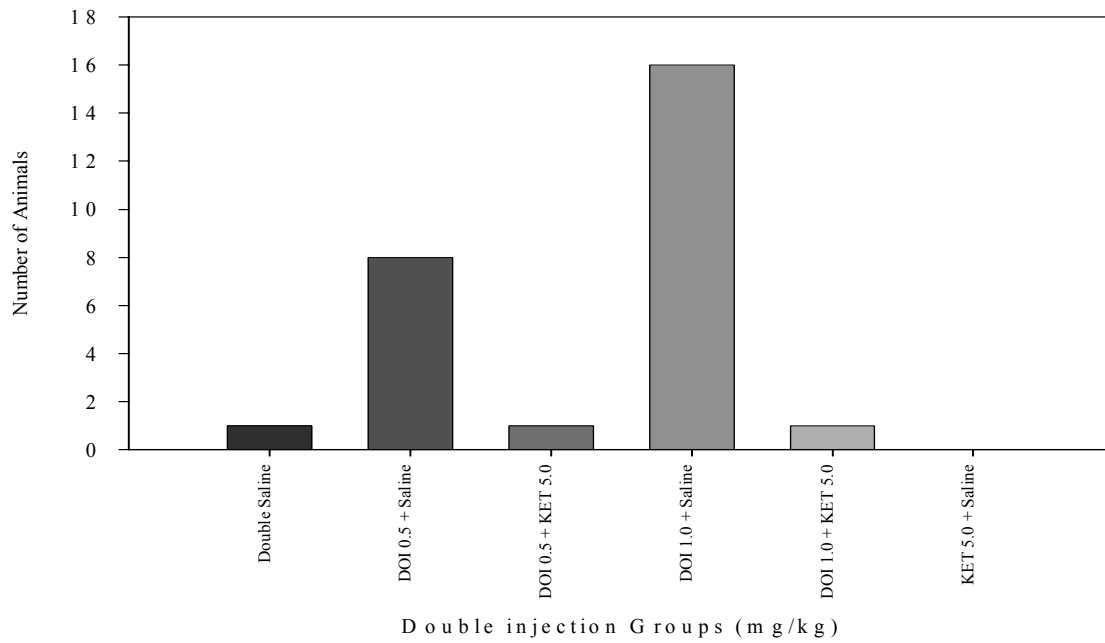


Figure 27: Flat Body Posture after Double Peripheral Injection

DISCUSSION

In general, the results of the five experiments supported the original hypotheses, showing a decrease in behavioral responding to stress with administration of the 5-HT_{2A/C} agonist DOI and a reversal of this decrease with administration of the 5-HT_{2A/C} antagonist KET (see Table 3 below). Administration of DOI resulted in significantly decreased behavioral responding to tail pinch stress in all five experiments, regardless of route of administration. Peripheral injection of DOI resulted in the maximal behavioral change (i.e., the highest number of dependent variables with significant behavioral reductions) in the tail pinch condition. Central + peripheral injection also resulted in significant reduction in several behaviors, but central injection alone reduced responding in only one behavior in the tail pinch test. Behavioral responding to tail pinch stress following peripheral injection of KET was increased compared to animals that were administered DOI and in some cases increased compared to the saline control. There was only one case in which behavior evoked by tail pinch stress was significantly decreased following administration of KET compared to saline. As hypothesized, concomitant peripheral administration of KET and DOI resulted in a reversal of the decrease in stress-evoked behaviors seen with administration of DOI alone that is, the return of behavioral responding to tail pinch stress significantly higher than the DOI groups and/or to a level equal to the saline control group.

DOI

The pattern of behaviors observed in the tail pinch test suggests that activation of 5-HT₂ systems centrally and/or peripherally results in an anxiolytic effect. This suggestion is similar to other findings in the literature. Compared to the saline control group, peripheral injection of DOI in both the single and double injection conditions (i.e., Experiments 1 & 5) resulted in significant dose-related reductions in oral behavior directed at food, the amount of food gnawed, as well as

Table 3: Summary of significant findings for Experiments 1-5.

Dependent Variables	Exp1 Peripheral DOI	Exp 2 Central DOI	Exp 3 Central + Peripheral DOI	Exp 4 Peripheral KET	Exp 5 Peripheral KET + DOI
Tail Pinch					
OS with food	DOI decreased compared to saline		DOI decreased compared to saline	KET increased compared to saline	KET reversed DOI effect
Eating			DOI decreased compared to saline		KET reversed DOI effect
Gnawing	DOI decreased compared to saline		DOI decreased compared to saline		
OS without food	DOI decreased compared to saline			KET <i>decreased*</i> compared to saline	
Grooming					
Vocalizations	DOI decreased compared to saline	DOI decreased compared to saline		KET increased compared to saline	KET reversed DOI effect
Fecal boli					
Open Field					
Rearing	DOI <i>decreased*</i> compared to saline			KET decreased compared to saline	KET reversed DOI effect
Line crossing					
Freezing				KET increased compared to saline	
Headshakes	DOI increased compared to saline	DOI increased compared to saline	DOI increased compared to saline		KET reversed DOI effect
Wet dog shakes	DOI increased compared to saline	DOI increased compared to saline	DOI increased compared to saline		
Flat body posture	DOI increased compared to saline	DOI increased compared to saline			KET reversed DOI effect

