$GABA_A$ alpha-5 subunit manipulation of maternal separation-induced anxiety in mice

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Abstract

Rodent models were used to examine the role of alpha-5 subunit containing $GABA_A$ receptors in modulating anxiety-like behavior through the maternal separation procedure, a reliable model for eliciting early life stress in rodents (Levine, 1957). In the brief separation procedure, subjects were separated from the dam and tested following subcutaneous injections of 0, 3, 5.6, 10, 17 or 30 mg/kg of the alpha-5 subunit preferential antagonist Xli-093, or injections of 0, 3 or 10 mg/kg of the alpha-5 subunit preferential agonist SH-053-2'F-R-CH_3. During testing, the number of ultrasonic vocalizations (USVs) was recorded, as well as locomotor activity and motor coordination. In the long-term separation procedure, subjects underwent timed, daily separations, while control subjects were left undisturbed until weaning. Both groups were tested as adults in an open-field paradigm using the Xli-093 compound. In both experiments, no significant differences were found between any of the dose groups or conditions; however a trend towards a peak in USV production occurring at the 5.6 mg/kg dose of Xli-093 in the brief separation experiment was observed. Further testing is required in order to determine whether modulation of the $GABA_A$ alpha-5 subunit has an effect on anxiety-like behavior.

Early life stress (ELS) occurs in children that are unable to cope when exposed to one or many stressful events, which can range anywhere from physical, sexual, and emotional abuse, to neglect and parental separation (Pechtel & Pizzagalli, 2010). In 2009, approximately 702,000 unique cases of child mistreatment that qualified as early life stress events were reported in the United States, 78.3% percent of which were cases of neglect (Administration of Children and Families, 2009). The occurrence and prevalence of these early life stress events is of the utmost concern, not only because of the acute emotional havoc that they wreak on the child, but the long-lasting effects that these events may have on brain development.

Early life stresses that occur during critical periods in brain development can cause disturbances in the psychological and physiological elements associated with later stress responses (Pechtel & Pizzagalli, 2010). While further examination is required in human subjects, preclinical studies have provided a preliminary hypothesis for the mechanisms underlying these disturbances. This hypothesis implicates the development of hypersensitivity in the corticotropin releasing-factor (CRF) system and other neurotransmitter systems associated with the stress response (Heim & Nemeroff, 2001). The changes in brain structure and function associated with this sensitization occur in brain regions that are also implicated in several mental illnesses, and may be the underlying mechanisms that connect early life stress events to the development of 44.6% of childhood-onset mental disorders, and 32% of late onset disorders (Green et al., 2010).

When examining the relationship between early life stress and anxiety, it was found that 32.4% of anxiety disorders could be traced back to early life stress events (Green et al., 2010). Due to the relationship between early life stress and anxiety

disorders, the present study seeks to examine the link between these events and the functions of the neurotransmitter systems that contribute to anxiety throughout the life of an individual. The prevalence of cases of parental neglect and separation has led the present study to pay particular attention to dissecting the relationship between these types of early life stress events and anxiety related behaviors.

Anxiety

Anxiety disorders are one of the most prevalent classes of mental illnesses in the world. In America alone, over 40 million people over the age of 18 are afflicted with at least one anxiety disorder (National Institute of Mental Health, 2010). The spectrum of common anxiety disorders is vast, and includes illnesses such as posttraumatic stress disorder (PTSD), obsessive compulsive disorder (OCD), generalized anxiety disorder (GAD), panic disorder, and phobias, (Oxford Handbook of Anxiety and Related Disorders, 2009). Each of these conditions can be disabling, and may occur concurrently with another anxiety disorder or an unrelated mental illness. Individuals with these disorders experience a pronounced decrease in their quality of life. Given the severity of anxiety disorders, it is important to study the neurobiological mechanisms underlying anxiety, and to use this knowledge to develop effective treatments.

Presently, behavioral therapies and pharmacological manipulations are used as treatments to help alleviate the symptoms of anxiety disorders (Rickels et al., 1993). While both of these methods seem to be efficacious, the present study focuses on the neurochemistry associated with pharmacological treatments. Benzodiazepines have become one of the leading treatment methods for anxiety disorders, especially for treating the symptoms associated with Generalized Anxiety Disorder (GAD) (National Institutes

of Mental Health, 2009). Much biochemical research targets the $GABA_A$ receptor in order to investigate how benzodiazepines decrease anxiety symptoms. The biochemical interactions between benzodiazepines and the $GABA_A$ receptor, how the receptor functions both in the presence and absence of these drugs, and the roles of the different components of the receptor in defining the various behavioral effects of benzodiazepines must be examined thoroughly in order to determine the reasons for their effects (Rudolph et al., 1999).

GABA Receptor Physiology and Function

γ-aminobutyric acid (GABA) is the primary fast inhibitory neurotransmitter in the central nervous system (Olsen, 1981). Its role in mediating neurochemical interactions is accomplished through its ability to bind to and subsequently alter specific GABA receptors, usually present on interneurons found throughout the brain (Rudolph & Mohler, 2006). The GABA receptor family is composed of three distinct types of receptors, $GABA_A$, $GABA_B$ and $GABA_C$, which differ in their effects on brain function, their modes of action and dispersion throughout the brain and related structures (Bormann, 2000). This study specifically targets the $GABA_A$ receptors, since previous research on the structures associated with the pathophysiology of anxiety disorders has implicated the $GABA_A$ receptor as being an integral player in modulating anxiety (Shephard, 1987).

The $GABA_A$ receptor is a heterogeneous, ionotrophic receptor composed of five protein subunits that cross the cell membrane of the neuron and form a chloride ion channel located in the middle of the subunits (Levitan, 1988). Binding of the ligand

(GABA) generates an inhibitory postsynaptic potential in the postsynaptic neuron (Bormann, 2000). There is a vast array of subunits that may be incorporated into the structure of the $GABA_A$ receptor, however the most common configuration includes two alpha subunits (α_1, α_2, α_3, or α_5) one gamma (Υ) and two beta (β_2 or β_3) subunits (Pritchett et al., 1989; Rudolph et al., 2001). The presence of the gamma subunit renders the receptor susceptible to modulation by benzodiazepines (Miczek et al., 2003). Studies have attributed each of the behavioral effects of benzodiazepines to the alpha-1, alpha-2, alpha-3 and alpha-5 subunits that may be present on the receptor. Two other kinds of alpha subunits, alpha-4 and alpha-6, also exist, but receptors containing these subunits have proven to be insensitive to classical benzodiazepines (Rudolph & Mohler, 2006).

Benzodiazepines and Anxiety

Anxiety disorder symptoms (primarily those associated with generalized anxiety disorder) can be reduced or alleviated by benzodiazepines. These drugs bind between the gamma and alpha subunits of the $GABA_A$ receptor, and serve as positive allosteric modulators, which act by increasing the likelihood that the chloride channels will open an inhibitory postsynaptic potential will occur (Rowlett et al., 2005). These compounds were popularized as effective pharmacological treatments in the early 1960s, following the synthesis of chlorodiazepoxide and its clinical introduction as the primary ingredient in the drug librium (Sternbach, 1979). Today, several kinds of benzodiazepines remain popular pharmacological treatments for anxiety, including xanax (alprazolam), klonipin (clonazepam), valium (diazepam), and ativan (lorazepam) (Trustees of the University of Pennsylvania, 2010).

Benzodiazepines are most frequently used for their anxiolytic properties; however they are known to have several other behavioral and physiological effects. These effects include sedation, muscle relaxation, cognitive impairment and memory impairment (Rudolph & Mohler, 2004). They have also been known to induce psychological addiction (Tallman et al., 1980). Due to the complex array of side effects, research has been conducted to determine the exact physiological interactions underlying each of the aforementioned behavioral and psychological effects incurred by these compounds (Savic et al., 2010). Researchers hope to identify any specific differences in the morphology or function of the $GABA_A$ receptors that are affected when these effects are occurring, and to be able to isolate the routes of action responsible for each of them. Once this is accomplished, they may be able to synthesize compounds that produce only the desired effects while eliminating all of the extraneous ones, and prove to be less addictive than traditional benzodiazepines (Rudolph & Mohler, 2006).

Rodents as Model Organisms in Anxiety Studies

Most preclinical psychopharmacological studies have been conducted using model organisms that are analogous to humans, due to the higher controllability of experiments. In particular, rodents have proven to be one of the most useful species to use in behavioral pharmacology studies involving anxiety, due to the similarities between the neuronal circuits responsible for mediating anxiety-like behaviors in rodents and those responsible for comparable behaviors in human beings (Rodgers, 1997). Various paradigms have been developed to induce and quantify anxiety-like behavior in rodents.

These paradigms can either rely on conditioned behavior, or behaviors that are induced through forms of operant conditioning that usually involve punishment, or unconditioned behavior, which are the spontaneous reactions of rodents to stimuli presented by the experimenter (Olivier et al., 1994).

One of the most widely used methods of testing unconditioned anxiety-like behavior is the open-field test, which capitalizes on the innately avoidant reactions of rodents to novel, open settings (Hall, 1934; Denenberg, 1969; Walsh, 1976). The animal is placed into a novel area, and behavioral reactions such as frequency of leaning or rearing, stretch-attend, latency and frequency of entering the center, thigmotaxis (the tendency of rodents to stay close to the walls of the arena) and locomotor activity are measured (Choleris et al., 2001). The most commonly analyzed behavior in this paradigm is thigmotaxis, which is positively correlated with anxiety levels. Animals that are more anxious tend to spend more time near the walls of the arena, and less time in the center (Belzung, 1999). Because this paradigm provides a reliable and quantifiable behavioral definition of anxiety in rodents, it has also been used to study the effects of various anxiety-modulating substances. Studies done to identify the behavioral effects of benzodiazepines, for instance, have revealed a decrease in most of the aforementioned behaviors associated with elevated levels of anxiety, as well as an increase in the time spent in the center of the open field arena (Prut & Belzung, 2003). The open field paradigm has helped make rodent models invaluable in studying anxiety, because they allow for the exploration of modulatory ligands of the benzodiazepine site on the $GABA_A$ receptor (in order to understand the neurochemical underpinnings of anxiety).

$GABA_A$ Alpha Subunit Findings in Rodent Models

Several breakthroughs about the physiology of anxiety and the effects of benzodiazepines have been made using rodent models. In these studies, $GABA_A$ receptors in mice are either pharmacologically rendered insensitive to or enhanced by benzodiazepines, or mice are bred with knock-in and knockout mutations that render specific alpha-subunit containing $GABA_A$ receptors insensitive to benzodiazepine binding (Rudolph & Mohler, 2006). Through these kinds of studies, researchers have been able to connect the different behavioral effects of benzodiazepines to the various alpha subunits.

