BEHAVIORAL ASSOCIATIONS WITH A SEROTONIN TRANSPORTER GENE POLYMORPHISM IN YOUNG RHESUS MACAQUES

by

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Abstract

Anxiety is a normal aspect of human personality which can manifest in a variety of disorders and other negative traits. The social cost of anxiety traits and disorders was 42.3 billion in 1990 alone. The primary treatment for anxiety is the class of drugs known as the selective serotonin re-uptake inhibitors (SSRI's), which bind to the serotonin transporter. The transporter is regulated by a variety of intrinsic and extrinsic modulators, including hormones, phosphorylation state, and genetic inheritance. Genetic polymorphisms are an important part in the study of human biology, and one particular polymorphism in the serotonin transporter, the 5HTTLPR, is thought to be involved in the genesis of anxious traits and disorders. To date, this link is controversial, since association studies are difficult to replicate. We hypothesize that this difficulty in replicating these 5HTTLPR studies lies in two possibilities, (1) diverse experiential and environmental backgrounds of the test subjects such as differences in age or lifetime experience or (2) that the 5HTTLPR is only involved in regulating very specific anxious behaviors. In order to address this hypothesis, we genotyped 128 infant and juvenille monkeys for the 5HTTLPR and analyzed the behavior of 90 of them. The behavioral analysis took the form of four standardized tests, a free play, remote controlled car, human intruder and novel fruit test. The s/s monkeys were found to be behaviorally inhibited in the free play test, engaged in more fear behaviors in the remote controlled car test, and threatened more in the stare portion of the human intruder test. This data indicates greater anxiety in the s/s monkeys only for certain behaviors and supports our hypothesis that the 5HTTLPR may play a role in regulating specific anxious behaviors.

Chapter 1: Introduction

Anxiety

At the intersection of psychology and biology is the field of personality research. It is clear, especially amongst higher primates, that personality traits are an important component of social interaction and cooperation, and thus in many cases survival. One unified theory that explains the underlying basis of personality and how individual traits and pathologies can arise is the tri-dimensional model by Cloninger (Cloninger 1987), that examines personality in terms of three basic stimulus-response characteristics, namely novelty seeking, harm avoidance, and reward dependence. The intersection of scoring in these three dimensions, especially in the extremes (high or low) of any particular dimension, relates closely to described personality disorders. One of the implications of this theory is that the same basic structure underlies both normal and pathological personality traits.

One such normally distributed personality trait, anxiety, has been described extensively in humans. Using the tri-dimensional model, humans with anxiety and affective disorders score highly on the harm avoidance scale (Ball et al. 2002). Anxiety is also defined medically as "apprehension of danger and dread accompanied by restlessness, tension, tachycardia and dyspnea unattached to a clearly identifiable stimulus" (Spraycar 1995). Anxiety also has a high human cost. Pathological anxiety manifests in a variety of disorders, including post-traumatic stress disorder, social anxiety disorder and generalized anxiety disorder (Ollendick and Hirshfeld-Becker 2002; Ballenger 2001; Shalev 2001). These types of anxiety disorders are also frequently co-

morbid with other mental disorders such as depression (Melartin et al. 2002). Overall, anxiety disorders had an estimated cost of 42.3 billion dollars in the US in 1990 alone (Greenberg et al. 1999; Grudzinski 2001). Clearly, it is very important to more fully understand and treat anxiety disorders.

Current methods of treating anxiety disorders rely primarily on the selective serotonin reuptake inhibitors (SSRI) and behavioral therapy (Charney et al. 2002; Greist et al. 2002), although other treatments such as norepinephrine reuptake inhibitors are also used (Versiani et al. 2002). However, these therapies are not always effective, and unpleasant side effects, such as sleep disorders or sexual side effects, can complicate treatment. Thus, there is a need to elucidate more pharmacological targets and to understand the biology of anxiety more thoroughly.

One important area of anxiety research is to determine how anxiety as a personality trait, and anxiety disorders in particular, develop. One possible factor is genetics. Using twin studies (Plomin et al. 1994), it has been found that 40-60% of the variation observed in anxiety related personality traits is inherited. Clearly, variations in particular genes will be very important in understanding how anxiety traits and disorders develop. Environmental and experiential factors also play an important role in anxiety traits, however, with factors such as maternal separation or parental psychopathologies contributing significantly to the development of anxiety disorders (Hessl et al. 2001; Feigon et al. 2001).

Further complicating the understanding of anxiety traits is the contribution of different systems in the brain to different aspects of anxiety. For example, different

aspects of a young monkey's response to a threatening stimulus were differentially regulated by the GABAergic and opioid systems (Kalin and Shelton 1989). In this study, the GABA agonist diazepam reduced barking, freezing and crouching in response to a human intruder, while the opioid agonist morphine and antagonist naloxone both reduced and increased, respectively, cooing behavior due to maternal separation while failing to affect barking, freezing and crouching in response to the human intruder.

Serotonin

The serotonergic nervous system originates in the midbrain. The soma of the neurons that project to forebrain regions are all located within the dorsal and median raphe nuclei (Paxinos et al. 2000). From these nuclei, axons project to most of the areas of the forebrain, including the cortex and hypothalamus. Serotonergic neurotransmission contributes to many physiologic functions such as motor activity, food intake, sleep, reproductive activity, and cognitive states relating to mood and anxiety (Chen et al.1992).

There are a number of proteins expressed in serotonin neurons and on post-synaptic targets that are critical for serotonin neurotransmission. One pivotal protein is tryptophan hydroxylase (TPH), that catalyzes the rate limiting step in the synthesis of serotonin. Allelic variations in TPH have been shown to be associated with a decreased clinical efficacy of antidepressants such as paroxetine (Serretti et al. 2001; Serretti et al. 2001[2]). The serotonin 1A autoreceptor (5HT1A) is a pre-synaptic receptor that mediates a negative feedback inhibition of serotonergic neurotransmission. Antagonism of 5HT1A with p-MPPI has anxiolytic effects with mice in the elevated plus maze (Cao

and Rodgers 1997). The serotonin re-uptake transporter (5HTT) has been the target of a great deal of interest since this protein regulates the magnitude and duration of serotonergic neurotransmission, and is the primary target of the SSRI antidepressants. Lastly, there are 14 different post-synaptic serotonin receptors that mediate the effects of serotonin on the target cells.

Through these various protein components, the serotonergic nervous system is regulated in a variety of ways. Hormones appear to be important regulators of the serotonin system. Estrogen has been shown to increase the mRNA levels of TPH, and decrease the mRNA levels of 5HT1A, 5HTT and monoamine oxidase A (MAO-A) (Bethea et al. 2002). Similarly, hydrocortisone has been shown to increase the catalytic activity level of TPH in the brains of rats (Park et al. 1989). Serotonin neurons have also been shown to be regulated by glutamatergic, GABAergic and norepinephrine neurons (Celada et al. 2001; Blier 2001). Lastly, serotonin neurons can be regulated intrinsically through variations in relevant genes. Genetic polymorphisms, such as in the 5HTT, have been shown to alter the prolactin response to fenfluramine challenge, which implies decreased serotonergic neurotransmission, decreased levels of 5HT2A/C receptors, or both (Reist et al. 2001). Such polymorphisms may have consequences for the development of serotonin related pathologies.

Brain Circuitry of Anxiety

Several brain systems have been strongly implicated in anxiety related traits. One such system is Neuropeptide Y, where it has been shown that direct application of NPY

to the lateral septal complex increases social investigatory behavior in the rat Social Interaction test model. Increases in social investigatory behavior is considered an anxiolytic type effect, and the action of NPY could be reversed with the Y1 receptor antagonist BIBO3304 (Kask et al. 2001). Another candidate system is norepinephrine, although it is possible that norepinephrine is acting through the serotonergic system, since SSRI treatment has been shown to decrease the firing rate of norepinephrine neurons (Blier 2001). Lastly, the serotonergic system has been strongly implicated. 5HT1B receptor stimulation with the specific agonist CP 94,253 decreases exploratory behavior in rats in the Elevated Plus Maze paradigm (Lin and Parsons 2002). Furthermore, administration of the serotonin releaser MDMA (Ecstacy), has been shown to have anxiogenic effects in rats in the Elevated Plus Maze, Social Interaction, and Emergence tests (Morley et al. 2001). Furthermore, the SSRI's, which target the serotonin re-uptake transporter (5HTT), are one of the primary pharmacologic treatments for anxiety disorders.

Brain Circuitry of Fear Conditioning

One important model for the elucidation of the circuitry of anxiety is classical, or Pavlovian, fear conditioning. In this fear paradigm, the organism learns to associate a fearful stimulus (i.e. electric shock) with a conditioned stimulus (i.e. audio tone), so that the conditioned stimulus alone will produce a negative reaction when no actual fearful stimulus is present. Conditioned fear has many paralells with both anxiety and anxiety disorders, such as the conditioned negative affect to public speaking associated with Social Anxiety Disorder.

In the model of fear conditioning developed by LeDoux and collaborators, the conditioned and unconditioned stimuli are transmitted to the lateral nucleus of the amygdala via the appropriate sensory pathways, such as the auditory cortex or auditory thalamus (Blair et al. 2001). Lesion studies have supported this initial pathway, in that lesions of the auditory cortex or thalamus have prevented or slowed the acquisition of conditioned fear (Shi and Davis 1999). Further evidence consists of single unit recordings demonstrating conditioning of amygdala neurons (i.e. long term potentiation) in response to auditory signals passed through these two pathways, with faster conditioning displayed in response to auditory signals mediated by the thalamic pathway (Quirk et al. 1995).

In the lateral nucleus of the amygdala, there is evidence that the association between the aversive and neutral stimuli is accomplished via long term potentiation, a specific form of neuronal plasticity that enhances synaptic transmission (Schafe et al. 2001; Rogan et al. 1997). This long term potentiation serves as a form of memory, which would appear in this case to preserve the association between the fearful and neutral stimuli for future retrieval. One piece of evidence to support this interpretation is the observation that the specific NMDA receptor subunit NR2B antagonist ifenprodil dose-dependently decreased the acquisition of conditioned fear (i.e. more trials would be required for the association to occur) in rats when directly applied to the lateral nucleus of the amygdala (Rodrigues et al. 2001). In a similar manner, application of the glutamate receptor subunit mGluR5 specific antagonist MPEP blocked the acquisition of long term potentiation in the lateral nucleus of the amygdala in an *in vitro* slice

preparation, as well as the acquisition of conditioned fear in rats (Rodrigues et al. 2002). Blockade of L-Type voltage gated calcium channels was shown to block the acquisition of long term conditioned fear in rats, as well as long term potentiation in an *in vitro* slice preparation, an effect mirrored by general NMDA blockade (Baeur et al. 2002). This evidence supports the interpretation that conditioned fear is mediated by excitatory glutamate neurons in the lateral nucleus of the amygdala, which induces long term potentiation mediated by L-Type calcium channels.

From the lateral nucleus, there is evidence that the signal is further processed by other nuclei of the amygdala, particularly the central nucleus of the amygdala, from which efferent projections leave the amygdala and project to other brain regions. The primary observations to support the role of other amygdala nuclei come from lesion studies. One particular study demonstrated that bilateral lesions of the lateral and central nuclei only eliminated the acquisition of conditioned fear (Nader et al. 2001). However, bilateral lesions of other amygdala nuclei such as the basal nucleus had no discernable effect. In a separate study, bilateral lesion of the basal nucleus alone had no discernable effect on the acquisition of conditioned fear (Amorapanth et al. 2000). In contrast, another study found that lesion of the basal nucleus inhibited the acquisition of both acoustic and contextual conditioned fear (Goosens and Maren 2001). The weight of the evidence would suggest that the lateral and central nuclei of the amygdala are necessary and sufficient for conditioned fear learning. This is the most parsimonious amygdala pathway, since the lateral nucleus connects directly to the central nucleus, which then projects out of the amygdala. The possible role of other amygdala nuclei such as the

basal nucleus remains undetermined, and these nuclei may have a modifying rather than a critical role in the circuitry of fear conditioning.