* indicates a significant finding in the unexpected (opposite of hypothesized) direction.

oral behavior not directed at food or self (cage licking, vacuous chewing, teeth chattering), and vocalizations in animals exposed to tail pinch stress. However, animals that were administered DOI centrally behaved differently from the saline group on just one variable: vocalizations. Simultaneous administration of central and peripheral injections of DOI resulted in significant behavioral reduction similar to those seen with peripheral injection alone – reduction was observed in oral stereotypy with food (due to both eating and gnawing), though the reduction in non-food directed oral behavior and vocalization seen in the peripherally injected animals was absent from the central + peripheral results. In no instance was behavior produced following any of the three modes of administration (peripheral, central, or central + peripheral) that indicated increased reaction to tail pinch stress. These findings are similar to previous reports, indicating that administration of DOI leads to reduced behavioral responding to stress, indicative of an anxiolytic response. Several studies found that DOI decreased escape behavior (Nic Dhonnchadha, Hascoet, Jolliet, & Bourin, 2003; Nic Dhonnchadha, Bourin, & Hascoet, 2003; de Paula Soares & Zangrossi, Jr., 2004) and increased exploratory behaviors (Peng et al., 2004) in the elevated plus maze. Others have reported similar results with a foot shock stressor, where both adult and young rats exhibited reduced ultrasonic vocalization following administration of DOI (De Vry, Benz, Schreiber, & Traber, 1993; Sanchez, 1993; Schreiber, Melon, & De Vry, 1998; Winslow & Insel, 1991). Njung'e and Handley (1991) found that DOI reduced marble burying, a behavioral measure of anxiety, and Nic Dhonnchadha et al. (2003b) reported DOI to be anxiolytic in the four plates test in mice.

The possibility that the reductions in responding observed in the current experiments are due to a nonspecific motor impairment preventing a response, rather than a decrease due to an actual anxiolytic action of DOI, is made less likely by the fact that administration of DOI did not

significantly affect grooming (in the tail pinch test), locomotion (as measured by line crosses in the open field test), or rotarod performance (test of motor function exclusively) at any of the doses or modes of administration tested. This finding confirms previous work (Raghavendra & Kulkarni, 2000b; Redrobe et al., 1997). However, increases (Darmani, Shaddy, & Gerdes, 1996; Granoff & Ashby, Jr., 1998) and decreases (Kaur & Ahlenius, 2000; Krebs-Thomson & Geyer, 1996) in locomotion following administration of DOI have also been reported.

The effect of DOI on stress responding to the open field stressor was consistent across all three modes of administration, with one exception: in both Experiments 1 and 5, peripherally injected DOI produced a decrease in rearing compared to the saline control, while neither of the other two modes of administration produced this effect. This behavioral decrease, indicative of an anxiogenic action of DOI, has been reported previously (Kaur et al., 2000), but in this case appears to be due to differences between the saline control groups rather than differences in behavioral responding to DOI. Rearing was substantially increased in the two peripheral saline control conditions compared to central and central + peripheral saline injections (Figure 28 below).

Rearing observed following peripheral administration of any dose of DOI was comparable to that observed after both central and central + peripheral injections. Conclusions about whether these differences in responding between the control groups are due to random variation or to differences in the injection protocols would require further investigation.

The animals that received the middle or high doses of peripherally injected DOI, the high dose of centrally injected DOI, and the central + peripheral injection of DOI consistently displayed significantly more head and wet dog shakes than the saline control group. Additionally, flat body posture was observed following peripheral and central injection of DOI. These behaviors are

known to be mediated by 5-HT₂ receptors (Heslop & Curzon, 1999; Takao et al., 1995),