Schofield et al., (1989) first identified the presence of the human alpha-1 subunit of the $GABA_A$ receptor, and enumerated its amino acid sequence. These alpha-1 subunit-containing receptors are particularly important, due to the fact that they account for approximately 60% of all $GABA_A$ receptors present in mammalian systems (Rudolph & Mohler, 2004). Using genetic knock-in mutations in mice, researchers have identified it as the subunit primarily responsible for the sedative and some of the amnesic effects of benzodiazepines (Rudolph et al., 1999; McKernan et al., 2000). Administration of diazepam and zolpidem to alpha-1 subunit knockout mutant mice supported the aforementioned finding by revealing that the absence of functional alpha-1 subunits did not hinder the ability of the animals to experience the anxiolytic or locomotor impairing effects of benzodiazepines, but hindered only the experience of the sedative and anticonvulsant effects of the drugs (Kralic et al., 2002).

Since the discovery of the heterogeneous nature of the $GABA_A$ receptor, much debate has occurred as to which of the alpha subunits mediates the anxiolytic properties

of benzodiazepines. The consensus seems to shift between implicating only the alpha-2 subunit to suggesting that the combined actions of both the alpha-2 and alpha-3 subunits are responsible for these effects (Dias et al., 2005). Selective antagonism of the alpha-2 and alpha-3 subunit containing $GABA_A$ receptors, for instance, implicated both of the subunits in mediating the anxiolytic effects of benzodiazepines in both rats and mice (Atack et al., 2005). When the roles of the individual subunits were examined, evidence for the involvement of both in anxiolysis were found. Subtype selective agonism of the alpha-3 subunit, as well as experiments involving genetically modified mice whose alpha-2 subunit containing $GABA_A$ receptors were rendered insensitive to benzodiazepines further implicated the alpha-3 subunit in the anxiolytic effects of benzodiazepines (Dias et al., 2005). Administration of benzodiazepines to knock-in point mutated mice devoid of functional alpha-2 subunits showed that the alpha-2 subunit was partially responsible for anxiolysis, as well as some muscle relaxant effects (Low et al., 2000). More recently, alpha-3 knock-in mice were used to show that anxiolysis occurred in the absence of a functional alpha-3 subunits following benzodiazepine administration (Rudolph & Mohler, 2004). Although some discrepancies still exist between studies that have set out to identify the subunit primarily responsible for expressing the anxiolytic effects of benzodiazepines, the most current research in the field has ruled out the alpha-3 subunit as the primary subunit responsible for anxiolysis, and instead implicates it as being primarily responsible for the muscle relaxant effects (Tan et al., 2011).

The $GABA_A$ Alpha-5 Subunit

The primary goal of the present study is to examine the role of the alpha-5 subunit in anxiety. So far, it is known that $GABA_A$ receptors containing alpha-5 subunits tend to be highly concentrated within the olfactory bulb and hippocampal areas of the brain, and unlike the other alpha subunits, are primarily extrasynaptic (Crestani et al., 2002). Previous research using rodents as experimental models has revealed important information regarding the role of the alpha-5 subunit in modulating certain effects of benzodiazepines. Traditionally, the alpha-5 subunit was thought of as being primarily responsible for mediating the cognitive and memory deficits associated with benzodiazepine use (Rudolph & Mohler, 2006). Mice lacking functional alpha-5 subunits demonstrated an inability to find the hidden platform in a Morris Water Maze task, implicating the absence of the subunit in the deficiency of hippocampal-dependent learning and memory necessary to complete the task (Collinson et al., 2002). In an associative learning task, mice lacking functional alpha-5 subunits showed a facilitation of freezing behavior following administration of diazepam when the task was altered to assess hippocampal-dependent processing. The lack of functional alpha-5 subunits produced no effects in task performance when it was altered so that the hippocampus was not necessary for fear conditioning (Crestani, et al., 2002). Based on their localization within the brain, it is not surprising that alterations in alpha-5 subunit function produce deficits in performance on hippocampus-dependent tasks and are subsequently connected to learning and memory (Rudolph & Mohler, 2004).

Several newly-synthesized agonists of the alpha-5 subunit were also tested in order to assess their effects on locomotor activity, and have implicated the alpha-5 subunit in mediating the sedative and muscle relaxation effects of benzodiazepines

(Rudolph & Mohler, 2004). Mice with various alpha subunit knock-in mutations were given diazepam daily in order to assess their ability to develop tolerance to sedative effects, and when compared to mice with knock-in mutations for the alpha-2 and alpha-3 subunits, those lacking functional alpha-5 subunits displayed constant levels of motor activity, indicating less of a sedative tolerance (van Rijnsoever et al., 2004). Theories for the reasons behind the involvement of the subunit in the experience of sedation predict that it may occur through an indirect relationship with alpha-1 subunit containing $GABA_A$ receptors (Savic et al., 2009).

Studies involving the alpha-5 subunit have proven to be difficult to undertake, due to the fact that the properties of the receptors containing these subunits prevent them from being easily manipulated by genetic modifications. This is evidenced by the fact that the alpha-5 subunit is the only one in which a knock-in mutation reduces the availability of proteins that form the alpha-5 subunit by about 20%, thus resulting in immediate cognitive and memory impairments in the absence of benzodiazepines (Balic et al., 2009; Crestani et al., 2002). For this reason, many of the findings about the alpha-5 receptor have come from pharmacological manipulations of the receptor using novel compounds, and not genetic knock-in studies.

Xli-093 and SH-053-2'F-R-CH_3

Since the invention of classical benzodiazepines, numerous novel compounds have been developed to serve as modulatory ligands to the $GABA_A$ receptor. While many of these compounds are unsuitable for use in the treatment of anxiety disorders, some of them have proven to be valuable in the realm of $GABA_A$ subunit research. These

compounds allow for the functions of $GABA_A$ receptors that contain specific subunits to be discerned through agonism and antagonism targeting the different alpha subtypes.

In order to overcome the difficulties associated with genetic manipulation studies, the present study performs its examination of the function of the alpha-5 subunit containing $GABA_A$ receptors during anxiety-evoking situations through the use of a novel alpha-5 subunit preferential antagonist of the $GABA_A$ receptor known as Xli-093 (Cook et al., 2009). The antagonist exerts very little effect on the function of the receptor itself, but blocks the receptor so that the agonist is unable to bind and potentiate neurotransmission (Rowlett et al., 2005). Xli-093 was used to investigate the cognitive impairment effects of diazepam in a water maze task, and it was discovered that Xli-093 potentiated a decrease in locomotor activity, as well as decreased cognitive impairment that is normally caused by diazepam during the water maze task. (Savi

et al., 2009). Due to the novelty of this compound, little else has been discovered about its effects *in vivo,* both when administered alone and when given in conjunction with benzodiazepines. Its selectivity for the alpha-5 subunit, however, makes it a crucial tool for use in the present study.

Testing with a novel alpha-5 preferential agonist was also included in the present study, in order to examine the function of potentiated receptors containing these subunits during exposure to anxiety-provoking stimuli. Cook et al., (2010) recently synthesized one such agonist known as SH-053-2'F-R-CH_3. Much like its antagonistic counterpart Xli-093, very little research has been done using the SH-053-2'F-R-CH_3 compound. One of the few studies using this compound *in vivo* was done in rhesus monkeys, and determined that the compound produced partial increases in suppressed responding,

despite administration of electrical shock during a conflict procedure. Although this finding was not statistically significant, the tendency of this drug to increase suppressed responding illustrates some anxiolysis during a learning task; a characteristic that is consistent with the normal effects of functional alpha-5 subunits on cognitive function, learning and memory (Fischer et al., 2010). The preferential nature of this compound, coupled with its ability to potentiate alpha-5 subunit-containing $GABA_A$ receptor function, make it a key compound for use as a manipulator of the desired subunit during maternal separation-induced anxiety situations in the present study.

Maternal Separation in Rodents

Studies done on the etiology of human mental illness suggest that many of the anxiety and affective disorders experienced by adults can be traced back to traumatic events that occurred early in the life of an individual (Millstein & Holmes, 2006). Parental neglect is a childhood traumatic event experienced by humans (Ploj, Roman & Nylander, 2003) that can be replicated and adapted effectively in animal models. Primate studies on parental neglect have been conducted (Gutman & Nemeroff, 2002), however rodents are by far the most extensively used models in experiments aimed at examining the long and short-term behavioral effects of parental neglect, and early life stress in general.

Early life stress is commonly simulated in rodents through the maternal separation paradigm. In this paradigm, rodent pups undergo timed, daily repeated physical separation from the dam during the "hyporesponsive period" of postnatal development, which occurs between postnatal days 1-14 (Levine, 1994). These separations can be

short-term, brief, or long term, and each type of separation has been linked to distinct behavioral changes that occur during adulthood (Hsu, 2003).

Short-term separation involves daily removal of the pups from the home cage for a period of several minutes (Levine et al., 1966). Although some studies have been unsuccessful in arriving at conclusive findings (Millstein & Holmes, 2006), various studies have found that, in adulthood, positive behavioral effects arise from the short-term separation procedure. Fifteen minutes of daily, brief maternal separation of rat pups from their dam resulted in adults that showed lower levels of anxiety-like behavior when presented with an elevated plus maze, and females that exhibited more maternal behavior when caring for their own pups (Boccia & Pedersen, 2001). Rats who have experienced short-term separation, at baseline, exhibit lower levels of corticotropin releasing factor mRNA in stress related regions of the brain (Plotsky & Meaney, 1993). By contrast, more corticotropin releasing factor was released in non-separated rats than in separated rats following restraint stress, suggesting a greater stress response in non-separated animals during acute stressful situations when compared to those that have undergone short-term maternal separation (Pryce & Feldon, 2003).

The long-term maternal separation procedure is similar to the short-term procedure, however it requires that pups be separated from the dam for approximately 180 minutes daily (Huot, 2001). Unlike the lasting effects of short-term separation, most studies have found adverse behavioral effects resulting from long-term separation. When confronted with stressful events in adulthood, pups that have undergone long-term maternal separation tend to show a higher level of activation of the hypothalamic-pituitary-adrenal (HPA) axis, and are thus more prone to experiencing anxiety- and

depression-like behaviors in anxiogenic testing conditions (Millstein & Holmes, 2006). In comparison to pups that had undergone short-term separation, 180-minute daily maternal separation caused more anxiety-like behaviors in adult rats when presented with the elevated plus maze (Boccia & Pedersen 2001). It is important to note that some variation in behavior was seen in the different sexes following separation. Female pups that were separated were less maternal than their undisturbed counterparts, and increases in anxiety-like and depression-like behaviors were more prominent in separated male rats than in separated females (Boccia & Pedersen, 2001). Male rats also showed a higher biological stress response in adulthood than their female counterparts following maternal separation (Wigger & Neumann, 1999).