Further evidence would suggest that the conditioned fear signal is passed onto and further modified by other distinct brain regions after the amygdala. Lesions of the dorsal and ventral aspects of the medial prefrontal cortex has been shown to enhance the expression of conditioned fear behavior (freezing in response to the tone) (Morgan and LeDoux 1995; Morgan et al. 1993). Furthermore, Morgan et al. (1993) demonstrated that the ventral lesions had no effect on the acquisition of conditioned fear, while the dorsal lesions enhanced conditioned fear acquisition (Morgan and LeDoux 1995). This evidence suggests a region specific role for the medial prefrontal cortex in different aspects of fear conditioning, such as acquisition for the dorsal aspect and behavioral reactivity for both dorsal and ventral aspects.

The hippocampus has been suggested to play a role in the contextual aspects of fear conditioning. Contextual conditioning involves the association of static, continually present stimuli such as an object in an animal's cage with an aversive stimulus. Contextual conditioning is further divided into foreground and background conditioning, with background contextual conditioning occuring with a conditioned stimulus present such as an auditory tone (i.e. the tone is the primary stimulus for conditioning) while background conditioning occurs in the absence of a conditioned stimulus (i.e. contextual cues become more important in the absence of a conditioned stimulus to focus on). Phillips and LeDoux (1994) demonstrated that lesions of the dorsal hippocampal formation in rats prevented the acquisition of background contextual conditioning, while

leaving foreground contextual conditioning unaffected. A separate lesion study using the same methodology also demonstrated the inhibition of contextual conditioning with hippocampal lesion, further supporting the role of the hippocampus in contextual fear conditioning (Phillips and LeDoux 1992). In other brain regions, lesions of the fornix, perirhinal and entorhinal cortices all appear to inhibit contextual conditioned fear learning in a similar manner to the hippocampus, although these studies did not discriminate between foreground and background conditioning (Corodimas and LeDoux 1995; Phillips and LeDoux 1995).

The last step in this model of conditioned fear is the passage of the modified conditioned fear signal to regions of the brain responsible for somatic effects, such as the hypothalamus. Electrolytic and ibotenic acid mediated lesions in the lateral hypothalamus eliminated the increase in arterial pressure in response to a conditioned stimulus, but not freezing behavior (LeDoux et al. 1988). This evidence is supported by an earlier study, also using ibotenic acid lesions in the lateral hypothalamus, which also found decreases in conditioned autonomic, but not behavioral, response. No change was discernable with lesions of the medial hypothalamus (Iwata et al. 1986). The interpretation of this evidence in LeDoux's model of conditioned fear indicates a role for the hypothalamus as an autonomic endpoint to conditioned fear associations accomplished in higher brain centers such as the amygdala.

A great deal of evidence has been amassed to support this model of conditioned fear and anxiety, especially for the role of the amygdala. Blockade of NMDA receptors in the lateral nucleus of the amygdala blocks the acquisition of conditioned fear

(Rodrigues et al. 2001). Similarly, blockade of glutamate receptors prevents the acquisition of conditioned fear *in vivo*, and prevents long term potentiation in the lateral nucleus of the amygdala in an *in vivo* slice preparation (Rodrigues et al. 2002). For the intracellular pathway, the evidence suggests that protein synthesis and protein kinase A is required to transduce glutamate excitation and calcium influx into long term potentiation changes. This was suggested by infusions of anisomycin, a protein synthesis inhibitor, and Rp-cAMPS, a PKA inhibitor, into the lateral nucleus of the amygdala, which blocked the acquistion of conditioned fear in rats (Schafe and LeDoux 2000). The MAP kinase pathway has also been suggested to be necessary for the acquisition of conditioned fear, as the MAP kinase inhibitor U0126 was able to block the acquisition of conditioned fear in rats when applied to the lateral nucleus of the amygdala, and prevented long term potentiation of the same in an *in vitro* slice preparation (Schafe et al. 2000).

On a more global scale of evidence, lesions of the amygdala in rhesus monkeys lead to decreases in anxiety and fear of objects and surroundings (i.e. a novel cage and its contents), and increases in social affiliation and confidence in the constrained and unconstrained dyad paradigm (Emery et al. 2001). However, the role of the amygdala appears to be affected by development, since amygdala lesions in neonates leads to decreased fear of objects, but increased social fear (Prather et al. 2001). In human volunteers with selective unilateral amygdalo-hippocampectomy, autonomic responses were absent when exposed to a conditioned stimulus (audio tone with pictures of negative facial expressions) in volunteers with either left or right side lesion (Peper et al. 2001).

A great deal of attention has also been focused on the medial prefrontal cortex as

evidence for this model of anxiety circuitry. Of particular predictive value, a right medial prefrontal cortex hyperactivity has been strongly associated with high levels of fear and anxiety in both monkeys (Kalin et al. 1998) and humans (Tomarken et al. 1990). Right medial prefrontal cortex hyperactivity was not only associated with fearfulness and anxiety, but was also strongly positively correlated with blood levels of cortisol (Kalin et al. 2000). Furthermore, EEG recordings of volunteers with Social Anxiety Disorder prior to an anxiety provoking stimulus (a public speech) revealed higher levels of right frontal lobe activity as compared to controls (Davidson et al. 2000).

Stress Sensitization Model of Anxiety

While LeDoux's model for the circuitry of conditioned fear is important, it represents only one type of anxiety in one context (paired auditory and footshock stimuli). Other models for the brain circuitry of anxiety are equally important to consider, especially since these models examine different types of anxiety, in different contexts. One such model is the sensitization of brain anxiety circuits to stressors due to stressful experiences or conditions early in life.

The primary experimental paradigm for this model is the newborn rat pup, removed from maternal contact for varying periods of time. With a 180 minute per day separation from postnatal day 2-14, these rats were found to have elevated levels of corticosterone, corticotropin releasing factor (CRF) and adreno-corticotropic hormone (ACTH) when compared to unseparated controls to restraint stress later in life (Heim and Nemeroff 2001). This chronic, increased sensitivity to stress was also reflected by changes in neural circuitry related to anxiety. These maternally separated rats were found

to have increased CRF mRNA in the paraventricular nucleus of the hypothalamus (PVN) and central nucleus of the amygdala, increased CRF receptor binding in the locus coeruleus and raphe nuclei, and increased levels of norepinephrine in the PVN (Heim and Nemeroff 2001; Francis and Meaney 1999). Furthermore, representing a decrease in inhibitory tone, these stress sensitized animals also displayed decreased GABA(A) receptor binding in the central and basal nuclei of the amygdala as well as the frontal cortex, decreased benzodiazapene binding site (γ2 subunit of the GABA(A) receptor) in the same, and decreased serotonin cell firing rate in response to citalopram (Heim and Nemeroff 2001; Meaney 2001).

The central brain system of this model are the CRF neurons. CRF neurons are grouped into two main populations. One resides in the PVN, and mediates the release of corticotropins such as ACTH into the portal blood stream. The other main population resides in the central nucleus of the amygdala, and projects primarily to the locus coeruleus. Thus, responding to stress sensitization as described above, increased levels of CRF may result in increased neurotransmission to the median eminence, resulting in higher levels of corticotropins in response to stress, and increased neurotransmission to the locus coeruelus. The locus coeruleus in turn projects to a variety of important regions such as the raphe nuclei and the PVN, as well as higher brain centers that may mediate the behavioral effects of anxiety. Decreases in inhibitory GABA tone mediated by early life stress may further enhance the sensitivity of this circuit to stressors, and may potentiate the expression of anxiety.

While they may appear quite different, the models of stress sensitization and fear

conditioning are mutually complementary and supportive. The elucidation of the CRF neurons as a critical mediator of stress and anxiety may provide the next part of the pathway after fear conditioning occurs in the lateral nucleus of the amygdala. The central nucleus of the amygdala has been shown to be necessary in the acquisition of conditioned fear, and the CRF neurons in this nucleus have been shown to respond to early life stress in a manner that suggests their importance in the brain circuitry of anxiety. A fear conditioned signal may be transmitted to other regions of the brain via the CRF neurons in the central nucleus, and mediate at least part of the behavioral and somatic effects of conditioned fear and anxiety via the locus coeruleus and PVN. These models are also complementary in providing an explanation for the development of anxiety disorders, as early stressors could sensitize this circuit and facilitate an abnormally high conditioned response to a stimulus, such as the abnormal levels of anxiety associated with normal social interactions with sufferers of social anxiety disorder.

Serotonin Modulation of the Circuitry of Anxiety

While not a direct component of this circuit, it is clear that serotonin neurons can modulate the acquisition and expression of conditioned fear and anxiety. The raphe nuclei have projections that terminate in important nodes in this circuit, such as the medial prefrontal cortex, the amygdala and the locus coeruleus. Serotonin neurons have been shown to have an inhibitory effect in the lateral nucleus of the hypothalamus through GABA interneurons (Stutzmann and LeDoux 1999). This indicates the possibility that serotonin can inhibit the long term potentiation that may be the basis for conditioned fear. Serotonin neurons also have been shown to inhibit the firing of

norepinephrine neurons in the locus coeruleus (Blier 2001), which may help to inhibit the expression of anxiety from CRF neurons through this nucleus. The 5HT1A autoreceptor has also been shown to be important in anxiety and fear, whereby the 5HT1A knockout has an anxiogenic effect in paradigms such as the forced swim and open field tests, whereas 5HT1A agonists such as buspirone have an anxiolytic effect (Gross et al. 2002; Gingrich and Hen 2001; Gross et al. 2000; Ramboz et al. 1998).

The effects of 5HT1A knockout or stimulation/inhibition points towards the anxiolytic effect of serotonin neurons being mediated by post-synaptic inhibitory 5HT1A receptors. This resolves the paradox of the anxiolytic effect of the SSRI's, which stimulate serotonin neuron firing, and the anxiolytic effect of 5HT1A stimulation, which inhibits serotonin neuron firing. A post-synaptic explanation harmonizes the effects of these two classes of drugs, whereby increased serotonin neuron activity, such as by SSRI treatment, inhibits the overactivity of the medial prefrontal cortex or the association of fear in the amygdala via release of serotonin into the synaptic cleft which binds to 5HT1A receptors, while 5HT1A agonists such as buspirone may bind to these post-synaptic receptors directly with an anxiolytic effect, despite a possible inhibition of serotonin neuron activity. This interpretation is supported by Gross et al. (2002), who found that tissue specific 5HT1A conditional rescue in the hippocampus and cortex only, and not the raphe, was sufficient to reduce the levels of anxiety in the 5HT1A knockout mouse back down to control levels.

Serotonin Neuron Physiology

Serotonergic neurons in general are categorized by a slow, regular discharge pattern in awake and intact animals. This activity is closely related to arousal level, in that the discharge is greatest during periods of high arousal, low in periods of awake quiescence, and nearly abolished during sleep (Jacobs and Fornal 1999). Although this discharge pattern might indicate a circadian relationship to the serotonin neural system, it has been shown that while serotonin can modulate the rodent circadian clock, the activity of serotonin is not required for melatonin entrainment (Slotten et al. 2000).

Regulation of Serotonergic Neuronal Activity

The activity patterns of serotonin neurons are also regulated by a variety of physiological inputs, among them the influence of other neurons. Serotonin neurons have been shown to express both subtypes of GABA receptor perisynaptically (Varga et al. 2002) and GABA agonists and antagonists, applied locally through microdialysis techniques in intact animals or bath application for brain slices, have been shown to alter the firing activity of serotonin neurons in both raphe nuclei (Varga et al. 2002; Bagdy et al. 2000; Liu et al. 2000; Levine and Jacobs 1992). GABA interneurons have been shown to regulate serotonin neurons both as afferents from other neuronal types, as well as tight feedback loops originating with serotonin neurons themselves (Liu et al. 2000).