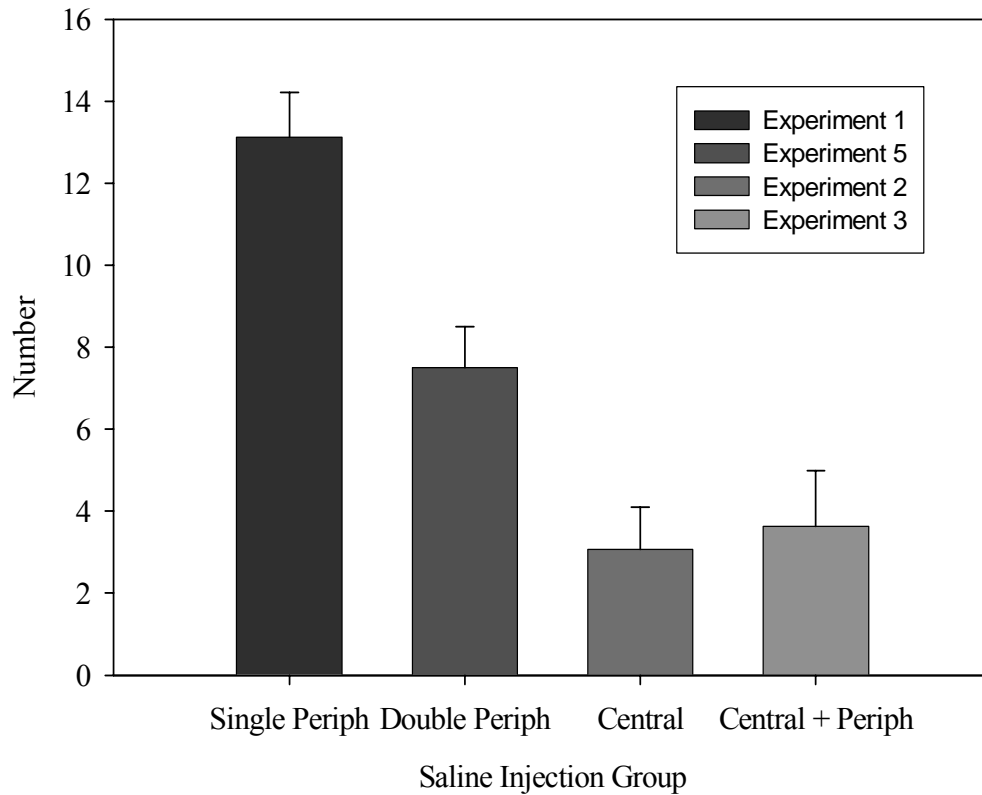


Figure 28: Difference in Rearing in Control Animals by Injection Group

indicating that the drug was still in effect at the time of the open field test (Benjamin, Knapp, & Pohorecky, 1993). While head and body shakes are usually thought of as a pharmacological effect, exposure to stress has been reported to influence the number of shakes elicited by DOI, with both increases (Brotto, Gorzalka, & Hanson, 1998; Chaouloff, Baudrie, & Coupry, 1994; Takao et al., 1995) and decreases (Izumi et al., 2002; Pericic, 2003; Yamada, Nankai, & Toru, 1993; Yamada, Watanabe, Nankai, & Toru, 1995) in shakes reported when animals were exposed to metabolic, restraint, swim, foot shock or tail pinch stressors prior to administration of DOI. These reports suggest the possibility that the number of head and body shakes observed in the open field could have been influenced by the animals' prior exposure to the tail pinch stressor. The lack of any

such effect on the saline control group, however, argues against this possible influence. Others (Van Oekelen, Megens, Meert, Luyten, & Leysen, 2003) have reported increased headshakes following cranial surgery, purportedly as a result of damage to the blood brain barrier. This finding was not replicated in the control conditions for the current Experiment 2 or 3, in which cranial surgery was performed.

The lack of significant change compared to the saline control in the number of line crosses and freezing following any injection of DOI suggests that, in contrast to the tail pinch stressor, stimulating 5-HT_{2A/C} receptors has little effect on behavioral responding to an open field stressor.

Ketanserin

Increased behavioral responding compared to saline-injected animals was seen in two variables in the tail pinch condition following KET injection, vocalizations and oral stereotopy with food, but this increase was not linearly related to dose. Increasing the dose of KET resulted in a U-shaped dose-response (i.e., middle dose resulted in biggest reduction in behavior) in the case of oral stereotopy with food, and an *inverted* U-shaped dose response (i.e., middle dose resulted in biggest increase in behavior) for the number of vocalizations emitted. This is in contrast to the findings following administration of DOI, where the level of responding evoked by tail pinch stress on these two dependent measures was consistently reduced as a function of increasing dose (i.e., highest dose resulted in the least amount of responding). In the open field, the time spent engaged in freezing behavior was significantly increased compared to saline control for animals that received the high dose of KET. While this effect was not replicated at a statistically significant level in the open field test for Experiment 5 (in which one group of animals received the high dose of KET + an injection of saline), those animals that received KET + saline did spend more time freezing than those that received saline (an approximately five-fold increase;

see Table 7 in the Appendix). Behavioral increases such as these following the administration of KET have been reported previously with exposure to stressors such as the light/dark paradigm and elevated plus maze (Zangrossi & Graeff, 1994; Nic Dhonnchadha et al., 2003).