A variation of the maternal separation paradigm involves brief separation of the litter from the dam with no reunification following testing, wherein the ultrasonic vocalizations (USVs) produced by the pups during separation provide an operational definition of anxiety (due to the fact that they can be manipulated by anxiolytic and anxiogenic drugs), and are quantified in order to assess the intensity of the anxiety that results from the separation. (Hofer, 1975; Olivier et al., 1998; Winslow et al., 1990). Greater numbers of USVs produced by the pups vary directly with the levels of anxiety (Hofer et al., 1993). Based on the time course of normal neonatal development, the production of USVs during periods of separation has proven to occur reliably, and has allowed for this variation of the paradigm to be used to assess the effects of different compounds on manipulating anxiety-related behavior (Winslow & Insel, 1991; Zimmerberg et al., 1993). The treatment of mouse pups with antidepressants, for instance, decreased the number of ultrasonic vocalizations produced during testing and supported

the evidence that connects selective serotonin reuptake inhibitors (SSRI) to anxiolytic behavioral effects (Fish et al., 2003). When the $GABA_A$ and glutamatergic receptors were manipulated using agonists and antagonists of both receptor systems, it was found that antagonism of low-affinity glutamatergic NMDA receptors resulted in an increase in the production of ultrasonic vocalizations, whereas agonism of the $GABA_A$ subunit resulted in decreases in ultrasonic vocalization production (Takahashi et al., 2009).

Short- and long-term maternal separation during postnatal development in rodents has provided experimenters with the ability to manipulate and measure anxiety related behaviors in response to acute stressors, in both the early stages of development and in adulthood (Miczek et al., 2008). Studies of brief separation have provided a valid means of quantifying anxiety-like behavior in early postnatal development, through the use of ultrasonic vocalizations in mouse pups (Olivier et al., 1998). The maternal separation approach has proven to be advantageous in comparative studies aimed at examining human ailments, since early traumatic events play a crucial role in the development of human anxiety disorders and stress (Pechtel & Pizzagalli, 2010). These methods of manipulating anxiety in mice allow for the study of the developmental and neurochemical changes in the networks associated with anxiety and how these changes relate to reactivity to stressors early in life as well as in adulthood.

Objective

The primary objective of the present study is to identify the role of the alpha-5 subunit of the $GABA_A$ receptor in modulating anxiety-like behavior in rodents following brief or long-term maternal separation. In order to examine this relationship, agonism and antagonism of these receptors will be performed using novel compounds that show a

preference to only those receptors containing alpha-5 subunits. This will allow for the identification of those behaviors that are specific to the alpha-5 subunit, as well as the determination of whether or not the subunit plays a role in other anxiety-like behaviors that it has not been linked to previously.

Based on previous research that found that general antagonism of the $GABA_A$ receptor in the absence of any additional drug administration increased anxiety (Sanders & Shekhar, 1994), it is hypothesized that following the brief separation conditions, the alpha-5 subunit antagonist will produce an anxiogenic effect during testing with acute stressors. This will hopefully be exemplified as higher numbers of USVs produced during brief maternal separation, particularly at the higher doses. Along those same lines, it is hypothesized that administration of the alpha-5 subunit preferential agonist will produce an anxiolytic effect during testing with an acute stressor (as seen in fewer USVs produced during testing, also at the higher doses). Based on findings that exclude the alpha-5 subunit from involvement in various behavioral changes associated with benzodiazepines, both drugs are not expected to influence the changes that occur in locomotor activity, motor coordination and other behaviors associated with modulation of the $GABA_A$ subunit at the benzodiazepine site (Tan et al., 2011).

In the long-term maternal separation experiment, it is hypothesized that those animals that have undergone separation will show higher levels of anxiety-like behaviors than those that were not separated. In conjunction, an interaction effect between the antagonist and long-term separation is predicted, wherein those animals that were subjected to long-term separation and are given the antagonist during testing will display more anxiety-like behavior than those animals that receive the antagonist and have no

history of separation and controls (Huot, et al., 2001; Wigger and Neumann, 1999; Millstein & Holmes, 2006; Sanders & Shekhar, 1994).

The present study will bolster the findings that suggest that maternal separation increases the occurrence of anxiety-like behavior during situations that involve acute stressors. It will also provide new information on the role of the alpha-5 subunit in modulating these behaviors

Methods

Experiment 1. Male and female mouse pups underwent a single, brief maternal separation. They were then tested with either an antagonist or an agonist that showed preference for the alpha-5 subunit-containing $GABA_A$ receptors in order to assess the role of that subunit in mediating the anxiety-like state induced under separation conditions (Savić et al., 2009; Fischer et al., 2010; Takahashi et al., 2008). The following schematic provides a timeline for the experiment.

Subjects

Seven-day-old Carworth Farm Webster (CFW) mouse pups were used, due to the substantial litter size, and their ability to produce large amounts of ultrasonic vocalizations (USVs) (Fish et al., 2000). They were obtained through the breeding of adult pairs of CFW mice originally purchased from Charles River Laboratories (Wilmington, MA, USA). The pups were housed with the dam and littermates in clear polycarbonate cages that were (46 x 24 x 15 cm^3). The cages were kept in a vivarium, which was kept at a constant temperature of 21.1 $\pm$ 2 ºC and a constant humidity of 30-

35%. Since mice are primarily nocturnal, and are more active at night, the vivarium had a reverse 12-hour light/dark cycle, where the lights went on at 7:00pm and were turned off at 7:00am. A total of 119 animals (n = 119) were used for this experiment. For testing with the antagonist, 19 subjects were in the vehicle group (n = 19), and 16 were in each of the five remaining dose groups (n = 16). For testing with the agonist, six pups (n = 6) were given the vehicle dose, seven (n = 7) were given the 3-mg/kg dose and six (n =6) were given the 10 mg/kg dose. The animals were cared for following the guidelines set forth by the "Guide for the Use and Care of Laboratory Animals" and the regulations enforced by the Institutional Animal Care and Use Committee (IACUC) of Tufts University. Immediately following the experiment, all animals were euthanized.

Apparatus

The experimental room was equipped with an 11.18x12.95 cm incubator that was set to keep the subjects at a constant temperature of 34 ± 1°C. Testing took place inside of a (49.5×38×34 cm) chamber, designed to block out ambient sound. Inside of the chamber, a 23x23 cm metal tray (marked with gridlines to create 3x3 cm squares) rested inside of a basin that was cooled to 19 ± 1°C using cold tap water and ice cubes. Pups were placed into the metal pan during screening and testing. The chamber also contained a high frequency microphone for USV recording, which was attached to the top of the chamber, and was connected to the SonoTrack control unit (Metris B.V., The Netherlands). SonoTrack software (Version 1.0, Metris B.V., The Netherlands) was used as a means of quantifying the amount of USV calls that each pup made during the screening and testing stages of the experiment. The software also converted the USVs from each session into a sonogram [see Figure 1], as well as produced an audible digital

recording of the USVs. The bandpass and peaks over threshold were adjusted to 30000-90000 Hz, and one (respectively), so that any artificial sources of high-frequency sound that were not USVs to would be excluded from the data.

Procedure

Brief Maternal Separation. Following the paradigm of Fish et al. (2000), animals were separated from the dam and their nest and placed into the incubator in the experimental room. They were kept with their littermates in the incubator with shavings from the nest, in order to reduce the effects of extraneous factors on their stress levels. Animals remained in the incubator for a period of 25 minutes prior to testing.

Screening. Animals were chosen at random for screening. Each animal was weighed using a standard digital scale, and then placed into the metal tray in the test chamber for 30 seconds while the number of USVs they produced were quantified using the SonoTrack software (Metris B.V., The Netherlands). Pups that produced a minimum of six USVs during screening were included in the testing phase of the experiment; otherwise they were omitted.

Administration of Drugs. Immediately following screening, pups were given a subcutaneous injection of 1ml/ 100 gram of 10mg/kg of one of the six dosages of XLi-093 or one of three doses of SH-053-2'F-R-CH_3. The Xli-093 doses included 0 mg/kg ("vehicle" dosage), 3 mg/kg, 5.6 mg/kg, 10 mg/kg, and 17 mg/kg and 30 mg/kg. Each of the doses was suspended in a vehicle of 50% propylene glycol and 50% distilled water (H_2O). The Sh-053-2'F-R-CH_3 doses included vehicle, 3 mg/kg, and 10 mg/kg. Each of the doses was suspended in a vehicle of 80% propylene glycol, 5% deionized water (H_2O), 5% 1N HCL, and 10% ethanol .To avoid the litter effect, individuals within the

litters were assigned to each of the different dose groups. After injection, pups were returned to the incubator, where they remained for 30 minutes, in order for the antagonist to take effect.

USV, Locomotor Activity and Motor Coordination Test. Prior to the start of the testing period, the body temperature of each pup was measured using a rectal thermal probe (YSI 555 N034, Yellow Springs Instruments, Yellow Springs, Ohio, USA) attached to an YSI-2100-Tele Thermometer (Yellow Springs Instruments). The pup was then placed onto the test pan in the testing chamber. Testing lasted for four minutes, during which time behavioral measurements were observed and quantified. USVs were recorded, as well as locomotor behavior and lack of motor coordination. Locomotor behavior was quantified by counting the number of times the animal's hind legs crossed one of the gridlines, and the lack of motor coordination was quantified by counting the number of times the back of the subject made contact with the tray (referred to as "body rolls"). Manual counters were used in order to ensure the accuracy of the counts for each of the behavioral categories. At the end of the testing period, the pup's rectal body temperature was taken again, and the subject was returned to the incubator with its littermates. The change in body temperature was calculated in order to eliminate the possibility that changes in behavior resulted from extreme changes in temperature.

Data Analysis. The number of USVs, the locomotor activity (grid crossings), body rolls and change in body temperature for each of the individuals assigned to the different dosage groups were averaged across litters, so that one data point for each dosage group in one litter was obtained. The averages were then recorded and compared between the dosage conditions. The mean and standard error of the mean for each of the

dosages were plotted onto a dose-effect curve using Sigma plot software. A one-way analysis of variance (ANOVA) was performed using Sigmastat software in order to determine whether any of the dosage groups produced significant effects in the animals.

Experiment 2. The goal of this experiment was to determine whether long-term maternal separation induced anxiety was modulated using the aforementioned preferential antagonist of alpha-5 subunit containing $GABA_A$ receptors. The experiment focused on determining the behavioral effects of long-term maternal separation, coupled with administration of the antagonist using the open-field paradigm. The following schematic depicts the timeline for this experiment.

Subjects

The subjects were pups obtained from pair-housed adult CFW mice (originally purchased from Charles River Laboratories, Wilmington, MA, USA). Each of the pairs' cages was checked twice a day for newborn litters, and if a litter was found, the birth date (postnatal day zero) was recorded. On postnatal day 21, the pups were weaned and placed into cages with up to four same-sex cohorts. The cages were kept under the aforementioned conditions in the vivarium. A total of 20 animals (n = 20) were used, half of them belonging to the control condition (n = 10) and half to the maternally separated condition (n = 10). Three subjects (n =3) from each condition were in each of the dose groups (5.6 mg/kg, and 10 mg/kg Xli-093), and five (n = 5) from each condition received the vehicle dose. The animals were cared for following the guidelines set forth by the

"Guide for the Use and Care of Laboratory Animals" and the regulations enforced by the Institutional Animal Care and Use Committee (IACUC) of Tufts University. Immediately following the experiment, the animals were euthanized.

Apparatus

Long-Term Maternal Separation. Litters of animals were removed from their home cages and placed into 460 ml covered plastic containers (Rubbermaid), with 1 cm holes in the lids of the containers for ventilation. The plastic containers were placed into a 40^3cm incubator, set to 32-34°C in order to avoid causing excess stress due to hypothermia. A thermal probe inside of the incubator allowed for the temperature to be monitored.