Excitatory neurotransmitters have also been shown to regulate serotonin neuron activity. Glutamate has been shown to increase the evoked response of serotonin neurons in awake cats (Levine and Jacobs 1992). Furthermore, postsynaptic dopamine receptors as well as intact striatal dopamine neurons were shown to be necessary for the evoked

increases in 5HT content in the striatum and prefrontal cortex of freely moving rats (Mendlin et al. 1999). These various regulatory neurons have also been shown to impact serotonin neurons in a variety of pathways, from the tight feedback loop mentioned above to a reciprocal innervation between the dorsal raphe and the medial prefrontal cortex (Juckel et al. 1999).

Despite these regulatory pathways however, serotonin neurons have been shown to be relatively insensitive to many physiological inputs. This is well demonstrated in the work of Dr. Barry Jacobs of Princeton University, who has demonstrated the insensitivity of serotonin neurons to a variety of physiological stimuli in the awake, behaving cat model. Raphe neurons were shown to be insensitive to changes in body temperature, both cold stress (Martin-Cora et al. 2000) and heat/fever stress (Fornal et al. 1987), despite the involvement of serotonin neurons in thermostatic regulatory mechanisms via hypothalamic inputs. Similarly, changes in baroreceptor input or blood pressure failed to impact serotonin neuronal activity (Fornal et al. 1990) as well as changes in the cardiac cycle (Morilak et al. 1986). Furthermore, a variety of traumatic stressors such as physical restraint or exposure to white noise failed to activate raphe neurons (Jacobs et al. 1990), and changes in blood glucose and insulin levels were similarly ineffective (Fornal et al. 1989). Although not a forebrain projecting nucleus, the nucleus raphe magnus was also shown to be insensitive to pain, noxious stimuli, morphine, and audio/visual stimuli (Auerbach et al. 1985; Fornal et al. 1985). Clearly, the serotonin nervous system is fairly insensitive to many forms of regulation, despite being influenced by a variety of hormones as well as other neuronal types such as dopamine or GABA neurons, as well as

the demonstrated influence of peripheral chemoreceptors (Singewald et al. 2000).

5HT1A Receptors

Another important area of study in serotonin neuron physiology is the impact of the 5HT1A autoreceptor on serotonin neuron firing activity and serotonin release. The 5HT1A autoreceptor has important ramifications for human health, since the activation of this receptor is a primary candidate for the long delay in treatment time with SSRI's, and the 5HT1A is also the target of several drugs of interest in the treatment of anxiety, such as buspirone. One such drug of interest is pindolol, which has been hypothesized as an antagonist of 5HT1A somatodendritic receptors and a possible potentiating agent for SSRI treatment. However, in the awake cat, pindolol has been shown to act as a 5HT1A agonist, which mediates decreases in dorsal raphe serotonergic firing (Fornal et al. 1999). Consistent with this finding, pindolol was unable to reverse the fluoxetine mediated acute inhibition of neuronal firing in the awake cat (Fornal et al. 1999 [2]). Furthermore, pindolol was shown to mediate both a decrease in dorsal raphe activity and an increase in serotonin release in the caudate nucleus in the same cat model (Fornal et al. 1999 [3]).

Other pharmacological agents have been shown to have similar effects when activating or inhibiting the 5HT1A autoreceptor, which is consistent with this receptor's inhibitory cellular actions. The 5HT1A antagonist p-MPPI was shown to mediate an increase in neuronal firing in the awake cat (Bjorvatn et al. 1998). Similarly, the 5HT1A antagonist spiperone was shown to mediate a similar effect, while the 5HT1A agonists 8-OH-DPAT, ipsapirone and buspirone all mediated decreases in neuronal firing (Fornal et al. 1994).

Changes in the activation state of the 5HT1A autoreceptor also have a clear relationship to the release of serotonin in the synaptic cleft. Treatment with the 5HT1A agonist 8-OH-DPAT was shown to decrease extracellular levels of 5HT in the hypothalamus and caudate of the freely moving cat as measured by microdialysis (Wilkinson et al. 1991). 5HT1A activation via 8-OH-DPAT in the anaesthetized rat was also shown to decrease the release of serotonin in the locus coeruleus (Kaehler et al. 1999).

Another drug of interest due to its use as a non-benzodiazepene anxiolytic is the 5HT1A partial agonist buspirone. Before the target was known, buspirone was shown to decrease the serotonergic firing activity in the dorsal raphe nucleus, which is consistent with 5HT1A agonism (Wilkinson et al. 1987). There is some confusion as to the actions of buspirone however, since buspirone was shown to potentiate the effects of paroxetine, an SSRI, on anxiety levels in mice, which is inconsistent with 5HT1A agonism (Hascoet et al. 2000). Similarly, buspirone was shown to decrease maternal aggression in the lactating rat, while 8-OH-DPAT had no effect (Ferreira et al. 2000). Context dependence or target heterogeneity may explain some of these conflicts, especially since other studies have definitively tied the anxiolytic action of buspirone with 5HT1A activation (Cervo et al. 2000). Recent studies have also demonstrated the effects of buspirone in decreasing aversive classical conditioning (Hellewell at al. 1999), which may be due to post-synaptic inhibition of CA1 pyrimidal neurons in the hippocampus (Tada et al. 1999).

Serotonin Re-uptake Transporter

The 5HTT protein is the primary means by which extracellular concentrations of serotonin are controlled (Blakely et al. 1994), and hence it is the focus of a great deal of serotonin related research. The 5HTT gene is composed of 14 exons spanning approximately 31 kb of DNA. The gene has been localized in humans to chromosome 17. The protein itself is a 630 amino acid protein with twelve putative transmembrane domain segments (Lesch et al. 1994).

The 5HTT protein is a member of the Na/Cl dependent cotransporter family, and thus Na and Cl are required for serotonin re-uptake (Blakely et al. 1994). One of the primary regulators of the 5HTT protein are 6 phosphorylation sites that can be activated by protein kinases C and A (PKC, PKA) (Blakely et al. 1998; Ramamoorthy et al. 1998). Phosphorylation of these sites promotes transporter internalization, which decreases membrane expression and serotonin uptake (Ramamoorthy and Blakely 1999). Phosphorylation of these sites is, in turn, regulated by the passage of serotonin through the receptor, and increased serotonin re-uptake has an inhibitory effect on 5HTT phosphorylation. Thus, it is clear that the 5HTT regulates serotonergic neurotransmission, and is itself regulated at several potential points. Another of these regulatory points is genetic variation, whereby genetic polymorphisms found distributed through a population can impact the function of the 5HTT, which in turn can regulate the function of the serotonin neuron and the development of anxious personality traits and disorders.

Genetic Polymorphisms

Physiologic regulators have documented effects in serotonin neurons, but gene polymorphisms could explain in part why some individuals are affected more than others. For example, a polymorphism in a gene promoter might limit the induction or repression of a specific serotonergic gene in response to endogenous regulators.

Serotonin is present from very early in embryogenesis and it plays a critical role in neural development and organization. Any perturbation in serotonin will have long lasting consequences for CNS function. The recognition that genetic influences on serotonin will be present from conception has heightened interest in the serotonin polymorphisms.

To date, polymorphisms have been detected in TPH, with a cytosine for adenine substitution polymorphism at position 218 associated with anxiety and schizophrenia (Du et al. 2001; Hong et al. 2001) as well as a decreased concentration of 5-hydroxyindole acetic acid in the cerebrospinal fluid of humans (Virkkunen et al. 1995). Another polymorphism in intron 7 of this gene has been linked with suicidality and alcoholism in a Finnish population (Nielsen et al. 1998), while a separate promoter polymorphism was also linked to suicidality in the same population (Rotondo et al. 1999). A polymorphism was also found in MAO-A in which a CA repeat found in intron 2 was associated with bipolar disorder (Preisig et al. 2000).

A number of serotonin receptors have also been found to have relevant polymorphisms. A G861C substitution in the 5HT1B autoreceptor has been associated with alcohol dependence (Fehr et al. 2000) and suicidality (New et al. 2001), as well as

with an inactive aldehyde dehydrogenase-2 (Hasegawa et al. 2002). A T102C substitution in the 5HT2A receptor has been implicated in a seasonal pattern of depression (Arias et al. 2001), and susceptibility to neuroleptic-induced tardive dyskinesia (Tan et al. 2001). A G1438A substitution was also found in 5HT2A that is associated with the possession of impulsive traits (Preuss et al. 2001) and bulimia nervosa (Nishiguchi et al. 2001). Similarly, a cysteine to serine substitution at position 23 of the 5HT2C receptor was associated with susceptibility to tardive dyskinesia (Segman et al. 2000). A proline to serine substitution at position 15 in the 5HT5A receptor was associated with schizophrenia (Iwata et al. 2001). In the 5HT6 receptor, a C267T substitution was associated with both bipolar disorder (Vogt et al. 2000) and Alzheimer's disease (Tsai et al. 1999).

Lastly, the 5HTT has been found to exhibit two polymorphisms with physiological significance. The first, and perhaps most important, is the 5HTTLPR, a tandem repeat polymorphism in the 5HTT promoter (Heils et al. 1996). The second polymorphism of interest in the 5HTT is a variable number tandem repeat (VNTR) polymorphism in the second intron. One allele of this polymorphism has been associated with an increased risk of migraine (Yilmaz et al. 2001).

Taken together, these studies indicate the importance of genetic polymorphisms in central serotonin function. However, a note of caution is due. While several studies have indeed linked several polymorphisms to diseases, many more studies have been unable to replicate those same linkages. For example, several large-scale studies were unable to replicate the finding linking the 5HTTLPR to autism (Zhong et al. 1999; Maestrini et al.

1999). Similarly, another large-scale study was unable to replicate the linkage of suicidality to TPH polymorphisms (Geijer et al. 2000). Thus, caution is needed when evaluating the physiological role of particular polymorphisms until a clearer consensus is achieved. One factor which could be complicating these studies is the variable nature of many of the diseases studied. For instance, to be diagnosed with a certain clinical psychological disorder, one must typically fullfil a certain number of criteria or symptoms from a larger list. Thus, heterogeneity can occur in that one individual may have a symptom affected by serotonin neurons, while another individual may have a different symptom not affected by serotonin neurons, and yet be diagnosed with the same condition.

In the future, attention needs to be moved beyond descriptive studies, such as disease associations, to studies on the functional consequences of these polymorphisms in serotonin neurons at the cellular and molecular level. Ultimately, we may be able to unravel the complex actions between endogenous regulators and the highly variable neuronal genome, which combine to influence the development of anxious personality traits and disorders.

5HTTLPR

The 5HTTLPR polymorphism was first described by Heils, et al. (1996). This polymorphism is located approximately 1 kb upstream of the transcription initiation site in humans and most other primates. In macaques, a second locus for length variation was found approximately 100 bp further upstream. The presence of this second locus does

not affect the distribution of 5HTTLPR genotypes or 5HTTLPR function in macaques. In humans, the polymorphism is usually comprised of 14 or 16 repeat elements, the short (s) and long (l) alleles respectively. Rarely, an 18 and 20 repeat allele can be described in humans. In contrast, the orangutan, gorilla and chimpanzee usually exhibit an 18 or 20 repeat allele. Also, the rhesus macaque was found to have an unusually large 5HTTLPR, with 23 or 24 repeat elements. The alleles were found distributed within each species as expected according to the Hardy-Weinberg Equilibrium irrespective of species differences in repeat length. The 5HTTLPR is not present in prosimians and other lower mammals (Lesch et al. 1997). Taken together, this indicates that the 5HTTLPR was introduced into the primate genome approximately 40 million years ago when the prosimian lineage split.