Administration of KET also resulted in *decreased* behavioral responding compared to the saline control in the tail pinch condition for oral stereotopy without food (all doses), as well as significantly decreasing rearing behavior in the open field (all doses). Like the DOI results described above, rotarod performance was unaffected, suggesting that these reductions in behavioral responding to stress are not attributable to a general motor effect of KET. A few previous reports have indicated that administration of KET can result in an anxiolytic effect on behavioral responding in animals exposed to stress. For example, published reports indicate that administration of KET reduced freezing behavior during conditioned fear stress in rats (Ishida-Tokuda et al., 1996), increased the threshold of aversive brain stimulation (in the periaqueductal gray) for eliciting escape behavior (Jenck, Broekkamp, & Van Delft, 1989), and reversed the hypophagic effect of restraint stress when injected into the periventricular nucleus of the hypothalamus (Grignaschi et al., 1993). However, no such reports have been published for tail pinch or open field stressors, and other studies have only found anxiolytic effects with KET under very particular circumstances. For example, Diaz-Veliz et al. (1997) found increased behavior consistent with an anxiolytic action of KET in the elevated plus maze (increased entries and exploration of open arms), but only in diestrous female rats; the effect was absent in males, ovariectomized females, and females in other stages of the estrous cycle. Da Rocha, Jr., Puech, and Thiebot (1997) reported a decrease in immobilization following swim stress in mice, but this effect was found only following the administration of an atypically large (32 mg/kg) dose of KET.

No differences were found on eight of the 13 variables measured in both the tail pinch and open field tests. This absence of effect of KET on behavior has also been previously reported following exposure to other stressors: e.g., it had no effect on behavior evoked by the elevated T-maze (de Paula Soares & Zangrossi, 2004), the light/dark paradigm, social interaction (Costall & Naylor, 1995), or conditioned fear stress (Inoue, Tsuchiya, & Koyama, 1996).

Variations in Reported findings with 5-HT_{2A/C} Agonists and Antagonists

It is well known that stimulation or blockade of 5-HT_{2A/C} receptors may lead to opposite effects on behavior depending upon the type of stressor employed (Millan, 2003). However, as reviewed above, administration of agents that manipulate activity at 5-HT_{2A/C} receptors has also resulted in variable findings in studies employing the same stressor. While more research is clearly needed to explain these differences, variations in methodology may explain at least some of the reported differences. The use of different doses of DOI or KET is one such variation – doses range widely from report to report and, as described above in the *5-HT as anxiolytic* section, at least one study has found decreased behavioral responsiveness to stress with low doses of DOI and increased behavioral responsiveness to stress following administration of high doses of DOI (Onaivi et al., 1995). Varying the route of administration has also been shown to have different effects on behavior; for example, Larson and Kondzielski (1982) found that 5-HT injected peripherally reduces gnawing behavior in response to a tail pinch stressor, but produces gnawing when injected intrathecally. Another possible methodological explanation for the inconsistent results between reports could be the length or chronicity of the stressor to which the animals were exposed. One example where variation in length of exposure to a stressor is common is restraint stress. Studies of 5-HT activity employing restraint stress have reported exposure to the stressor varying from five minutes (e.g., Uphouse et al., 2003; Saphier et al., 1995) to up to two hours

(e.g., Whitton, Sarna, Datla, & Curzon, 1991; Kennett, Chaouloff, Marcou, & Curzon, 1986). Conditioned fear stress requires, by definition, repeated exposure to the stressor – it is possible that this repeated exposure causes some enduring physiological change that alters the drug effect at the time of testing or that the number of exposures vary between studies, and that this variation has some measurable impact on responding following drug administration. While there are currently no reports of direct manipulations of stressor chronicity or length of single exposure relating to 5-HT activity and behavioral responding to stress, the number of 5-HT₂ receptors increases following exposure to a chronic, but not an acute restraint stressor (Takao et al., 1995). This provides indirect evidence of a possible mechanism by which differences in characteristics of the stressor may result in contradictory findings.

In addition to the methodological differences suggested above, some property of the 5-HT_{2A/C} receptor itself may be responsible for these contradictory reports. For instance, variations in responsiveness at different points in the circadian cycle could be responsible for the differences observed in behavioral responding to stress. Studies employing the same stressor, route of administration, and dose of DOI or KET, but reporting contradictory results, may have tested the animals at different times of day. Nagayama and Lu (1996) conducted a between-group study in which rats were injected peripherally with DOI at four-hour intervals throughout the day. Wet dog shakes were observed at a significantly higher rate in the early morning (0400) than in the afternoon (1600), an effect replicated with central injection of DOI as well. This behavioral measure was made in the absence of a stressor; however, if baseline behavioral responding is affected by circadian rhythm, it is possible that behaviors evoked by stressors could be affected by the same rhythms. Unfortunately, many studies fail to report the time of day testing was performed, making the possible influence of circadian cycles difficult to determine.

DOI + Ketanserin

KET systematically reversed the behavioral reductions observed with administration of DOI in the tail pinch condition and open field, as hypothesized. Significant blockade of the DOI effect was observed in the measurement of oral stereotopy with food, eating, and vocalizations in the tail pinch condition, as well as rearing, headshakes, and flat body posture in the open field. These results provide strong evidence that the changes in behavioral responding observed following injection of DOI are, in fact, due to activation of the 5-HT_{2A/C} receptors.