Open Field Test. Animals were placed into 52 x 36 x 32 cm open fields (Rubbermaid). Video tracking of their locomotor activity in the open field, as well as the ratio of times that they spent in the center and surround of the arena were done using a PC-based data acquisition system (Ethovision, VTMAS v 1.80, Noldus, Wageningen, Netherlands) that received video recordings of the animal via a camera (Cohu, Model 4815-211/A209) placed 164 cm above the open field.

Procedures

Long-Term Maternal Separation. Separation procedures were adapted from Cruz, Quadros, Planeta & Miczek, 2008. On postnatal day one (PND 1), animals began long-term maternal separation (LMS). Litters were removed from the home cage (along with a small amount of shavings) and were placed into the covered plastic containers.

They were then put into the incubator, which was located in a separate room, and turned on prior to separation in order to ensure that it maintained the correct temperature before the animals were placed inside. The animals were separated every day from PND 1 to PND 14 for a total of 180 minutes each morning, between 0900-1400 hours (during their dark phase). Immediately following separation, the litters were returned to their nest and reunited with their parents.

Control litters were left undisturbed, and were only handled when their cages were cleaned once a week. Breeding pairs contributed several litters to the study, and the litters alternated between maternal separation and control (i.e. if the first litter underwent maternal separation, the second litter was used as a control). On PND 21, both control and LMS litters were weaned, and group housed (by sex) in clear polycarbonate cages (46 x 24 x 15 cm^3). After PND 60, they were housed individually in cages with identical properties as the ones described above, and began receiving intraperitoneal injections of saline daily for three days (in order to allow them to habituate to injections and handling).

Open Field Testing. After the injection and handling habituation period, the animals were assessed using the open field paradigm. All open field tests were conducted during the dark phase (in red light only). Four animals were tested at the same time in separate tubs during each open field session. Animals were first placed into the open field tubs for 40 minutes, in order to allow them to habituate to the arena, and their activity was recorded. Immediately following habituation, the animals were returned to their cages and given an intraperitoneal injection of either vehicle, 5.6 mg/kg or 10 mg/kg doses of Xli-093 (suspended in a vehicle of 20% 2- hydroxylpropyl β-cyclodextrine). Drug doses and time-courses for testing were determined via pilot testing (see Appendix

for supplemental methods). They were then placed back into the tubs, and their locomotor activity, as well as the amount of time that they spent in the center and surround of the open field arena was recorded for 60 minutes. At the end of the testing phase, the animals were returned to their home cages.

Data Analysis. The ratio of time spent in the center versus time spent in the surround, as well as the total distance traveled were averaged for the controls and the maternally separated mice for each of the dosage groups. The averages were then recorded and compared between the dosage conditions. The mean and standard error of the mean for each of the dosages were plotted onto a dose-effect curve using Sigma plot software. A two-way analysis of variance (ANOVA) was performed using Sigmastat software in order to determine whether any of the drug doses produced significant effects in either of the two conditions.

Results

Experiment 1: Brief maternal separation

Xli-093. A dose-effect curve was plotted in Figure 2 for all of the doses of the antagonist. The greatest number of USVs was seen at the 5.6 mg/kg dose (M = 210.875, SEM = 25.172), especially when compared to the USVs produced at the vehicle dose (M = 154.605, SEM = 22.305). There was no significant difference between any of the dose groups in the number of USVs produced during testing (F (5,93)= 1.376, p> 0.05).

The dose groups did not differ in the number of grid crossings (F (5,93) = 0.931, p > 0.05) [See Figure 3] or body rolls (F (5,93) = 0.676, p > 0.05) [See Figure 4]

observed during testing. There was also no difference between the dose groups in the change in body temperature (F (5,93) = 0.552, $p > 0.05$).

SH-053-2'F-R-CH3. The dosage groups did not differ in the number of USVs produced (F (2, 16) = 0.146, $p > 0.05$) [See Figure 5], the number of grid crossings (F (2,16) = 0.278, p > 0.05) [See Figure 6], or the number of body rolls (F (2,16) = 1.567, $p > 0.05$) [See Figure 7]. There was also no difference between dose groups in the change in body temperature (F (2,16) = 0.234 p > 0.05). The effects of this drug, as well as Xli-093 are compared to classical agonists and antagonists in Table 1.

Experiment 2: Long-term maternal separation

The preliminary findings for this experiment include a total of only twenty (n = 20) subjects that were tested in the open field following the long-term maternal separation protocol. Of those animals, half were controls and the other half had undergone maternal separation. Three subjects (n =3) from each condition were in each of the dose groups (5.6 mg/kg, and 10 mg/kg Xli-093), and five (n = 5) from each condition received the vehicle dose. The different groups were assessed based on the amount of time (measured in seconds) spent in the center of the open field during the test session.

There was no difference between the control and maternally separated animals in the amount of time spent in the center of the open-field (F(1,14) = 0.0925, $p > 0.05$). Animals in the vehicle group spent more time in the center of the open-field (M = 2204.536, SEM = 487.671) than those in the 5.6 mg/kg group (M = 1357.497, SEM = 563.114) and the 10 mg/kg group (M = 381.113, SEM = 563.114). These findings are suggestive of a difference, although they do not reach conventional levels of confidence (F(2,14) = 3.001, p = 0.082). There was also no interaction effect found between the dose

of Xli-093 given and the separation condition (control versus maternally separated) on the amount of time spent in the center of the open-field ($F(2,14) = 0.168$, $p > 0.05$). Figures 8 and 9 illustrate the emerging trend seen in the effects of each of the doses on the amount of time spent in the center and surround (respectively) of the open field for both the control and maternally separated conditions.

Discussion

The preferential alpha-5 antagonist Xli-093 had differing effects at each of the doses following brief maternal separation. Compared to the vehicle dose, the lowest dose (3 mg/kg) suppressed USV production. A peak in USV frequency was observed at the next highest dose (5.6 mg/kg), followed by a dose-dependent reduction in the number of USVs at the 17 mg/kg and the 30 mg/kg doses. Although the differences in USV frequencies were evident, they were not statistically significant. Thus the results do not reliably support the hypothesis that a potentiation of USVs (indicating an anxiogenic effect) would occur following administration of Xli-093. The lack of significance is surprising, given the large sample size included in each of the dose groups.

As predicted (Collinson et al., 2002; Crestani, et al., 2002), none of the doses of Xli-093 had an effect on locomotor activity or motor coordination. There was also no impact of Xli-093 on the change in body temperature, indicating that the behavioral effects that occurred during testing were likely not due to hypothermia or temperature related reactions.

The preferential alpha-5 agonist SH-053-2'F-R-CH_3 had no significant effects on the number of USVs produced. This finding did not support the hypothesis that agonism of the alpha-5 subunit containing $GABA_A$ receptors would result in anxiolysis. Due to the

novelty of the compound, very little information is known about its pharmacodynamics *in vivo*. Since SH-053-2'F-R-CH_3 has not be used in rodents, the doses used in the present study were adapted from Fischer et al., (2005), a study in which the agonist was used in primates to alter suppressed behavior in a conflict paradigm. It could be that the translation of the doses from a primate to a rodent model yielded doses that were ineffective for manipulating anxiety-like behavior.

Classic studies have revealed that differences between rodents and primates exist in their behavioral responses to benzodiazepine-site ligands (Ninan et al., 1982). Rowlett et al., (1999) illustrated this difference in responses to drug administration using a task where primates were trained to identify zolpidem, an alpha-1 subtype selective benzodiazepine-site agonist, as a discriminative stimulus (DS) versus saline. In previous studies, rats trained in this paradigm were found not to respond to zolpidem in the drug-appropriate manner observed following administration of conventional agonists such as pentobarbital and triazolam (Rowlett & Woolverton, 1997). Rowlett et al., found that the compound did, however produce the expected responses in baboons and monkeys. This difference suggests heightened sensitivity in primates to benzodiazepine site-specific compounds when compared to rodents, which may explain the lack of effect observed in the present study for SH-053-2'F-R-CH_3. In the future, testing with SH-053-2'F-R-CH_3 could be done using higher doses, in order to circumvent the possible insensitivity of rodents to the doses used in the present study.

Fischer et al., (2005) also noted that following administration of SH-053-2'F-R-CH_3, a trend towards an increase in suppressed responding was detected, but it was not found to be statistically significant. This might mean that, in general, this particular

preferential agonist of the alpha-5 subunit has some moderate effects on anxiety-like behavior, but not enough to trigger a distinct behavioral response. This may be due to the novelty of the compound, and further testing could be needed to determine the true extent of its efficacy *in vivo*.

SH-053-2'F-R-CH_3 did not have an effect on locomotor activity or motor coordination, which was expected given the general dissociation of the alpha-5 subunit from modulating these behaviors (Collinson et al., 2002; Crestani, et al., 2002). As with the antagonist testing, there was no significant difference between the dose groups in change in body temperature, suggesting that changes in the observed behaviors were not the result of temperature changes.

While the second experiment is only in its preliminary stages, there appears to be an emerging trend towards an inverse relationship between anxiety-like behavior in the open field and the dose of Xli-093 administered. At the vehicle dose, all animals, regardless of whether they were maternally separated or controls, spent more time in the center of the open field than in the periphery. The time spent in the center decreased at the intermediate dose (5.6 mg/kg), and was most reduced at the highest dose (10 mg/kg). This suggests that an anxiogenic effect may occur following administration of the antagonist, and may increase in magnitude with the drug dose.

When comparing the controls to the maternally separated subjects, there was no visible trend indicating a difference between groups in the time spent in the center of the open field. There was also no notable interaction between the drug dose and condition; however the current findings suggest an interaction effect at the 5.6 mg/kg dose could emerge with more extensive testing. The current findings for the 5.6 mg/kg dose group

show that maternally separated animals spent less time, on average, in the center of the open field than the controls. Findings from the first experiment also showed an increase in anxiety-like behavior (USV production) at the 5.6 mg/kg dose. If, with further testing, significant effects are found to occur at the 5.6 mg/kg dose in both experiments, an interesting parallel may eventually be drawn between the functions of the alpha-5 subunit both in premature and mature $GABA_A$ receptor systems.

Due to the small sample size in all of the dose groups in the second experiment, it was impossible to infer any true differences between the separation conditions or Xli-093 dose groups. There was also no interaction effect between dose and condition as it related to time spent in the center of the open-field. The emerging pattern in behavior at certain doses is promising, and perhaps will hold true following more testing.