The 5HTTLPR was found to regulate the expression of the 5HTT protein *in vitro*. The short allele of this polymorphism was found to decrease transcriptional efficiency of the 5HTT when transfected into JAR (Heils et al. 1996) and lymphoblast cells (Lesch et al. 1996), and is also associated with an attenuated prolactin response to fenfluramine challenge in humans (Reist et al. 2001). The prolactin response to fenfluramine challenge has long been considered an indicator for overall central serotonin function, and the short allele of the 5HTTLPR seems to decrease overall serotonin function. Consistent with this hypothesis are the findings that the short allele of the 5HTTLPR is associated with increased severity of autism (Tordjman et al. 2001), increased incidence of antidepressant induced mania in bipolar disorder (Mundo et al. 2001), and attention deficit hyperactivity disorder (Manor et al. 2001). Moreover, depressed patients with the

long variant of the 5HTTLPR, either l/l or l/s, showed a better response to fluvoxamine than homozygotes for the short variant (Smeraldi et al. 1998). In rhesus monkeys, the short allele has been associated with diminished orientation, lower attentional capabilities, and increased affective response, although environment has been shown to mitigate these effects to a degree (Champoux et al. 2000).

5HTTLPR and Anxiety

Individuals homozygous for the short allele (s/s) of the 5HTTLPR have been reported to display higher anxiety scores, increased neuroticism, higher muscle tension, a lack of assertiveness, increased shyness, increased harm avoidance, increased violence, increased likelihood to commit suicide and lower levels of agreeableness, compared to individuals homozygous or heterozygous for the long allele (l/l or l/s) (Lesch et al. 1996; Bondy et al. 2000; Courtet et al. 2001; Osher et al. 2000).

While these results are controversial, since several studies have been unable to consistently replicate some of the results (Jorm et al. 2000; Kumakiri et al. 1999), they hint that the 5HTTLPR is involved in the development of anxious traits and disorders. There is, however, a paradox inherent in the 5HTTLPR data. As described above, the short allele is associated with decreased transcription of the 5HTT. This would logically indicate less 5HTT protein expressed on the cell surface, and more serotonin in the synpatic cleft since the uptake rate would be less. This would seem to mimic the effects of the SSRI's, which inhibit the 5HTT and decrease uptake. However, the s/s genotype in individuals is anxiogenic, while the SSRI's are anxiolytic.

This apparent paradox could be explained by several factors. The first is that the effects of SSRI's have primarily been demonstrated in neuropsychiatric patients, who might have an underlying serotonergic dysfunction that is ameliorated by the SSRI's, while the studies above were usually carried out in general populations. Another factor is that the SSRI's may have other pharmacologic effects such as binding to the norepinephrine transporter which could contribute to the therapeutic effects (Blier and de Montigny 1994). Lastly, much like knock-out animals, the effects of a lifelong difference in 5HTT gene transcription as mediated by the 5HTTLPR, such as in early brain development (Shuey et al. 1992), could lead to the differential effects of the 5HTTLPR as compared to a relatively acute SSRI treatment later in life.

Another important consideration is the number of genetic variables that contribute to anxiety. As mentioned above, genetic inheritance has been estimated to account for 40-60% of the variation observed in anxiety related traits. The 5HTTLPR has been estimated to account for 7-9% of the genetically related variance (Lesch et al. 1996). Clearly, a number of other gene products are involved. If the percentages for the 5HTTLPR are assumed to be representative for any particular gene, then there could be 9 or 10 genes involved in the variation of anxious traits. If the percentages for the 5HTTLPR are not representative, then there could be literally any number of genes involved. Clearly then, the search for the genetic components of anxiety does not end with the 5HTTLPR. However, the 5HTTLPR could be an important factor in elucidating further mechanisms of anxiogenesis, as well as future targets for clinical intervention.

A method is needed to reconcile the lack of replication observed with anxiety

association studies. There are two possible hypotheses that could explain this difficulty. The first is that there could be a difference in the linkage between the 5HTTLPR polymorphism and behavioral affect in different populations with diverse experiential or genetic backgrounds. The second is that there could be a linkage between the 5HTTLPR and specific forms of anxious behavior, but not other forms of anxious behavior.

To begin to address these two possibilities we utilized young monkeys (3 months-1 year of age) all raised in similar semi-natural environments to minimize the influence of differences in experiential background on the potential links between 5HTTLPR polymorphism and behavior. We examined the relationship between anxious behaviors and the 5HTTLPR polymorphism using four standardized temperament tests that examine specific aspects of anxiety: (1) a Free Play test-which is used to examine propensity to explore a new environment, (2) a Remote-Controlled Car test-which is used to examine reactivity to a novel, nonsocial stimulus, (3) a Human Intruder test-which is used to examine reactivity to a novel, social stimulus, and (4) a Novel Fruit test-which is used to examine reactivity to a ecologically relevant nonsocial stimulus that has a reward value.

Significant correlations were found between 5HTTLPR polymorphism and degree of exploration in the Free Play test (s/s animals were less likely to explore), the number of fear and lipsmacking displays in the Remote Controlled Car test (s/s animals engaged in more fear and lipsmacking displays) and the likelihood to display threats in the Human Intruder test (s/s animals were more likely to threaten the stranger). There were no correlations between 5HTTLPR polymorphism and other anxious behaviors such as reactivity to a nonsocial stimulus or ecologically relevant stimulus. These findings support the hypothesis that there

may be a linkage between the 5HTTLPR polymorphism and very specific forms of anxious behavior.

Chapter 2: Materials and Methods

Animals

128 infant and juvenille rhesus monkeys from the Oregon National Primate Research Center (ONPRC) breeding colony were genotyped for the 5HTTLPR. These monkeys were divided into three groups based on age and upbringing. The first two groups were comprised of infants from 3-6 months of age raised either in outdoor corrals or caged indoors. The third group was composed of yearling monkeys raised in the outdoor corrals. Of these genotyped monkeys, 90 were chosen from the two corral groups for use in this behavioral study. The corral monkeys were used because the indoor monkeys behaved differently in the behavioral tests in a manner unrelated to genotype, which confounded the analysis. The outdoor reared monkeys lived in one of several one acre outdoor corrals, each containing approximately 100 monkeys, at the ONPRC. Corrals contained stable groups of reproductive age females, their offspring, and some adult males. Monkeys were fed commercial monkey chow twice daily, and water was available *ad libitum.*

For performance of the temperament tests, the monkeys were "rounded up", a procedure in which animals are brought from the corral to single cages in an attached building (the catch area), and are housed there for several days. Mothers usually carry their young during these round-ups. Infants were housed with their mothers in the catch area.

Behavioral Tests

All tests were videotaped and behaviors scored using a computer program (Observer Video Pro, version 4.0, Noldus Information Technology, The Netherlands) by a person blinded to the 5-HTTLPR polymorphism status of the monkeys. The Observer program assists in recording both behavioral states that occur over spans of time, such as locomotion or freezing behavior, as well as behavioral events such as vocalizations. The program can then be used to calculate the frequency of the behavioral events and states for the time periods designated.

Three of the four tests were adapted from designs by Dr. Hill Goldsmith and colleagues in the Laboratory Temperament Assesment Battery (Lab-TAB), Locomotor Version 3.0 (Goldsmith and Rothbart 1996). These tests were originally designed for use with human children, and provide a quantitative and repeatable assessment of anxious, fearful and inhibited behaviors in various contexts.

All test animals received their standard morning meal approximately two hours prior to the advent of the testing, in order to remove the confound of hunger during the tests, particularly in the Novel Fruit test. Approximately 10 minutes prior to testing, the mother was sedated with 5 μg/kg of Ketamine HCl, given as an intramuscular injection. The yearling monkeys were accompanied by an unrelated female surrogate, sedated in a like fashion, since their mothers had given birth to new infants since the time they had been born. The mother/surrogate was present in some of the behavioral tests in order to avoid the confound of separation anxiety, but sedated to prevent interference in the tests.

Once the sedative had taken effect, the monkeys were transported to the site of the

first behavioral test, the playroom. Infant monkeys were transported with their mothers, which took approximately five minutes, and were placed in the playroom together. Yearling monkeys were transported separately, and were released into the playroom with the surrogate already present. Once the Free Play and Remote Controlled Car tests had been completed, the monkeys were separated from the mother/surrogate, and placed into a novel room alone in a standard monkey cage for the Human Intruder and Novel Fruit tests. Once these tests had been completed, the infant monkeys were returned to their mothers, and all the monkeys were then transported back to the catch area.

Free Play Test

The Free Play test is designed to assess the degree of exploratory behavior versus inhibition to explore in an unstructured novel environment containing novel objects. This test is based on the Free Play episode in the Lab-TAB manual, which was originally designed for use in 12 month old human infants. Since the monkey's movements were unrestrained, this test assesses an intrinsic curiosity and drive to explore, rather than a reaction to situational constraints.

The playroom itself was 2.4 x 3.0 m, and contained a climbing/play structure (127 cm x 61 cm x 198 cm) as well as nine novel toys arranged in a semi circle. The monkey was videotaped through a one way mirror which faced the play structure, and the floor was crosshatched in order to assist in determining the monkey's distance from the mother/surrogate. The sedated mother/surrogate was placed in an infant car seat approximately 0.5 m from the climbing structure in the back right corner of the room, and

the yearling monkeys were introduced to the room through a small door on the far left.

Once the monkey had been introduced to the playroom, either with the mother for the infants or through the door on the left for the yearlings, their behavior was taped for 5 minutes. Special care was taken to observe the facial expressions of the monkey, especially in regards to fear or threat responses such as lipsmacking and fear grimaces. Two additional five minute epochs were recorded, from 15-20 minutes and 30-35 minutes.

During the data analysis portion of this experiment, 13 variables were scored from the videotapes. These variables included general measures of activity (time active, toy play) and measures of anxiety (vocalizations, time away from mother, latency to leave mother) measured during all three time epochs. For the latency to leave the mother variable, it should be noted that the yearling monkeys could not be scored for this behavior, since they were introduced separately from the surrogate. The time active, toy play, and time away from mother variables were all recorded as a percentage of the total time that the monkeys engaged in these behaviors. The vocalization and latency to leave the mother variables were recorded as a frequency and in seconds, respectively.

Remote-Controlled Car Test

This test assesses behavioral responsiveness to a novel, and potentially frightening, non-social stimulus. This test is modeled after the Remote Controlled Spider test in the Lab-TAB manual. Immediately after the completion of the Free Play test, a bright, yellow remote controlled car (5.2 cm x 7.9 cm x 9 cm, Radio Shack Incorporated, Fort Worth, TX) entered the room through the small door on the left side of the room that the yearling

monkeys had been introduced through. The test consisted of five epochs, the first of which was the entrance of the car into the room, upon which the car paused for 10 seconds (CAR IN). For the next epoch the car advanced to within 0.3 m of the monkey, and again paused for 10 seconds (FORWARD 1). The car then retreated approximately 1 meter and paused for 10 seconds (BACK) and once again advanced to within 0.3 m of the infant and paused for 10 seconds (FORWARD 2). For the final epoch, the car left the room through the door in which it entered, taking approximately 5 seconds (OUT).

During all epochs of the test, the monkey was carefully filmed, taking special care to note the facial expressions and actions of the monkey in response to the car. For the analysis of this test, seven variables were recorded across all five epochs. These variables included time away from the mother, escape behavior (rapid movement away from the car), vigilant observation of the car, retreat (slower movement away from the car), as well as vocalizations, signs of fear (such as the fear grimace) and lipsmacking.

Human Intruder Test

This test assesses behavioral responsiveness to both a threatening and a non-threatening social stimulus. This test was originally used by Kalin and colleagues in rhesus monkeys (Kalin and Shelton 1989) and an analogous test, the Stranger Approach test, is used in the Lab-TAB manual. This test assesses the behavioral response of the monkey to three stressful conditions: being alone in an unfamiliar cage, and being confronted with a threatening stimulus (novel human) in both a social (direct stare) and a non-social (profile) manner. The stereotypic response of the rhesus monkey to the above situation as described by Kalin and Shelton (1989) includes freezing during the profile threat, and threatening during

the stare threat.