While this is the first report describing the reversal by KET of the effect of DOI on behaviors evoked by tail pinch and open field stressors, reversal of decreased stress responding has been reported previously in different stress paradigms following injection of DOI as well as other 5-HT₂ agonists. Schreiber et al (1998) reported that KET blocked the anxiolytic effects of DOI in the ultrasonic vocalization test. KET reversed the anxiolytic effect of aniracetam in the social interaction test with mice and reversed headshakes induced by benzodiazepine injection (Nakamura & Kurasawa, 2001; Tadano et al., 2001). De Paula Soares and Zangrossi (2004) found that decreased escape behaviors in the elevated T-maze were reversed by KET injection, and Graeff, Brandao, Audi, and Schutz (1986) reported that the anti-aversive effect of 5-HT injection in the dorsal periaqueductal gray was blocked by KET. Like the findings from Experiment 5, these reports also seem to suggest that administration of KET consistently reverses the behavioral decreases caused by 5-HT₂ agonists administered in the presence of a stressor.

However, when injected alone in Experiment 4, KET did significantly increase behavioral responding on two of these measures in the tail pinch test: oral stereotopy with food and vocalizations. These observed increases create the possibility that KET could be acting indirectly, through some competing process, to elevate behavioral responding rather than directly blocking

or reversing the DOI effect. While possible, this scenario seems unlikely considering the striking, complete reversal of the pharmacological effect of DOI on headshakes and flat body posture in the open field, behaviors thought to result exclusively from activity of the 5-HT_{2A/C} receptors.

Central vs. Peripheral Site of Action

The question of whether the observed behavioral changes resulted from manipulation of central or peripheral 5-HT_{2A/C} receptors is not easily determined from the results of this series of experiments. The consistent effect of DOI on head and wet dog shakes, behaviors known to be centrally mediated, following central and central + peripheral modes of administration, argues for a central site of action, and has been suggested by others (e.g., Dey, 1994; Nankai, Yamada, Muneoka, & Toru, 1995). On the other hand, the lack of effect of DOI following central injection on four of the eight behavioral variables that were significantly affected by peripheral injection would suggest a peripheral site of action. It is unlikely that the failure of centrally administered DOI to significantly affect these oral behaviors in the tail pinch condition was due to an insufficient concentration of DOI, as the 200 µg dose employed represents the maximum solubility of the drug in water (10 µg/µl). It is notable, however, that while not *significantly* reduced by central DOI injections in the tail pinch condition, the data suggested a dose-related trend toward reduction in the amount of time animals engaged in oral behavior directed at food, the amount of food gnawed, and oral behavior not directed at food or self (see Table 5 in the Appendix). Additionally, the low peripheral dose and the high central dose, neither of which produced an effect that was significantly different from saline when injected alone, did produce significant reductions in the time animals engaged with food, the amount eaten, and the amount gnawed during tail pinch stress when combined. The fact that these reductions in behavior were similar to those seen with the bigger peripheral doses of DOI suggests that the effect of DOI

could be both centrally and peripherally mediated. This suggestion is also supported by the results of Experiment 5, which showed reversal of the DOI effect by peripheral administration of KET. Previous reports provide strong evidence that manipulation of central 5-HT activity is likely following peripheral administration of 5-HT agents, including DOI specifically (e.g., Amer, Breu, McDermott, Wurtman, & Maher, 2004; Chaouloff, 1993).

It has been reported that 5-HT ligands injected into specific brain sites (rather than the lateral ventricles as in Experiment 2) result in a more pronounced effect on behavior (McCall & Clement, 1994). As discussed above (sections 5-HT Involvement in Stress Responding: CNS pathways, and 5-HT as anxiolytic), Graeff and colleagues have suggested that increased 5-HT activity in the ascending dorsal raphe pathway innervating the periaqueductal gray, the tectum of the midbrain, and the amygdala, inhibits panic reactions (Zangrossi, Jr. et al., 2001), and that this effect is blocked by central administration of KET (Brandao, Lopez-Garcia, Graeff, & Roberts, 1991). The possibility that DOI or KET injected into these areas might alter behaviors evoked by tail pinch and open field stress remains to be investigated.

Limitations

Power

The number of animals included in each group represents the final number of animals included in analysis following histology. MANOVA requires more cases per cell than dependent variables being measured in the design to be sufficiently powered (Tabachnick & Fidell, 2001). Following this rule, more animals than dependent variables were entered into each drug group for each individual experiment, however, due to attrition from improper ICV implantation, tail damage, or infection of peripheral injection site, final n for the experiments was, in some cases, lower.