The findings from both experiments suggest that, although the alpha-5 subunit is not traditionally implicated in the anxiolytic properties of benzodiazepines (Tan et al., 2011), there might be some relationship between alpha-5 subunit-containing $GABA_A$ receptors and anxiety-related behavior. Administration of the preferential antagonist Xli-093 produced interesting changes in anxiety-related behavior. As an antagonist, the primary function of Xli-093 is to bind to the receptor and block the agonist from binding and subsequently potentiating neurotransmission (Dingledine, Iversen & Breuker, 1978). Commonly, antagonism studies investigate simultaneous administration of an agonist. The binding of these antagonists produce effects that are not supposed to alter behavior (Sanders & Shekhar, 1995); however Xli-093 produced an anxiogenic effect that seemed as though it could become statistically significant. The fact that Xli-093 produced some changes in anxiety-like behavior in the absence of benzodiazepine administration suggest

that the effects of the alpha-5 subunit antagonist may have arisen from an interaction with existing endogenous benzodiazepines that normally bind to the receptor, and that the alpha-5 subunit could somehow be implicated in the experience of anxiety in the absence of any drug administration (Izquierdo & Medina, 1991).

The existence of endogenous positive modulators of the benzodiazepine site has been disputed since the discovery of the binding site itself, and through the use of techniques such as radioimmunoassays with antibodies for known benzodiazepines, several compounds were identified as possible candidates (Izquierdo et al., 1990). Nicotinamide, a compound isolated from acetone extracts extracted from bovine and rat brains, is one such endogenous modulator. It was shown to have a low affinity for the benzodiazepine site, a trait that is not uncommon in the endogenous ligands that have been identified thus far; however administration of nicotinamide restored punishment-suppressed behavior in rats (Tallman et al., 1980). Its ability to bind and cause anxiolysis may explain why some antagonist studies found anxiogenic effects (Sanders & Shekhar, 1995) in the absence of benzodiazepine administration. The apparent effects of the alpha-5 preferential antagonist Xli-093 on anxiety-like behavior suggest that some sort of alpha-5 subtype specific endogenous agonist exists and may be responsible for the effects seen in the present study.

When thinking about the clinical relevance of the present study, it is important to consider that one of the overarching goals of most $GABA_A$ alpha subunit research on anxiety is to identify the role of each of the alpha subunits (Rudolph & Mohler, 2004). Findings from studies targeting specific subunits contribute to the creation of compounds that produce therapeutic effects, without inducing any nonspecific changes in behavior or

function (Rudolph & Mohler, 2006). If a pharmacological treatment modeled after benzodiazepines is developed for anxiety, and includes an alpha-5 subunit antagonist to alleviate the cognitive impairments of benzodiazepines, it is important to know whether or not the presence of the antagonist will also have an anxiogenic effect. A concrete involvement of the alpha-5 subunit in anxiogenesis cannot be confirmed at this time (Tan et al., 2011), however, results from the present study necessitate future research using a combination of Xli-093 and a benzodiazepine-site agonist. This research would clarify whether or not specificity and the desired level of anxiolysis from benzodiazepine-like compounds can be achieved in the presence of an alpha-5 antagonist.

Several problems arose during the course of this study. Following the completion of experiment one, translation of the Xli-093 drug from pups to adults showed that the vehicle used in the brief separation procedure produced a markedly sedative effect in the adult mice when administered during open-field testing. Pilot studies (see Appendix) were conducted in order to determine a means of eliminating this vehicle effect, and eventually led to the use of 20% 2- hydroxylpropyl β-cyclodextrine for adult intraperitoneal injections of Xli-093. Previous research has shown that 2- hydroxylpropyl β-cyclodextrine is commonly used to overcome solubility issues in relatively insoluble compounds via molecular encapsulation and is relatively biologically inert (Pitha et al., 1986).

The discovery of the ability of the 20% 2- hydroxylpropyl β-cyclodextrine vehicle to suspend the Xli-093 compound improved data collection for the adult mice, however the change in vehicles complicated the possibility to compare these findings with pup results. In order to eventually draw parallels between adult mice and pups, testing should

be repeated in the pups with all of the doses of Xli-093 used in experiment one, but suspended in the 2- hydroxylpropyl β-cyclodextrine vehicle. This may also change the effects of the different doses on the locomotor behavior and motor coordination if the previous vehicle had a sedative effect on the pups as well as the adults. If the trend from previous studies holds true, no significant differences should become apparent between any of the doses.

In order to make this study more reliable, several measures could have been implemented to improve data collection during the brief maternal separation procedure. When examining the effects of the agonist and the antagonist on the locomotor behavior and motor coordination of the pups, manual counters were used to record the occurrence of each behavior. The experimenter was reliable at identifying and recording these behaviors, but to ensure that each instance of the behaviors was noted, video recordings of the test session could have been used. By allowing the experimenter to video record the sessions as well as measure the behaviors manually during testing, the occurrence of the behaviors could have been checked to make sure that the final counts for each instance of behavior were recorded. Video recordings of the test sessions could also have allowed for the inclusion of new factors for analysis such as the duration of body roll behavior, which would have provided a more accurate behavioral description of the effects of each of the compounds. Video recorded data would have served to eliminate the possibility of oversights in data collection due to errors on the part of the experimenter, as well as provide a more in-depth picture of the behavioral changes associated with administration of the various compounds.

One of the most interesting aspects of the experimental design was the use of the maternal separation paradigm to elicit anxiety-like behavior in both experiments. This paradigm of early-life stress in rodents allowed for the examination of the connections between early adverse event-related developmental changes and behavior (Caldji, Diorio & Meaney, 2000). In the brief separation procedure, the maternal separation paradigm was used to induce quantifiable anxiety-related behavior that could be pharmacologically manipulated (Hofer et al., 1993). The trend towards increases in USVs following administration of the antagonist provided support for the findings of previous studies that claimed that maternal separation could be used engender "anxiety" in developing systems, and that USVs were a legitimate means of quantifying this because they were susceptible to manipulation via anxiety-altering compounds (Winslow & Insel, 1991; Zimmerberg et al., 1993).

The second experiment provided a venue through which the long-lasting behavioral effects of maternal separation, coupled with pharmacological manipulations in an acute, anxiety-eliciting situation could be assessed. Subjecting the pups to long-term separation and testing them in conjunction with non-separated animals in adulthood proved useful in assessing changes in adult behavior. It allowed for the identification of any differences that existed between the groups in the level of susceptibility to anxiety-related behavior following exposure to an acute stressor. Findings from this experiment were inconclusive, but with further testing, evidence of increased thigmotaxis behavior in separated animals compared to non-separated animals would provide support for the existing notion that early life stress begets higher levels of "anxiety" in adulthood (Boccia & Pedersen, 2001; Wigger & Neumann, 1999). Further, if a difference is seen

between the separated and non-separated animals in anxiety-related behavior following administration of Xli-093, perhaps a specific neurotransmitter structure that was previously thought to be unrelated (i.e. alpha-5 subunit containing $GABA_A$ receptors) could be identified as one of the structures that undergoes long-lasting alterations following exposure to early -life stress (Rudolph & Mohler, 2006). If future testing determines that some anxiogenesis occurs in the presence of the antagonist, and that early-life stress potentiates this antagonist effect, the clinical use of compounds targeting the alpha-5 subunit containing receptors could improve treatments based on the patient's history of exposure to these kinds of events.

The interaction between biological and environmental factors illustrated in the present study has been a prominent topic of study in psychological research, and is implicated in the susceptibility of individuals to a vast array of mental illnesses (Heim & Nemeroff, 2001). Studies in humans are difficult to perform, due to the lack of controllability necessary to infer any causal relationships between brain-structure changes and environmental events, however several studies have found some structural and functional neurobiological differences in individuals that were subjected to early life stress (Gunnar & Quevedo, 2007). The most likely candidates for neurodevelopmental alterations following early life stress are the corticotropin releasing factor and $GABA_A$ neurotransmitter systems (Heim & Nemeroff, 2001). In particular, studies point to hypersecretion and a general overabundance of corticotropin releasing factor, whose actions on the hypothalamic-pituitary-adrenal axis and within other regions of the central nervous system are crucial to the stress response (Baker et al., 1999). A decrease in the density of $GABA_A$ receptors in the frontal cortex, locus coeruleus and several nuclei of

the amygdala has also been noted in both postmortem clinical and preclinical studies (Heim & Nemeroff, 2001). This interaction is just one of the few factors that contribute to the predisposition of an individual to anxiety and affective disorders.

Genetic variability also plays an important role in the etiology of mental illness (Jaffee & Price, 2007). One of the most well known examples of the power of the genetic and environmental interaction is the serotonin transporter gene (5-HTT). Caspi et al., (2003) examined a functional polymorphism of the 5-HTT gene as it relates to the development of depression, and found that individuals who had one or two copies of the short allele of the polymorphism were more likely to develop depression following exposure to a certain number of stressful life events than those individuals that were homozygous for the long allele. Additionally, the study found that exposure to early-life stress events, such as childhood maltreatment, predicted the development of depression in adulthood in individuals who were heterozygous or homozygous for the short allele.

Findings on the genetic variability associated with the $GABA_A$ receptor system and mental illness are less concrete than those observed for serotonin; however some key findings have been noticed as well. Variations in the genes that code for the beta-1 and alpha-1, 3, 5, and 6 subunits have been noted in unipolar and bipolar treatment-resistant depression and anxiety. Altered expression of $GABA_A$ receptor genes has also been observed, suggesting a decrease in receptor density in the cortex of depressed patients (Kalueff & Nutt, 2007).

Both the 5-HTT gene polymorphism data as well as the genetic differences observed in $GABA_A$ receptors that have been linked to depression and anxiety disorders help to highlight the idea that a complex combination of factors can contribute to the

development of these disorders. Some individuals may be more susceptible to illness following the same exposure to environmental and biological changes, simply on the basis of genetic differences (Caspi et al., 2003).

The overall goal of the present study was to examine the influence of the interaction between environmental events and biological factors on anxiety. Using the maternal separation paradigm, the effects that exposure to stressful environmental factors early in life had on anxiety related behavior in adulthood were examined. The present study was not able to account for the possibility that genetic differences may also contribute to the alterations in anxiety-like behavior demonstrated by the subjects in the second experiment.

Findings from this study contributed to the behavioral information about certain biological structures and how they relate to environmental manipulations, but did not analyze the specific neurodevelopmental changes associated with early life stress. The paradigms used in the study were not designed to map the trajectory of neurodevelopmental changes that contributed to the behaviors observed in adulthood. As a future direction, it may be beneficial to follow subjects throughout the course of their development and to examine morphological, functional and behavioral changes related to anxiety at various points in their lifetimes, as opposed to testing subjects at specific time points in their development. This way, the effects of specific subunit manipulation, in conjunction with environment-related changes over time could be directly connected to anxiety-related behaviors. Once this is accomplished, the same neurodevelopmental pathway could be investigated in humans in order to solidify our understanding of the interaction and how it relates to the development of anxiety.