Immediately after the end of the Remote Controlled Car test, the monkey was separated from the mother/surrogate and transported to a novel test room adjacent to the play room. The monkey was placed into a standard monkey cage (61 cm^3) and allowed to acclimate for 10 minutes with no interference. The monkey was then videotaped from behind a blind for 2 minutes with no human present (ALONE 1). An unfamilar human then entered the room and approached to within 0.3 m of the cage, taking care not to make eye contact with the monkey. The human presented a profile to the monkey, who was recorded for another 2 minutes (PROFILE). The human then left the room, during which the monkey was filmed for another 2 minutes (ALONE 2). Finally, the human re-entered the room, approached to within 0.3 m of the cage, and made continuous, direct eye contact for another 2 minutes (STARE).

For the analysis of this test, 26 variables were recorded across all four epochs. These variables included freezing behavior, exploratory behavior, locomotion, vocalizations, fear responses, teethgrinding, lipsmacking and threats.

Novel Fruit Test

This test is designed to test the monkey's reaction to an ecologically relevant novel object with reward value (i.e., a piece of unfamiliar fruit). Two minutes after the end of the Human Intruder test, the same human entered the room again and placed a small white box (3.8 cm x 13.3cm x 9.5 cm) through the feeding hole in the cage, upon which a slice of novel fruit (kiwi) was immediately placed. The human then left the room, and the infant was videotaped for five minutes. After five minutes, the human re-entered the room and placed a slice of familar fruit (apple) onto the box, and the monkey was videotaped for

another five minutes. The apple was placed onto the box even if the kiwi was still present.

The only variables recorded and scored for this test was the latency to inspect, touch and eat the kiwi and the apple, measured in seconds. The touch had to be judged intentional; incidental contact was not scored as a touch. If the monkey never inspected, touched or ate the fruit, a maximum latency score of 300 seconds was given. During the familar fruit portion of the test, if the kiwi still remained, interaction with the kiwi was not scored.

Genomic DNA Extraction and PCR

2-3 days after the end of behavioral testing, blood was obtained from each animal under ketamine anesthesia for genotyping. 5 ml of whole blood was collected by femoral venupuncture into Vacutainer brand vacuum tubes. The blood was then stored at -20°C until genomic DNA extraction.

Genomic DNA was extracted from the whole blood using a Qiagen QIAmp DNA Blood Maxi Kit. There were no differences in the extraction protocol from the kit instructions. Genomic DNA was stored at -20°C until the PCR.

The genotyping PCR protocol was based on a protocol obtained from Dr. Rainald Moessner at the University of Wuerzburg, Germany. All PCR reagents were obtained from Gibco BRL. The primer sequences were MutI (forward): 5'-TCG ACT GGC GTT GCC GCT CTG AAT GC-3' and IntI (reverse): 5'-CAG GGG AGA TCC TGG GAG GGA-3'. The reaction was begun by adding 2.5 μL of 10X PCR Buffer, 0.5 μL of 50 mM MgCl2, 0.6 μL of

10 mM dNTP mix, 1.0 μL of both the MutI and IntI primers at a working concentration of 0.1 nmole/μL, 1.0 μL of the Taq polymerase, 1.0 μL of the template genomic DNA diluted to a working concentration of 50 ng/μL, and 17.4 μL of sterile water to a PCR tube. The reaction was overlayed with mineral oil, and run in a thermal cycler on the following program: 95°C for 5 min., 95°C for 30 sec., 60°C for 30 sec., 72°C for 1 min., 30 cycles from the second step, 72°C for 15 min., and a final holding temperature of 4°C.

Once the PCR was complete, the reaction products were run on a 3.5% agarose gel cast with ethidium bromide at 23 V for approximately 6-7 hours, or until the bands had migrated for at least 4 cm. The bands were visualized under ultraviolet illumination and photographed. Genotype was assesed by the presence of a slow running band at 419 bp (l/l), a fast running band at 398 bp (s/s) or the presence of both bands (l/s).

Statistical Analyses

The data recorded in the tests, with the exception of the time active, toy play, and time away from mother variables in the Free Play test, was transformed into a standardized scale for the purpose of statistical analysis. In this scale, a score of 100 was considered uninhibited or unfearful, while a score of 0 was considered inhibited or fearful. The monkey with the most uninhibited or unfearful behavior in each variable (i.e. made the fewest fear displays or spent the most time away from the mother) received a standardized score of 100, while the other monkeys were scored proportionately along the scale, with the most fearful or inhibited monkey (i.e. never inspected the novel fruit or spent the least time active) receiving a standardized score of 0. This standardized scale was used in order to facilitate the analysis

without altering the statistics, and the standardized scores are not reported in the results.

The standardized data from the behavioral tests was then tested for normality and homoscedacity. Log+1 transformations were used to help normalize the data. None of the behavioral variables were found to be normally distributed, which required the use of non-parametric statistical methods.

The Kruskal-Wallis non-parametric test was used to analyze the behavioral data. This test is the non-parametric equivalent of a one-way ANOVA, is ordinal in nature, and is a relatively sensitive test. 5HTTLPR genotype was used as the primary grouping variable, however, age and gender were also used as grouping variables in order to account for the variability due to these factors. Statistical power, the probability of avoiding a Type II error, was also calculated for each variable. Power was calculated using a standard one-way ANOVA formula, and multiplying by a factor of 0.95 in order to account for the slightly lower power of the Kruskal-Wallis test.

The population data for the genotypes recorded was also analyzed. The genotype frequencies observed were compared to the frequencies expected from the Hardy-Weinberg Equilibrium using a Chi-square Goodness of Fit test.

Chapter 3: Results

For this study, 128 animals were genotyped for the 5HTTLPR over the course of a two year period. The PCR protocol generated fragments of 419 bp for the L allele and 398 bp for the S allele. These sizes are specific to rhesus macaques since the rhesus have a higher number of repeat units in the 5HTTLPR (23 or 24 repeats) than the human (14 or 16 repeats)

or most other great apes (18 or 20 repeats). Since the fragments were close in size, a high concentration agarose gel, 3.5%, was used. The products were run at a low voltage, 23 V, for a long period of time, 6-7 hours, to minimize distortions and other problems inherent in a high concentration gel. Genotypes were then assigned based on the pattern of bands observed, which is demonstrated in Figure 1. A dual band pattern, as observed in lanes 3 and 9, is categorized as a heterozygote (l/s). Using a known heterozygote for comparison, the other patterns were categorized as one fast running band (shorter fragment), as shown in lane 6, or one slow running band (longer fragment). The presence of only a short amplified product indicates the animal is homozygous for the short allele, or s/s. The presence of only the long amplified product indicates the animal is homozygous for the long allele, or l/l.

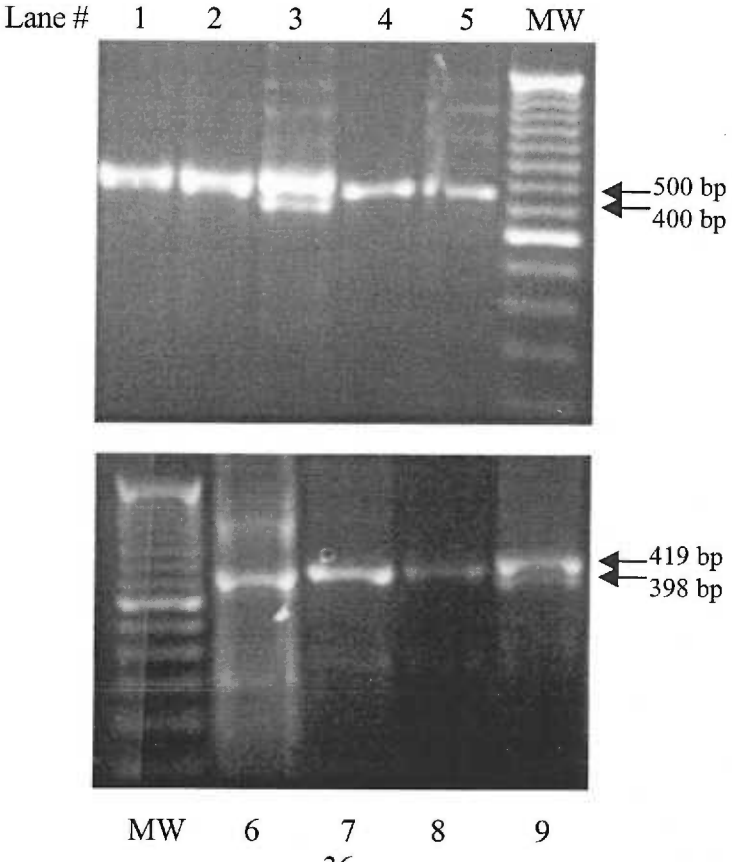

Figure 1: Agarose Gel Electrophoresis of PCR Products

PCR products were run on a 3.5% agarose gel for 6-7 hours at 23 V. The size of the L allele is 419 bp, the size of the S allele is 398 bp. Genotypes were assigned on the basis of one band at 419 bp (l/l), one band at 398 bp (s/s), or two bands at both 419 and 398 bp (l/s). Two representative gels are shown above. Lanes 3 and 9 contain heterozygotes (l/s). Lanes 1,2,4,5,7 and 8 contain l/l homozygotes, and lane 6 contains an s/s homozygote.

Once all of the individual monkeys had been genotyped, the data was analyzed as a group for distribution patterns. The data was analyzed in two separate cohorts, 1999 and 2000 with an n-value of 83 and 45 respectively, as well as a combined total (Table 1). The number of genotypes observed was used to calculate the percentage of the total, which was in turn compared to the expected percentage value. The expected percentage values were calculated using the Hardy-Weinberg Equilibrium, which models the distribution of genetic alleles throughout a population. A Chi-square Goodness of Fit test was then used to compare the frequencies observed compared to the frequencies expected for the combined total cohort. A low alpha level ($p<0.01$) was selected in order to minimize the possibility of a Type I error, due to the relatively low n values used in this study for the purpose of population genetics. Using the Chi-square test, the frequencies observed were not found to be different from the frequencies expected ($X^2=8.28$, $p>0.01$, $df=2$, $n=128$). We thus conclude that our population is representative with regard to the 5HTTLPR.

# of Genotypes observed	% of Total	% Expected
1999 Cohort, n=83		
34 L/L	41	37
25 L/S	30	48
24 S/S	29	15
2000 Cohort, n=45		
21 L/L	47	37
21 L/S	47	48
3 S/S	7	15
Combined Total, n=128		
55 L/L	43	37
46 L/S	36	48
27 S/S	21	15

Table 1: Genotype Distribution of the 5HTTLPR

The table above shows the distribution of the 5HTTLPR genotypes determined amongst the monkeys in this study. The total numbers of each genotype are shown for both cohorts of monkeys genotyped in 1999 and 2000, as well as the combined totals. The percentage of each genotype compared to the total number of monkeys is compared with the Hardy-Weinberg equilibrium values that would be expected to be shown in this population. A Chi-square Goodness of Fit test was used to compare the observed and expected categories, which were not found to be different (X^2=8.28, p>0.01, df=2, n=128). 90 of the above animals were used for behavioral testing.

Out of the 128 individuals genotyped, 90 were scored in the behavioral analysis. These consisted of 45 l/l, 30 l/s and 15 s/s. There were two groups of these monkeys, infants (3-6 months of age) that had been raised in the outdoor corrals, and yearling monkeys raised in the corrals. Age and gender were used as grouping variables in a separate statistical

analysis, which found no significant interactions between the variables reported here and either age or gender, although age was found to interact with other variables not reported here. The similar upbringing of the animals also worked to minimize differences in environmental and experiential influences. The l/l and l/s monkeys were grouped together for the statistical analysis, since these monkeys performed similarly in our behavioral tests.