In order to evaluate the effect reduced n may have had on the analyses performed, several steps were taken post hoc to investigate whether or not the design employed was sufficiently powered to test the hypotheses. First, estimates of observed power for all univariate tests were calculated. These values ranged from 0.448 to 1.00 for Experiments 1 & 4 (with four of the dependent variables with observed power $<.80$), from .069 to 1.00 for Experiment 2 (with 11 of the dependent variables with observed power $<.80$), from .051 to .985 for Experiment 3 (with ten of the dependent variables with observed power $<.80$), and from 0.256 to 1.00 for Experiment 5 (with seven of the dependent variables with observed power $<.80$). Eighty percent power is generally thought of as the cutoff for an acceptable level of power in statistical tests; that several of the univariate tests in each experiment fell below 80% observed power indicates an insufficient sample size.

Second, a commonly used table of sample sizes was consulted (Hinkle, Wiersma, & Jurs, 1998). This table allows for determination of sample size based on power, effect size, and number of treatment levels. According to this table, 203 animals would be required for Experiments 1 and 4 (actual number of animals tested = 79), 92 for Experiment 2 (actual = 55), 34 for Experiment 3 (actual = 23), and 163 for Experiment 5 (actual = 96) to achieve 80% power to detect a mid-range effect on behavior in these experiments.

Third, a trial and error method was employed to test what effect doubling the sample size in each experiment would have had on the outcome. In Experiments 1, 4, and 5, doubling the sample size resulted in every dependent variable measured gaining significance. Results from Experiments 2 and 3, however, required a quadrupled sample size to approximate the results in the other three experiments. Taken together, these findings indicate that the results of this experiment are limited by reduced power due to insufficient sample size. However, the fact that

several variables were found to be significantly different between the drug groups, most notably in the least powered test (i.e., for Experiments 1 & 4), speaks indirectly to the strength of the effects reported.

Conclusions

This is the first report of the influence on behaviors evoked by tail pinch or open field stress of centrally and peripherally administered DOI, and the reversal of that influence by the 5-HT_{2A/C} antagonist ketanserin. Despite the limitation of low power, the results from this series of experiments support the hypothesis that stimulating 5-HT_{2A/C} receptors results in an anxiolytic effect, reducing behaviors evoked by tail pinch stress (specifically oral behavior directed at food), and that peripherally administered KET reverses these effects. While these findings also suggest that DOI has little effect on behaviors elicited by the open field test in rats, the analysis of power and hypothetical manipulation of sample size described above indicate that this lack of effect could be due to insufficient sample size. While the question of whether this effect is centrally or peripherally mediated is unresolved, current theory would predict a central site of action, with brain areas that contain projections from the dorsal raphe, such as the periaqueductal gray, being important to the control of 5-HT on behavioral responding to a tail pinch stressor.

Future Directions

Given the results of these experiments, there are three logical follow up studies that should be conducted. First, replicating these experiments with a larger sample size will help clarify the full impact of DOI on behavioral responding during exposure to the tail pinch and open field stressors, and the ability of KET to reverse that effect. Second, to help determine whether this is a centrally or peripherally mediated effect, a central manipulation in which DOI is administered peripherally and KET is administered centrally should be conducted. If the results from this

second study were to argue for a central site of action, a third study in which measurement of behavioral responding to stress following administration of DOI to specific brain areas (e.g., periaqueductal gray and/or amygdala) would be appropriate. The data yielded from such an investigation would determine whether the results from the peripheral studies performed in the current report can be replicated with site-specific central injections, as opposed to the ICV central injections employed in Experiment 2. Building on the knowledge established in the current report, these suggested manipulations would help further elucidate the role of the 5-HT_{2A/C} receptors in behavioral responding to stress.

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APPENDIX: NON-SIGNIFICANT RESULTS

Table 4: Non-significant findings from Experiments 1 & 4: Peripheral Injection.

Dependent Variable	Group	Mean (S.E.)	Degrees of Freedom	F	Probability Value (p)
Grooming	Saline	23.80 (5.93)	6, 72	1.831	.105
	DOI 0.1 mg/kg	13.61 (7.66)			
	DOI 0.5 mg/kg	8.05 (4.69)			
	DOI 1.0 mg/kg	9.02 (6.63)			
	KET 0.5 mg/kg	20.42 (6.93)			
	KET 2.5 mg/kg	14.67 (7.26)			
	KET 5.0 mg/kg	31.01 (7.26)			
Boli in tail pinch	Saline	1.07 (.28)	6, 72	1.400	.226
	DOI 0.1 mg/kg	.89 (.36)			
	DOI 0.5 mg/kg	.54 (.22)			
	DOI 1.0 mg/kg	.000 (.31)			
	KET 0.5 mg/kg	.82 (.32)			
	KET 2.5 mg/kg	.70 (.34)			
	KET 5.0 mg/kg	.20 (.34)			