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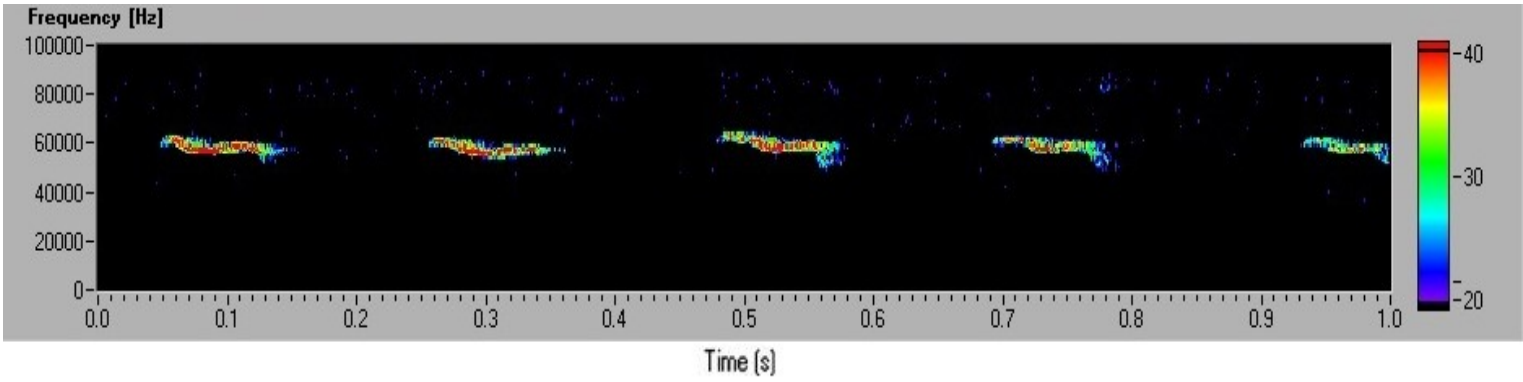

Figure 1. Sonogram of the ultrasonic vocalizations (USVs) produced during testing of the Xli-093 compound in 7-day-old CFW mouse pups. The frequency (Hz) of each of the calls, as well as their relative intensities over a period of one second (s) is depicted.

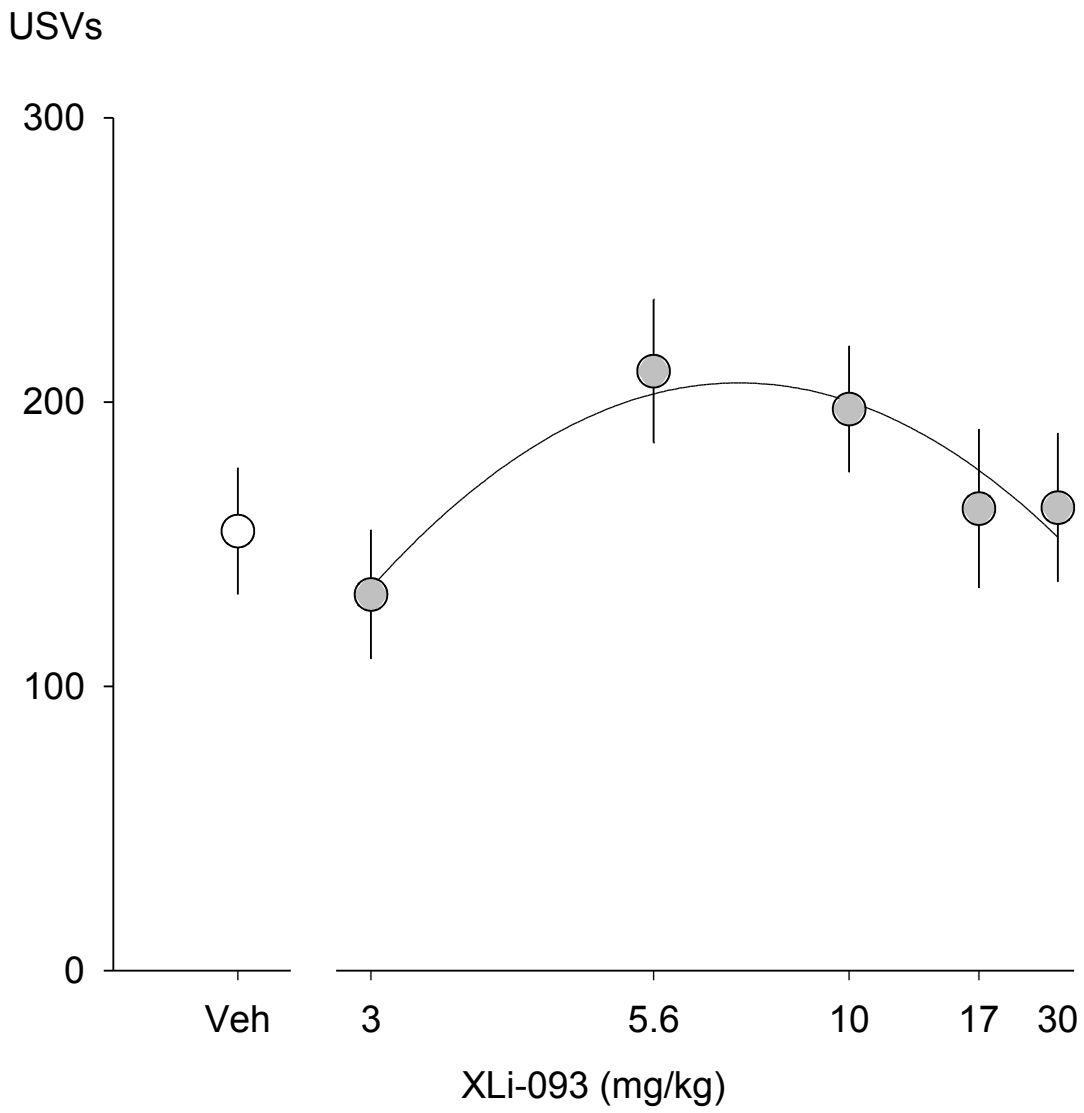

Figure 2. Amount of ultrasonic vocalizations (USVs) produced during testing for each of the dose groups. No significant difference was found between the doses in the amount of USVs produced (F (5,93)= 1.376, p> 0.05). Standard deviations are represented in the figure by the error bars attached to each point.

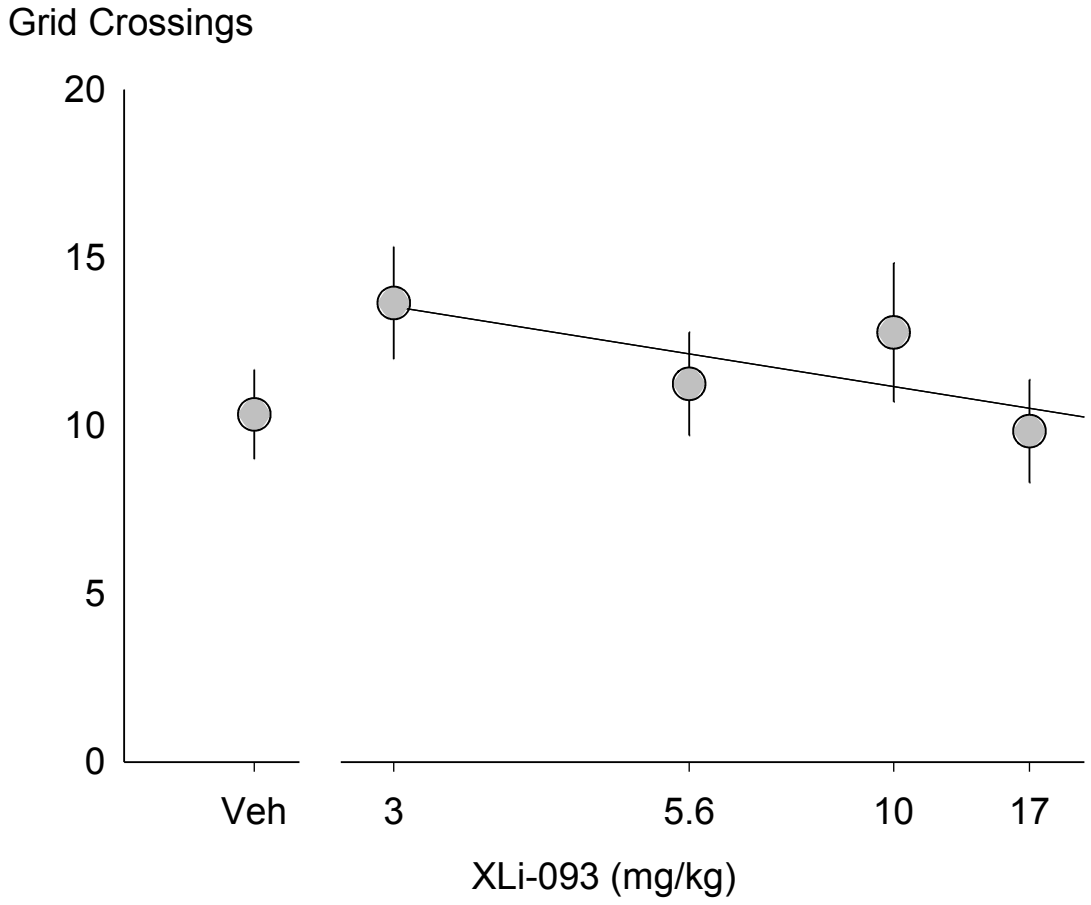

Figure 3. Locomotor activity, quantified as the number of grid crossings made during testing for each of the dose groups. No significant difference was found between the doses in the amount of grid crossings ($F(5,93) = 0.931$, $p > 0.05$). Standard deviations are represented in the figure by the error bars attached to each point.

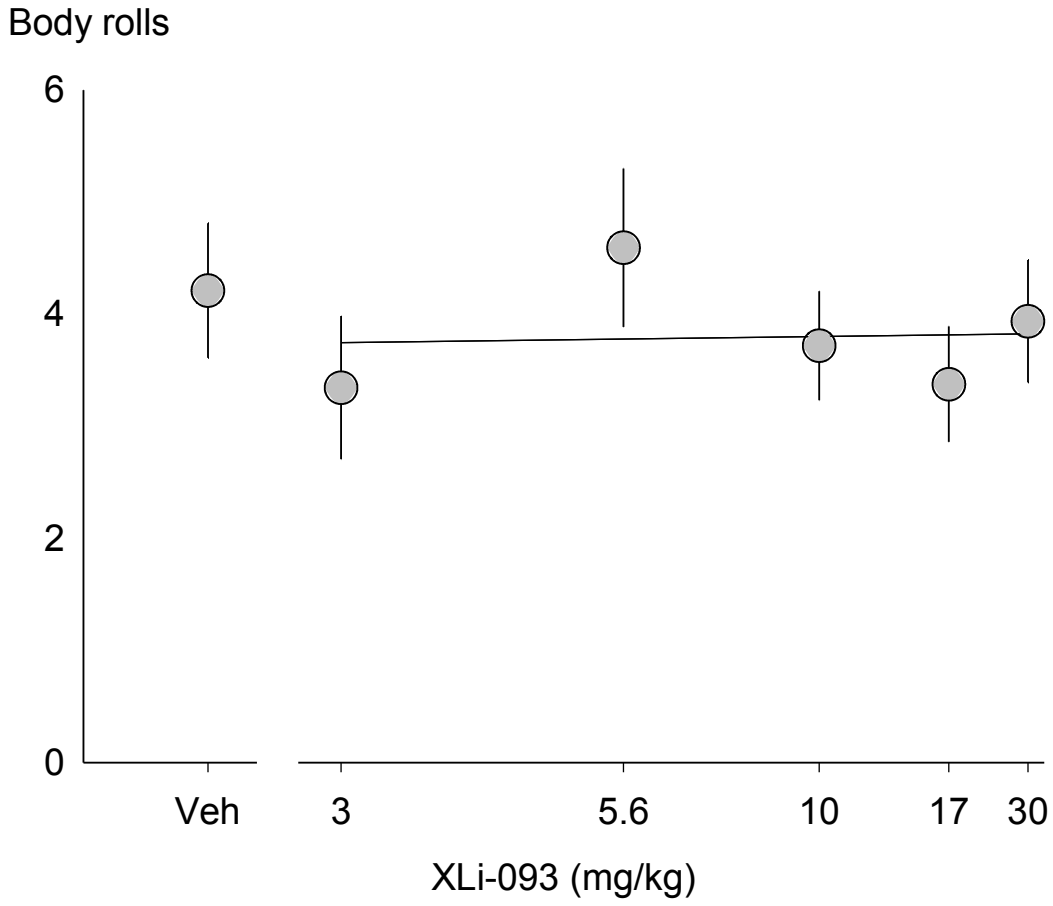

Figure 4. Motor coordination, quantified as the number of body rolls observed during testing for each of the dose groups. No significant difference was found between the doses in the amount of body rolls ($F(5,93) = 0.676$, $p > 0.05$). Standard deviations are represented in the figure by the error bars attached to each point.