In the Free Play test, the variables measured as explained above were all analyzed for differences. Of these variables, a significant difference was found in the amount of time the monkeys spent active during the third recording epoch of the Free Play test, 30-35 minutes after being put into the playroom (Figure 2, $p=0.019$, $H=5.479$, $df=1$, $n=85$, Power=0.798). Activity, for the purposes of this test, includes locomotor activity such as movement or toy play, as well as active observation while stationary (i.e. monkey has head raised, and is actively looking around the room). It should also be noted that the sample sizes reported for each test are not 90 in all cases because data was not available for all monkeys in all tests. No significant differences were found in the amount of time spent active in the first and second epochs (0-5 minutes and 15-20 minutes after entry into the playroom, respectively), which suggests that once the monkeys had acclimated to their novel environment differences in activity influenced by genotype could be detected. It is also clear that genotype did not influence all types of activity, even in the third epoch of the test. Figure 3 demonstrates the percentage of time that the monkeys spent playing with the novel toys, which was not found to be different between the genotypes ($p=0.21$, $H=1.571$, $df=1$, $n=85$, Power=0.088). The results of this test argue for very specific differences in behavioral inhibition, namely general activity level in a novel environment after an acclimation period, in the s/s monkeys.

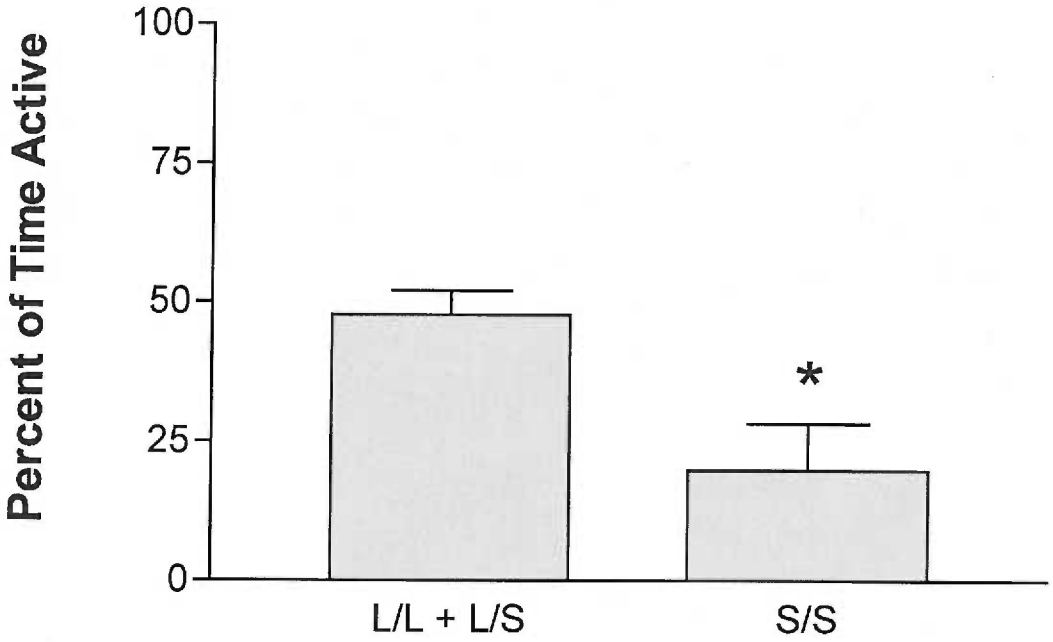

Figure 2: Free Play Test Activity Level

The s/s monkeys were found to spend less time active in the third recording epoch ($p=0.019$, $H=5.479$, $df=1$, $n=85$, Power=0.798). Such differences were not found in the first and second epochs, which suggests that the s/s monkeys are more behaviorally inhibited in regards to activity level in a novel environment after an acclimation period.

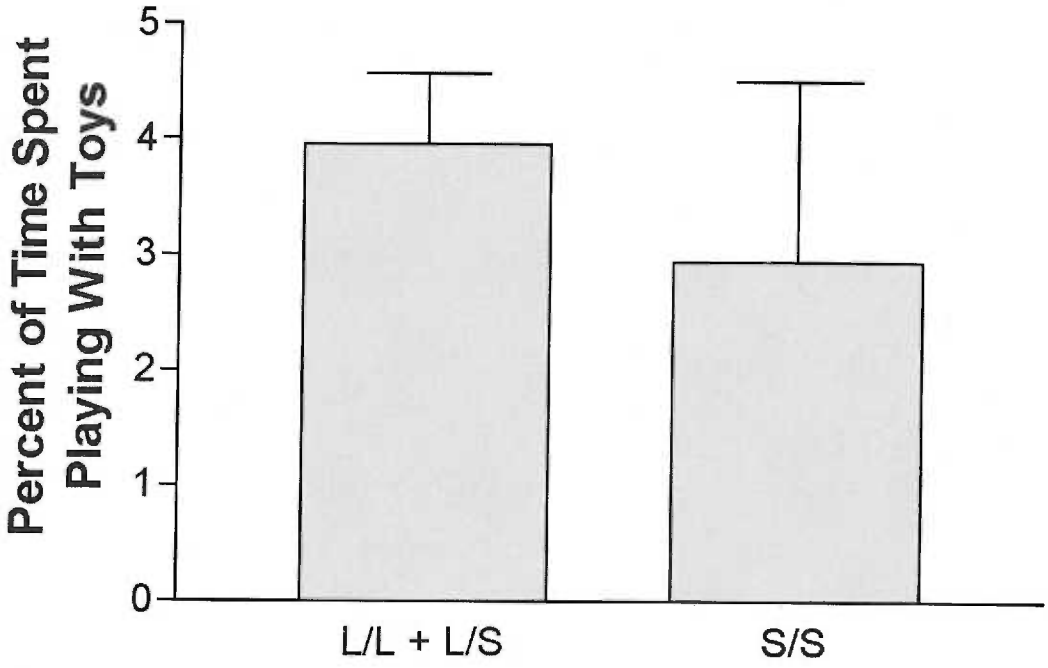

Figure 3: Free Play Test Toy Play

No differences were found between the genotypes in regards to the amount of time spent playing with the novel toys in the third recording epoch (p=0.21, H=1.571, df=1, n=85, Power=0.088). This suggests that the s/s monkeys, even in the third epoch, are only inhibited in certain measures of activity.

The Remote Controlled Car test was performed at the end of the Free Play test, when the monkey would be approached by a novel and potentially threatening object. The nature of the threat is also non-social in nature. During this test, most monkeys were observed to huddle near the mother/surrogate, and no differences were found between the genotypes in the percentage of time spent away from the mother (Figure 4, p=0.085, H=2.967, df=1, n=81, Power=0.409). A note of caution is due at this point, however. With the relatively low p

value (0.085) as well as the relatively low power in this test (0.409) the possibility of committing a Type II error is present. Thus, care should be taken in interpreting this result, as it might be a "false negative".

While the time spent away from the mother was not found to be different, significant differences were found in the number of fear displays as well as the percentage of time spent in lipsmack displays. Fear displays include obvious facial displays of fear, including the "fear grimace", which is characterized by a facial grimace that exposes the teeth in a non-threatening manner. The s/s monkeys were found to engage in a greater number of fear displays (Figure 5, $p=0.037$, $H=4.365$, $df=1$, $n=81$, Power=0.219). Lipsmacking is characterized by a rapid movement of the lips without opening the mouth, and can indicate uncertainty, anxiety or submissive behavior. The s/s monkeys were found to spend a greater percentage of time in lipsmacking episodes (Figure 6, $p=0.010$, $H=6.658$, $df=1$, $n=81$, Power=0.235). The overall results of this experiment suggest that the s/s monkeys are not more "anxious" in regards to one specific measure of anxiety, time spent away from the mother, but are more anxious as measured by displays of fear and uncertainty in response to a threatening, non-social stimulus.

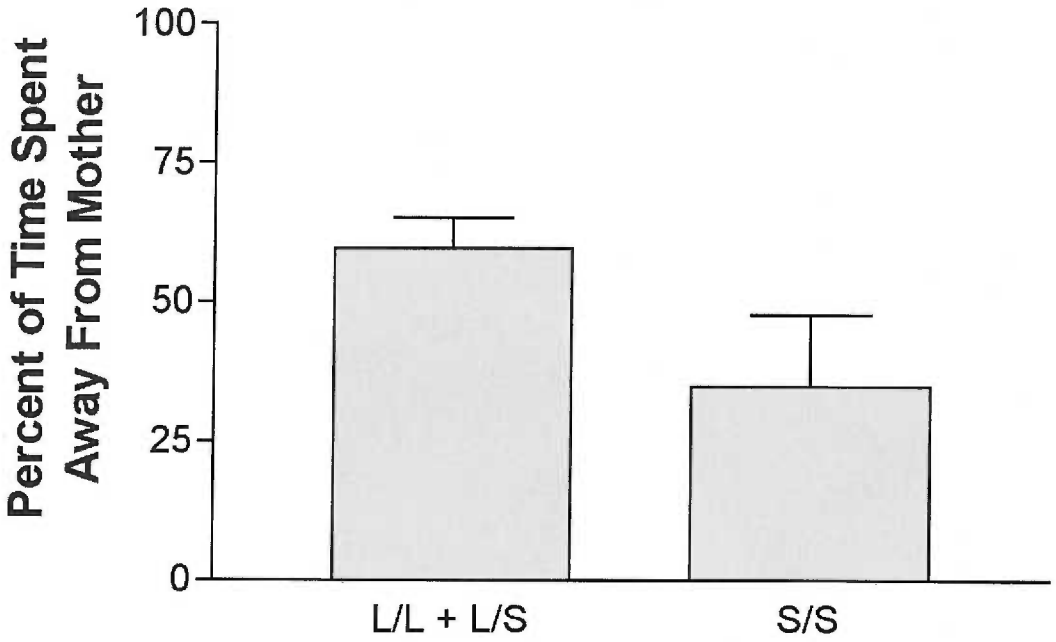

Figure 4: Remote Controlled Car Test Time Away From Mother

During the Remote Controlled Car Test, most of the monkeys spent the majority of their time huddled near the mother, and no significant differences were observed between the genotypes ($p=0.085$, $H=2.967$, $df=1$, $n=81$, Power=0.409). This finding suggests that the s/s monkeys are not more anxious in regards to the time spent away from the mother when confronted with a threatening, non-social stimulus.

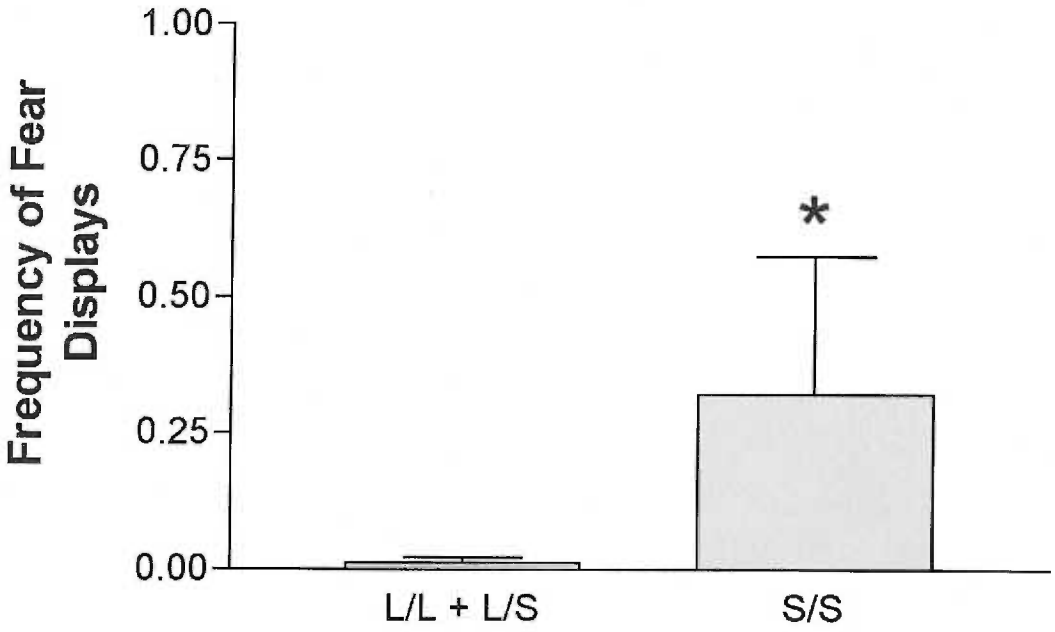

Figure 5: Remote Controlled Car Test Fear Displays

The s/s monkeys were found to engage in a greater number of fear displays in response to this threatening, non-social stimulus ($p=0.037$, $H=4.365$, $df=1$, $n=81$, Power=0.219). This result suggests that the s/s monkeys are more fearful in response to this non-social, threatening stimulus.