Dependent Variable	Group	Mean (S.E.)	Degrees of Freedom	F	Probability Value (p)
Eating	Saline	.31 (.22)	6, 72	1.213	.309
	DOI 0.1 mg/kg	.14 (.29)			
	DOI 0.5 mg/kg	.12 (.17)			
	DOI 1.0 mg/kg	.02 (.25)			
	KET 0.5 mg/kg	.80 (.26)			
	KET 2.5 mg/kg	.51 (.27)			
	KET 5.0 mg/kg	.71 (.27)			
Line crosses	Saline	16.00 (1.77)	6, 72	1.448	.209
	DOI 0.1 mg/kg	14.22 (2.28)			
	DOI 0.5 mg/kg	18.75 (1.40)			
	DOI 1.0 mg/kg	15.58 (1.98)			
	KET 0.5 mg/kg	15.18 (2.06)			
	KET 2.5 mg/kg	16.20 (2.17)			
	KET 5.0 mg/kg	11.80 (2.17)			
Rotarod	Saline	26.64 (1.88)	6, 69	.586	.74
	DOI 0.1 mg/kg	28.06 (2.34)			
	DOI 0.5 mg/kg	28.06 (2.12)			
	DOI 1.0 mg/kg	27.70 (2.12)			
	KET 0.5 mg/kg	23.78 (2.12)			
	KET 2.5 mg/kg	27.31 (2.22)			
	KET 5.0 mg/kg	25.01 (2.22)			

Table 5: Non-significant findings from Experiment 2: Central Injection.

Dependent Variable	Group	Mean (S.E.)	Degrees of Freedom	F	Probability Value (p)
Oral stereotypy with food	Saline	53.77 (17.88)	3, 50	.227	.877
	DOI 20µg	43.96 (15.28)			
	DOI 100µg	37.43 (17.47)			
	DOI 200µg	38.63 (13.93)			
Oral stereotypy without food	Saline	49.77 (11.46)	3, 50	.829	.484
	DOI 20µg	54.31 (14.95)			
	DOI 100µg	35.10 (10.26)			
	DOI 200µg	25.91 (7.15)			
Grooming	Saline	3.28 (1.67)	3, 50	1.779	.163
	DOI 20µg	7.34 (2.34)			
	DOI 100µg	3.41 (1.63)			
	DOI 200µg	1.94 (1.14)			
Boli in tail pinch	Saline	.86 (.49)	3, 50	.641	.592
	DOI 20µg	1.54 (.50)			
	DOI 100µg	1.29 (.44)			
	DOI 200µg	1.64 (.45)			
Eating	Saline	.12 (.04)	3, 50	.111	.953
	DOI 20µg	.10 (.03)			
	DOI 100µg	.10 (.05)			
	DOI 200µg	.10 (.03)			
Gnawing	Saline	5.14 (2.12)	3, 50	1.32	.278
	DOI 20µg	2.04 (.93)			
	DOI 100µg	1.72 (1.03)			
	DOI 200µg	2.56 (.88)			
Linecrosses	Saline	7.71 (1.88)	3, 50	1.821	.155
	DOI 20µg	12.62 (2.64)			
	DOI 100µg	8.57 (2.07)			
	DOI 200µg	12.64 (2.13)			
Rears	Saline	3.07 (.79)	3, 50	.964	.417
	DOI 20µg	4.31 (.99)			
	DOI 100µg	1.86 (.60)			
	DOI 200µg	3.36 (1.51)			
Freezing	Saline	35.09 (9.54)	3, 50	2.161	.104
	DOI 20µg	18.97 (10.39)			
	DOI 100µg	57.51 (7.61)			
	DOI 200µg	19.90 (19.12)			
Rotarod	Saline	26.04 (2.20)	3, 39	.868	.466
	DOI 20µg	26.85 (2.20)			
	DOI 100µg	27.02 (2.43)			
	DOI 200µg	22.70 (2.10)			

Table 6: Non-significant findings from Experiment 3: Central + Peripheral Injection.

Dependent Variable	Group	Mean (S.E.)	Degrees of Freedom	F	Probability Value (p)
Oral stereotypy without food	Saline	28.69 (8.15)	1, 21	.014	.908
	DOI 200µg+0.1 mg/kg	25.61 (8.38)			
Grooming	Saline	10.98 (4.99)	1, 21	.479	.496
	DOI 200µg+0.1 mg/kg	6.79 (3.19)			
Vocals	Saline	5.67 (3.02)	1, 21	.163	.690
	DOI 200µg+0.1 mg/kg	7.36 (2.89)			
Boli in tail pinch	Saline	.67 (.26)	1, 21	3.047	.095
	DOI 200µg+0.1 mg/kg	1.36 (.31)			
Line crosses	Saline	13.92 (2.11)	1, 21	.003	.960
	DOI 200µg+0.1 mg/kg	14.09 (2.72)			
Rears	Saline	4.33 (1.26)	1, 21	.138	.714
	DOI 200µg+0.1 mg/kg	3.64 (1.40)			
Freezing	Saline	32.80 (16.72)	1, 21	.368	.550
	DOI 200µg+0.1 mg/kg	19.81 (12.90)			
Flat body posture	Saline	11 absent 1 present	1	$\chi^2 = .004$.949
	DOI 200µg+0.1 mg/kg	10 absent 1 present			
Rotarod	Saline	26.80 (2.91)	4, 65	1.20	.318
	DOI 200µg+0.1 mg/kg	19.84 (2.74)			

Table 7: Non-significant findings from Experiment 5: Double Peripheral Injection.