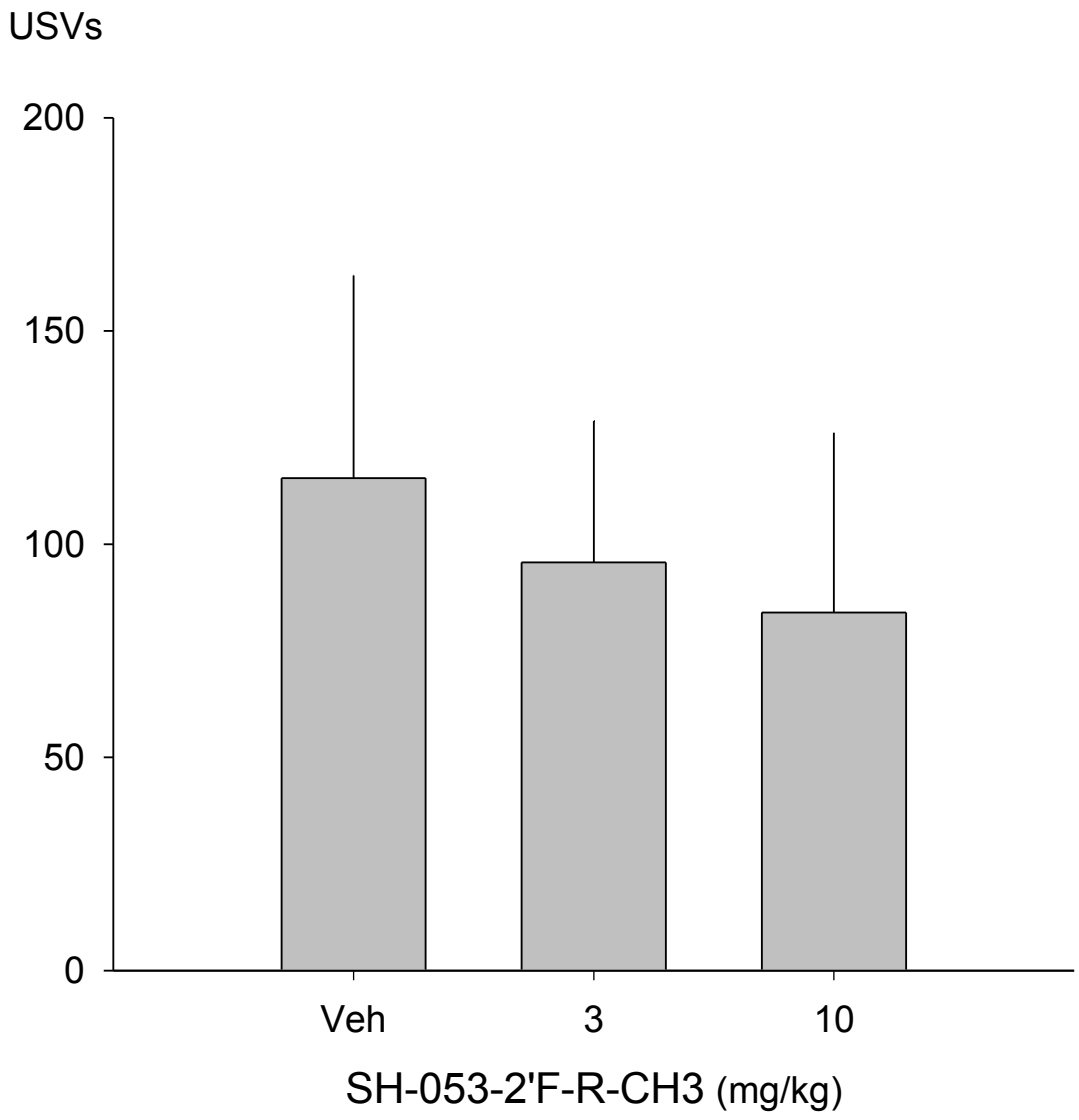

Figure 5. Number of USVs produced during testing for each of the dose groups. No significant difference was found between the doses in the amount of USVs ($F(2,16) = 0.146$, $p > 0.05$). Standard deviations are represented in the figure by the error bars on each bar.

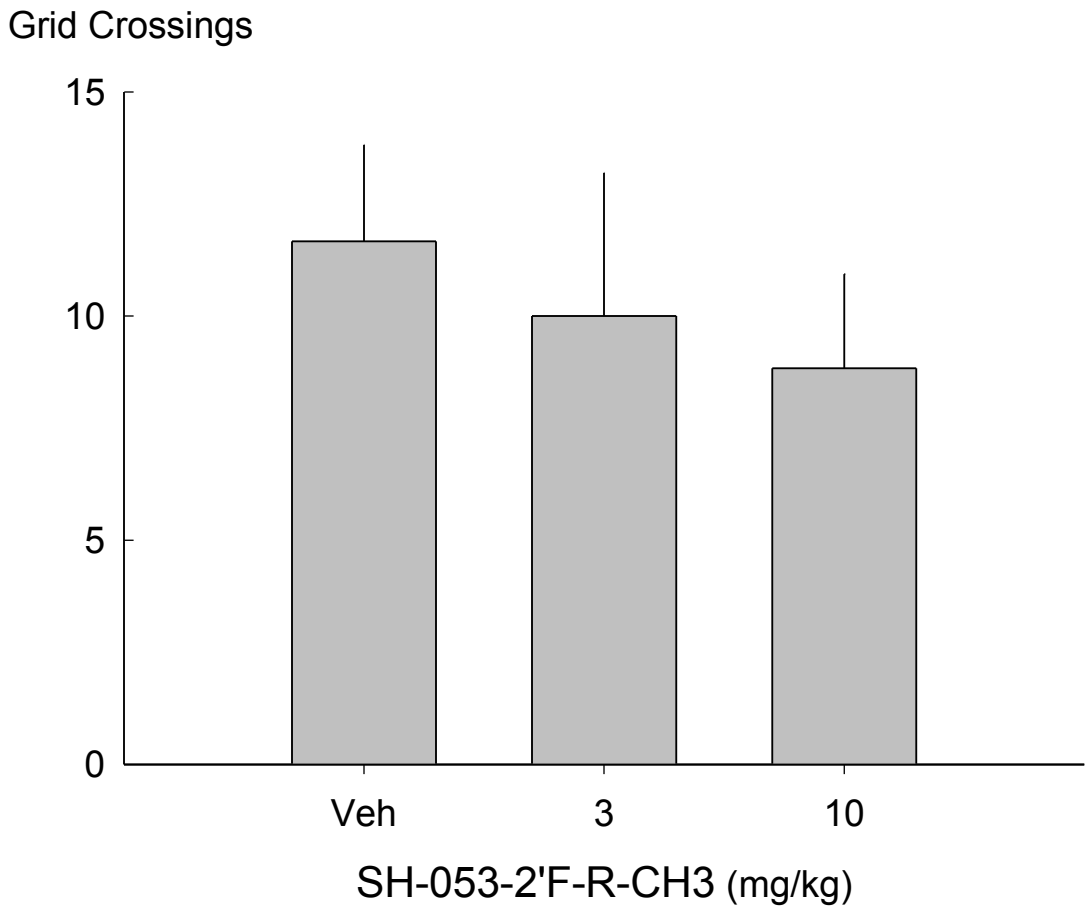

Figure 6. Locomotor activity, displayed as the number of grid crossings made during testing for each of the dose groups. No significant difference was found between the doses in the amount of grid crossings ($F(2,16) = 0.278$, $p > 0.05$). Standard deviations are represented in the figure by the error bars on each bar.

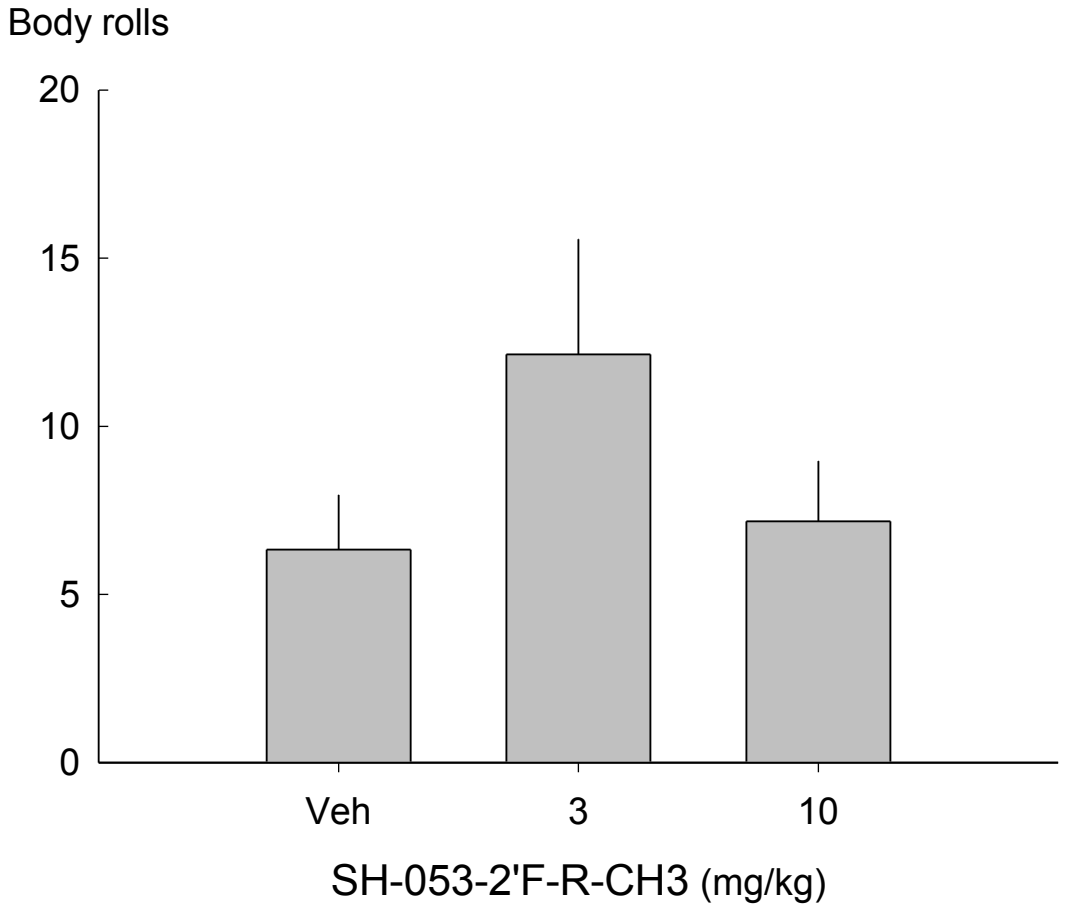

Figure 7. Motor coordination, quantified as the number of body rolls made during testing for each of the dose groups. No significant difference was found between the doses in the amount of body rolls ($F(2,16) = 0.234$, $p > 0.05$). Standard deviations are represented in the figure by the error bars attached to each point.