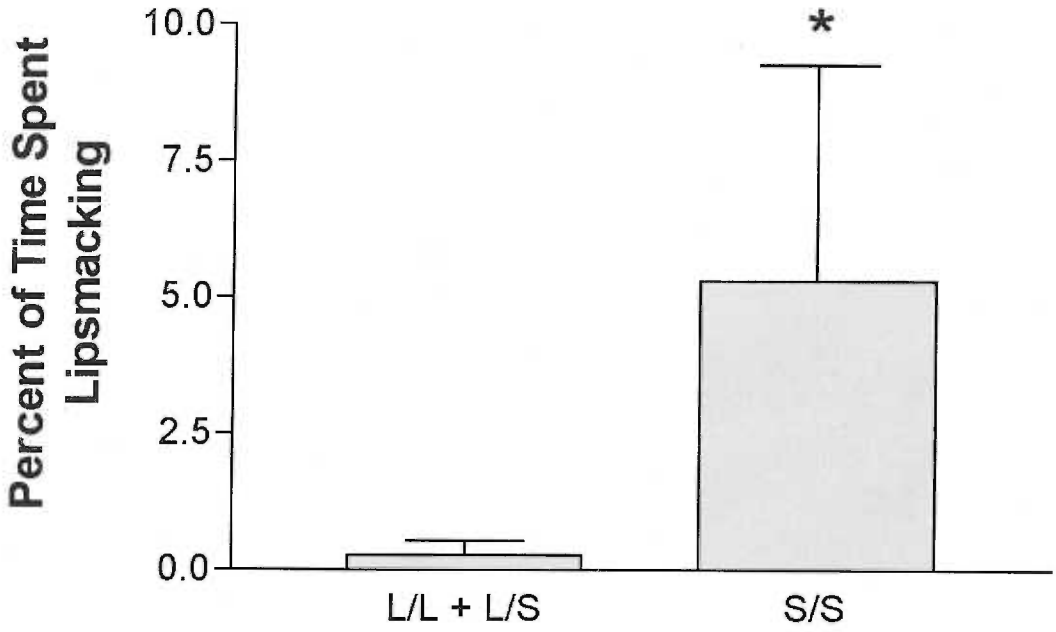

Figure 6: Remote Controlled Car Test Lipsmacking Displays

The results of this test indicate that the s/s monkeys spent a greater percentage of time in lipsmacking displays in response to this threatening, non-social stimulus ($p=0.010$, $H=6.658$, $df=1$, $n=81$, Power=0.235). These results suggest that the s/s monkeys are more anxious or uncertain in response to the remote controlled car.

The Human Intruder test was performed after the Remote Controlled Car test in a novel room in a standard monkey cage. This test is designed to be a threatening response that is directly social in nature, unlike the Remote Controlled Car test. After a 10 minute acclimation period, the test was started, and it consisted of four epochs. These epochs were all 2 minutes in duration, and consisted of two alone periods, and two periods with a human intruder presenting a profile as well as engaging in a direct stare. The profile and stare epochs

were separated by a second alone epoch.

No differences were found in the number of fear displays evoked by the human intruder in either the profile or stare epoch. The results of the stare epoch are shown in Figure 7 ($p=0.569$, $H=0.325$, $df=1$, $n=90$, Power=0.158). Similarly, no differences were found in the percentage of time spent lipsmacking in either epoch, and the results of the stare epoch are shown in Figure 8 ($p=0.994$, $H=0.000$, $df=1$, $n=90$, Power=0.200). In contrast, a difference was detected in the number of threatening displays in the stare epoch. The s/s monkeys were found to make more threats in response to the direct social challenge (stare) than the l/l + l/s monkeys (Figure 9, $p=0.022$, $H=5.216$, $df=1$, $n=90$, Power=0.331). The results of this test indicate that the s/s monkeys are not more fearful or anxious as measured by fear displays and lipsmacking, but do express a greater level of anxious behavior by threatening the human intruder more than the other genotypes.

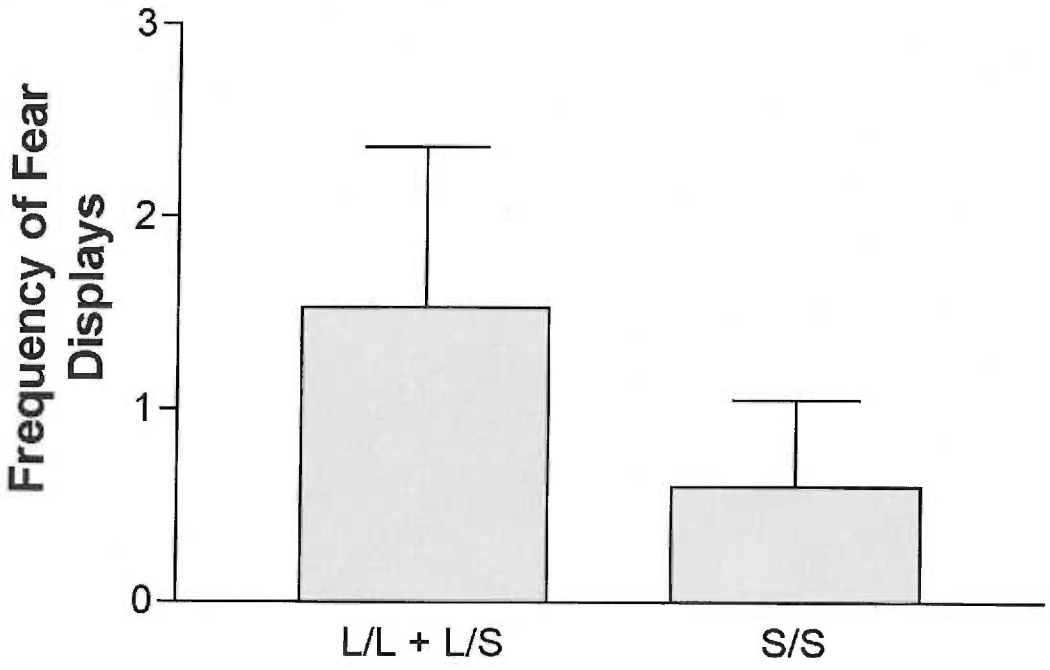

Figure 7: Human Intruder Test Fear Displays

No differences were found between the genotypes in the number of fear displays during the stare epoch of the Human Intruder test ($p=0.569$, $H=0.325$, $df=1$, $n=90$, Power=0.158). In contrast to the Remote Controlled Car test, the results of this test indicate that the monkeys are not more fearful in response to a threatening stimulus that is social in nature.

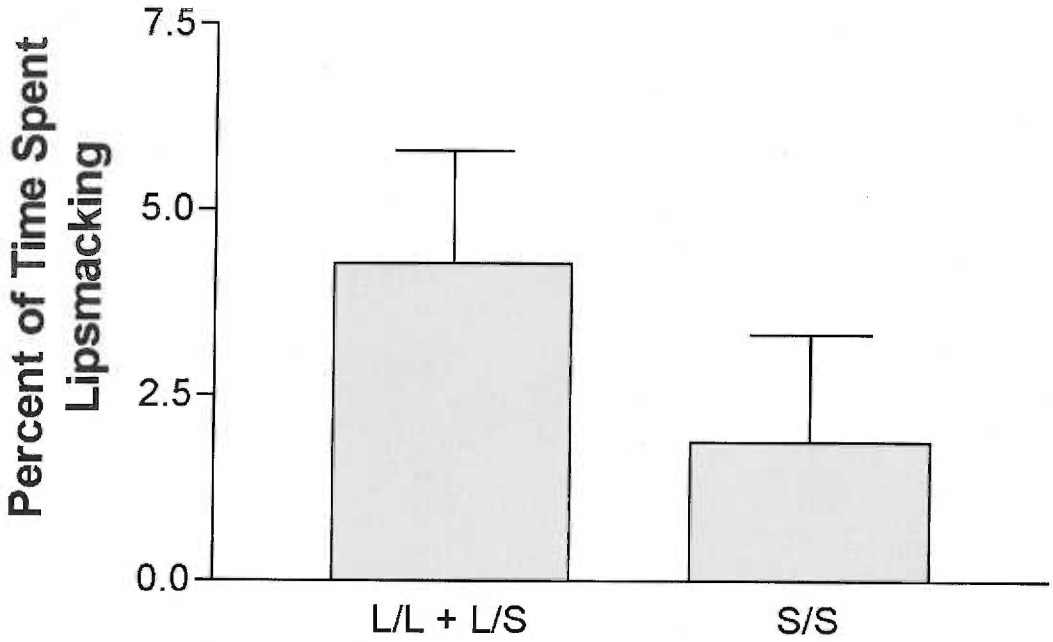

Figure 8: Human Intruder Test Lipsmacking Displays

No differences were found between the genotypes in the percent of time spent lipsmacking during the stare portion of the Human Intruder test ($p=0.994$, $H=0.000$, $df=1$, $n=90$, Power=0.200). In contrast to the Remote Controlled Car test, the results of this test indicate that the s/s monkeys are not more anxious or uncertain in response to this threatening stimulus as measured by lipsmacking.

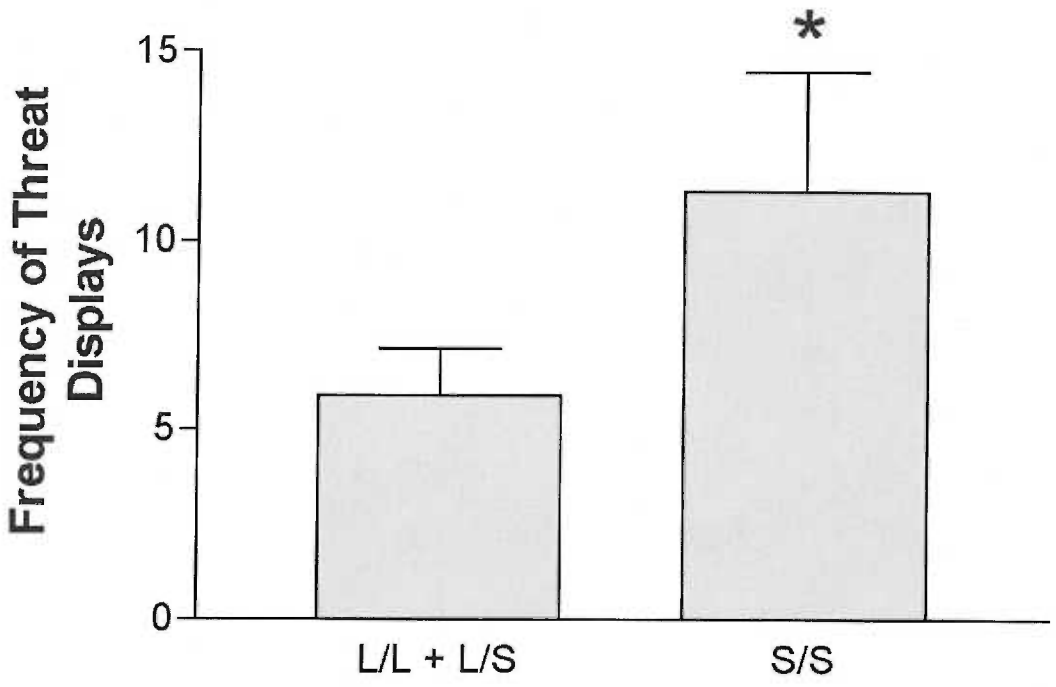

Figure 9: Human Intruder Test Threat Displays

During the stare epoch of the Human Intruder test, the s/s monkeys were found to engage in a greater number of threat displays than the other genotype monkeys ($p=0.022$, $H=5.216$, $df=1$, $n=90$, Power=0.331). The results of this test indicate that the s/s monkeys are more anxious in regards to a social, threatening stimulus as measured by threat displays, but not displays of fear or uncertainty as demonstrated in Figures 7 and 8.