Dependent Variable	Group	Mean (S.E.)	Degrees of Freedom	F	Probability Value (p)
Oral stereotopy without food	Double Saline	40.55 (9.71)	5, 90	1.616	.164
	DOI 0.5 mg/kg + Saline	42.93 (9.71)			
	DOI 1.0 mg/kg + Saline	21.62 (9.71)			
	KET 5.0 mg/kg + Saline	11.15 (9.71)			
	DOI 0.5 mg/kg + KET 5.0 mg/kg	28.07 (9.71)			
	DOI 1.0 mg/kg + KET 5.0 mg/kg	20.38 (9.71)			
Grooming	Double Saline	15.43 (5.82)	5, 90	2.213	.060
	DOI 0.5 mg/kg + Saline	3.45 (5.82)			
	DOI 1.0 mg/kg + Saline	4.69 (5.82)			
	KET 5.0 mg/kg + Saline	21.17 (5.82)			
	DOI 0.5 mg/kg + KET 5.0 mg/kg	6.34 (5.82)			
	DOI 1.0 mg/kg + KET 5.0 mg/kg	22.97 (5.82)			
Boli in tail pinch	Double Saline	.56 (.27)	5, 90	1.839	.113
	DOI 0.5 mg/kg + Saline	1.13 (.27)			
	DOI 1.0 mg/kg + Saline	.44 (.27)			
	KET 5.0 mg/kg + Saline	.50 (.27)			
	DOI 0.5 mg/kg + KET 5.0 mg/kg	.94 (.27)			
	DOI 1.0 mg/kg + KET 5.0 mg/kg	1.31 (.27)			

Dependent Variable	Group	Mean (S.E.)	Degrees of Freedom	F	Probability Value (p)
Gnawing	Double Saline	1.31 (.63)	5, 90	1.614	.164
	DOI 0.5 mg/kg + Saline	.69 (.63)			
	DOI 1.0 mg/kg + Saline	.20 (.63)			
	KET 5.0 mg/kg + Saline	2.08 (.63)			
	DOI 0.5 mg/kg + KET 5.0 mg/kg	2.27 (.63)			
	DOI 1.0 mg/kg + KET 5.0 mg/kg	1.12 (.63)			
Line crosses	Double Saline	13.56 (1.45)	5, 90	.734	.593
	DOI 0.5 mg/kg + Saline	14.63 (1.45)			
	DOI 1.0 mg/kg + Saline	15.00 (1.45)			
	KET 5.0 mg/kg + Saline	14.63 (1.45)			
	DOI 0.5 mg/kg + KET 5.0 mg/kg	11.81 (1.45)			
	DOI 1.0 mg/kg + KET 5.0 mg/kg	12.81 (1.45)			
Bodyshakes	Double Saline	.06 (.08)	5, 90	1.481	.204
	DOI 0.5 mg/kg + Saline	.25 (.08)			
	DOI 1.0 mg/kg + Saline	.19 (.08)			
	KET 5.0 mg/kg + Saline	5.55 ^{E-17} (.08)			
	DOI 0.5 mg/kg + KET 5.0 mg/kg	.00 (.08)			
	DOI 1.0 mg/kg + KET 5.0 mg/kg	.13 (.08)			

Dependent Variable	Group	Mean (S.E.)	Degrees of Freedom	F	Probability Value (p)
Freezing	Double Saline	1.76 (5.70)	5, 90	1.897	.103
	DOI 0.5 mg/kg + Saline	21.37 (5.70)			
	DOI 1.0 mg/kg + Saline	12.69 (5.70)			
	KET 5.0 mg/kg + Saline	8.40 (5.70)			
	DOI 0.5 mg/kg + KET 5.0 mg/kg	14.75 (5.70)			
	DOI 1.0 mg/kg + KET 5.0 mg/kg	1.12 (5.70)			
Rotarod	Double Saline	28.41 (1.42)	4, 65	1.203	.318
	DOI 0.5 mg/kg + Saline	*Group not included in analysis			
	DOI 1.0 mg/kg + Saline	25.36 (1.42)			
	KET 5.0 mg/kg + Saline	29.34 (1.64)			
	DOI 0.5 mg/kg + KET 5.0 mg/kg	28.77 (1.64)			
	DOI 1.0 mg/kg + KET 5.0 mg/kg	26.68 (1.52)			

*The rotarod data for the group that was administered the low dose of DOI + Saline was not available for analysis (due to a data collection/recording error), however, it is not anticipated that these data would have shown any difference from the control group as neither the high dose of DOI + Saline nor the 0.5 dose of DOI alone (from Experiment 1) resulted in motor impairment.

VITA

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