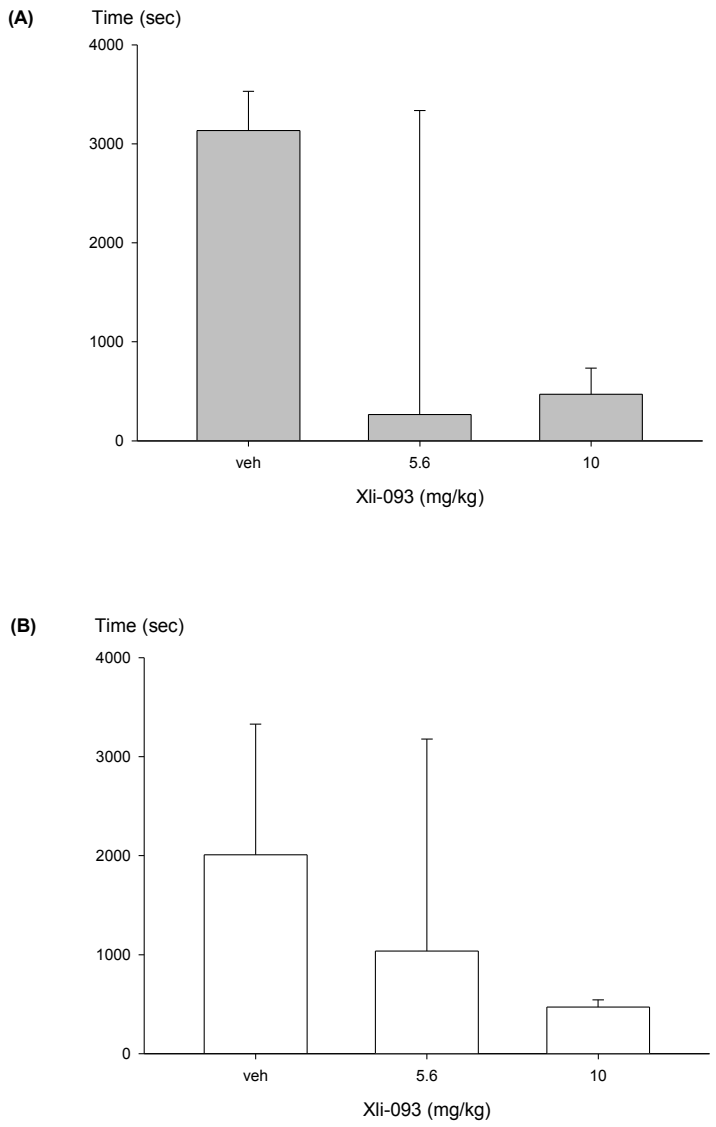

Figure 8. Median amount of time (seconds) spent in the center of the open-field following vehicle or drug injection for maternally separated (A) and control subjects (B). No significant difference was found between the doses or the conditions. There was no significant interaction between the dose group and the condition ($F(2,14) = 0.168$, $p > 0.05$). Bars indicate the inter-quartile range for each of the points.

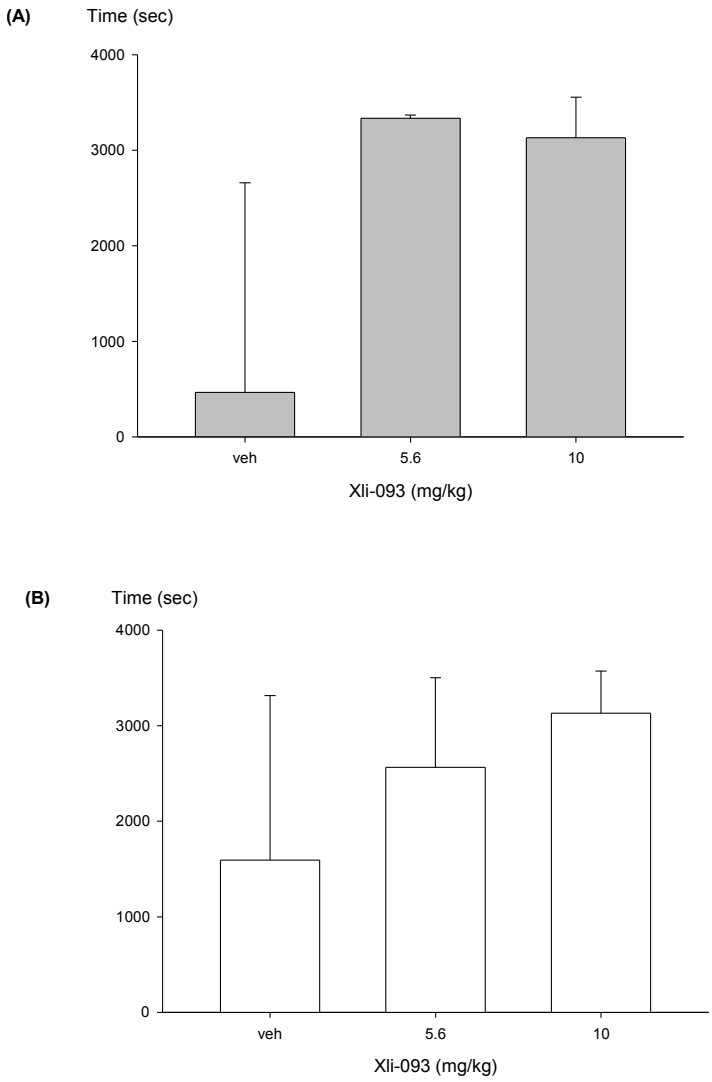

Figure 9. Median amount of time (seconds) spent in the surround of the open-field following vehicle or drug injection for maternally separated (A) and control subjects (B). No significant difference was found between the doses or the conditions. There was no significant interaction between the dose group and the condition ($F(2,14) = 0.168$, $p > 0.05$). Bars indicate the inter-quartile range for each of the points.

Table 1. The effects of classical benzodiazepine site ligands on USVs, locomotor activity and motor incoordination

Treatment	Role	Ultrasonic Vocalizations (USVs)	Locomotor Activity	Motor Incoordination	Reference
Fluintrazepam	Nonselective agonist	↓	↓	↑	Takahashi et al., 2009
Bromazepam	Nonselective agonist	↓	↓	↑	Takahashi et al., 2009
Chlorodiazepo-xide	Nonselective agonist	↓	↓	↑	Takahashi et al., 2009
Diazepam	Nonselective agonist	↓	↓	↑	Miczek et al., 2008
Zolpidem	α1 Preferential agonist	↓	N/A	↑	Rowlett et al., 2001
Triazolam	Nonselective agonist	↓	N/A	↓	Rowlett et al., 2001
Flumazenil	Nonselective antagonist	↑	N/A	↓	Rowlett et al., 2001
β-cct	α1 Preferential antagonist	↑	—	—	Rowlett et al., 2001

Description of behavioral findings from maternal separation studies using classical benzodiazepine site agonists and antagonists in both rats and mice. All antagonists were administered in conjunction with a corresponding agonist. "↑" symbols indicate increases, "↓" indicate decreases, and "—"indicate negligible changes.

Appendix

Pilot Experiment

Subjects

The subjects used in the first portion of the experiment were male Carworth Farm Webster (CFW) mice obtained from Charles River Labs (Wilmington, MA, USA), each ranging in age and size and group housed in cages of up to 11 same sex cohorts.

Apparatus

Animals were placed into 52 x 36 x 32 cm open fields (Rubbermaid). Video tracking of their locomotor activity in the open field, as well as the ratio of times that they spent in the center and surround of the arena were done using a PC-based data acquisition system (Ethovision, VTMAS v 1.80, Noldus, Wageningen, Netherlands) that received video recordings of the animal via a camera (Cohu, Model 4815-211/A209) placed 164 cm above the open field.

Drugs

In order to identify a dosage that was ideal for producing the desired behavioral effects without causing heavy sedation, various dosages of Xli-093 and different vehicles were tested. Subjects were given either a drug dose of either 5.6 mg/kg or 10 mg/kg of Xli-093 dissolved in 50% Propylene Glycol and 50% distilled H_2O, a vehicle dose of 50% Propylene Glycol and 50% distilled H_2O, a drug dose of 10mg/kg of Xli-093 suspended in 20% (2-Hydroxypropyl) β-cyclodextrine (dissolved in 10ml of distilled H_2O), or a vehicle dose of 20% (2-Hydroxypropyl) β-cyclodextrine. All compounds were given via intraperitoneal injection, and each animal received 10 mg/kg of drug (based on their body weight).

Procedure

Four subjects were single housed, and given injections of saline every day for three to five days prior to the test session. The injection served as a means of acclimatizing the mice to the handling and injection process. New subjects were used for each of the four experiments, in order to ensure that they were naïve to open field environment. The subjects were moved into the room containing the open field apparatus on the day of testing, and were tested simultaneously in one of four open field arenas.

The first phase of testing was the habituation phase, where animals were placed into the open field arena for 30-40 minutes, and their locomotor behavior was recorded. Following the habituation phase, they were removed from the open field arenas and placed back into their home cages. They were then given an injection of vehicle (either 50% propylene glycol and 50% distilled H_2O or 20% or 20% (2-Hydroxypropyl) β-cyclodextrine) and their locomotor activity in the open field was recorded for 15 minutes. The subjects were removed from the open field replaced in their home cages again, and were given another injection of either one of the aforementioned vehicles, 5.6 mg/kg or 10 mg/kg of Xli-093 in either of the two vehicles. Their locomotor activity in the open field was recorded for 60 minutes, after which they were returned to their home cages.

Data Analysis. For each of the phases of the experiment, the mean distance traveled by each of the four mice and the standard deviations were calculated. In order to visually compare the activity levels of each of the mice in response to the compounds that they received in each phase, a graph was generated using Sigmaplot software.