Directly after the end of the Human Intruder test, the Novel Fruit test was performed. This test consisted of the same human intruder entering the room and placing a piece of novel fruit (kiwi) into the cage with the monkey. Behavior was recorded for 5 minutes, when the human would re-enter the room and place a piece of familiar fruit (apple) in the same area as

the kiwi, and behavior was recorded for an additional 5 minutes. The only variables recorded were the latency in seconds to inspect, touch and eat each fruit. A maximum latency score of 300 was given if the monkeys failed to inspect, touch or eat the fruit.

The analysis of the Novel Fruit data indicates that there were no differences between the genotypes in the latency to inspect, touch or eat either fruit. Most monkeys rapidly inspected, touched and ate the fruit. The data for the latency to inspect the kiwi is shown in Figure 10 ($p=0.076$, $H=3.153$, $df=1$, $n=89$, Power=0.152) and the latency to touch the kiwi is shown in Figure 11 ($p=0.125$, $H=2.348$, $df=1$, $n=89$, Power=0.200). The low p value and low power of the latency to inspect the kiwi test indicates the possibility of a Type II error, however, it is unlikely that a difference in inspection latency alone with no differences in touch or eat latency would be meaningful. The results of this test indicate that the s/s monkeys are not more anxious than the other genotypes in response to a non-threatening, ecologically relevant stimulus that has reward value.

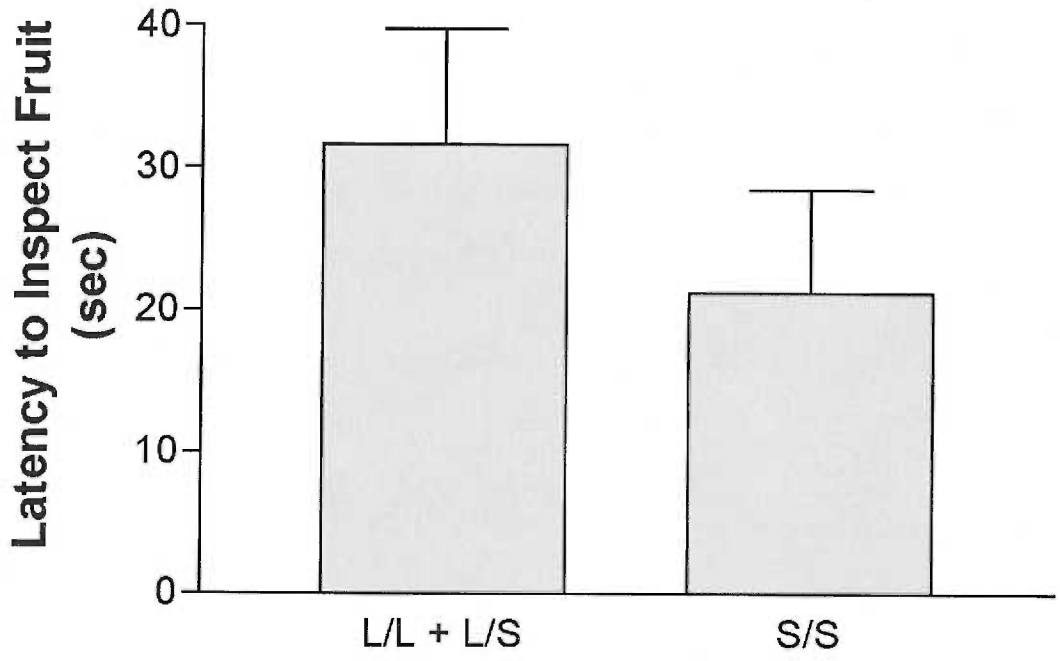

Figure 10: Novel Fruit Test Inspection Latency

The results of this test indicate no differences in the kiwi inspection latency between the genotypes (p=0.076, H=3.153 df=1, n=89, Power=0.152). The s/s monkeys do not appear to be more anxious in regards to this ecologically relevant stimulus.

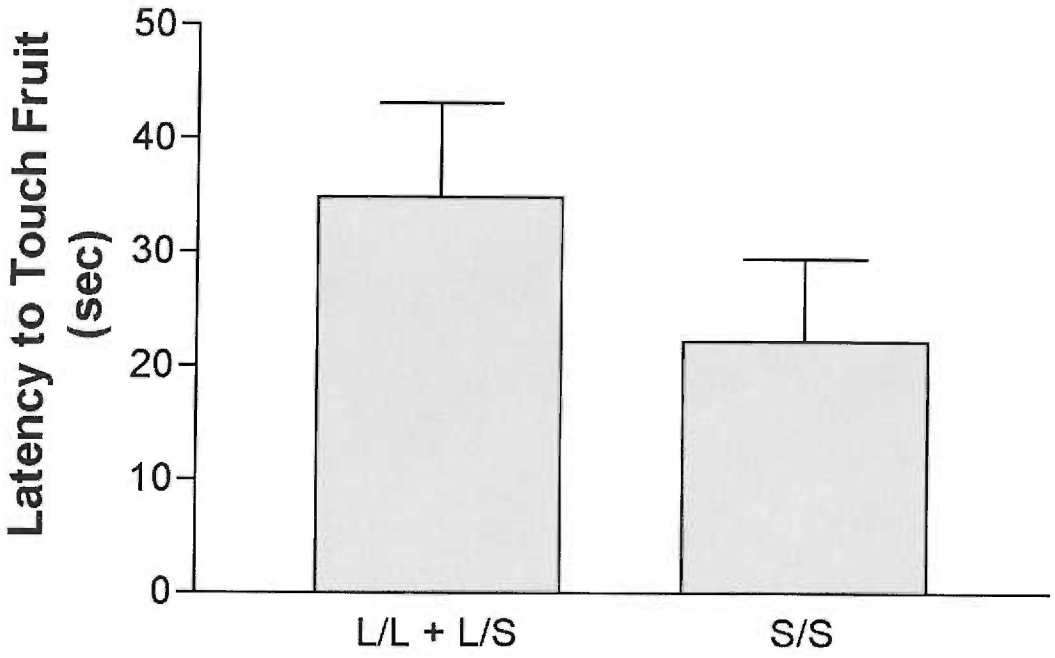

Figure 11: Novel Fruit Test Touch Latency

No significant differences were found between the genotypes in the latency to touch the kiwi (p=0.125, H=2.348, df=1, n=89, Power=0.200). The results of this test further support the contention that the s/s monkeys are not more anxious in regards to this ecologically relevant stimulus.

Chapter 4: Discussion

In this study we found an association between the 5HTTLPR short allele and very specific forms of anxious behavior in a representative population of infant and juvenile rhesus macaques. There remains controversy over 5HTTLPR association studies due to inconsistency amongst some human studies (Lesch et al. 1996; Jorm et al. 2000; Kumakiri et al. 1999). In human studies in which an ethnically and socially diverse group of humans is enlisted, it is difficult to control for diverse environmental and experiential factors such as life experiences that should comprise about 40-60% of the variation observed in anxiety research. In addition, the 5HTTLPR may influence only very specific anxious behaviors, without affecting others.

Using infant and juvenile rhesus macaques raised together in the same institution with standard practices of husbandry and protocol, it is possible to minimize environmental and experiential influences. Thus, we could examine the hypothesis that the 5HTTLPR influences very specific behaviors.

Using four standardized behavioral tests, we have found that the s/s monkeys are more anxious in response to a novel, non-threatening environment; a novel, threatening, non-social stimulus; and in response to a threatening, social stimulus. They are not more anxious in response to a novel ecologically relevant stimulus with reward value. Using these four tests, it has recently been demonstrated that context is critical in the expression of anxious traits (Coleman et al. submitted). Specifically, anxious individuals may not score as "anxious" for each test, and one type of anxious behavior (i.e. threat display) may not be displayed in all tests. Thus, a monkey that is "anxious" in one test may not be "anxious" in another. The

results of this study showed that the s/s monkeys somehow scored as "anxious" for three of the four behavioral tests. However, what those anxious behaviors were differed sharply between tests. For instance, the s/s monkeys engaged in a greater number of fear grimaces and spent a greater percentage of time lipsmacking in the Remote Controlled Car test (Figures 5 and 6). However, in another measure of anxious behavior in the same test, the s/s monkeys were not different in the percentage of time spent away from the mother (Figure 4). Thus, an "anxious" monkey in one test does not display every type of anxious behavior. Similarly, while the s/s monkeys engaged in more lipsmacking and fear grimacing in the Remote Controlled Car test (Figures 5 and 6), they were not different in these very same behaviors in the Human Intruder test (Figures 7 and 8). These results, as well as previous work by Coleman and others, support the interpretation that the 5HTTLPR is only influencing very specific aspects of anxious behavior, such as fear grimaces or threat displays, the expression of which is dependent on context.

One caveat, however, is in regards to the low statistical power for all but one of the tests performed. Statistical power is the probability of discarding a true difference (i.e. a false negative). Two variables in particular which were not significantly different may in actuality represent a true difference. These variables are the latency to inspect the fruit in the Novel Fruit test ($p=0.076$, Power=0.152) and the time away from mother variable in the Remote Controlled Car test ($p=0.085$, Power=0.409). These variables combine an alpha level just above significance ($p<0.05$) and a low power. While other variables had low power scores, the alpha levels were generally high enough that a Type II error was unlikely. One way to resolve this difficulty would be to use a higher sample size in future experiments, which

would increase the power and decrease the possibility of a Type II error. Calculating for sample size, an increase of power to 0.80 (approximately the highest value for any test in this study) for the time away from mother variable would require 41 animals each of the L hetero- and homozygotes and S homozygotes. This is actually fewer animals than was used in this study, however, a greater number of S homozygotes are required for higher power due to the smaller sample size of that group. An increase of power to 0.95 would only require 72 L animals and 71 S animals. Clearly, this variable could be addressed in the future with an increase in sample size. However, for the latency to inspect the fruit variable, an increase of power to 0.80 would require 391 L animals and 156 S animals. Clearly, this variable cannot realistically be addressed by increasing the sample size. Even an increase to a power of 0.50 would require 171 L animals and 69 S animals. One mitigating factor for the latency to inspect the fruit variable is that it would be unlikely to find a difference in the latency to inspect, but not the latency to touch, since these actions usually occur very closely together.

The finding that the 5HTTLPR is only associated with specific forms of anxiety in specific contexts is consistent with other studies (Lesch et al. 1996; Bondy et al. 2000; Courtet et al. 2001; Osher et al. 2000). These studies have described cases in which the 5HTTLPR is associated with specific anxiety related traits, such as suicidality, and not with others. Using a set of standardized tests, Lesch et al. (1996) described an association between the 5HTTLPR and the specific subcategories of neuroticism, namely anxiety, angry hostility, depression and impulsiveness, but not self-consciousness or vulnerability. This sort of specificity is similar to what is seen with this study, in that specific anxious behaviors, such as threats or fear grimaces, were expressed in specific contexts, such as increased fear grimaces against a non-

social threatening stimulus, but not against a social threatening stimulus.

One contextual factor that may influence the outcome of the human based association studies is the heterogenous nature of psychiatric diagnosis. Taken from the Diagnostic and Statistical Manual IV, to be diagnosed with Generalized Anxiety Disorder a patient must express at least three of the following symptoms: feels restless, edgy, keyed up; tires easily; has trouble concentrating; irritability; increased muscle tension; and insomnia. Several of these symptoms could easily be linked to changes in serotonin function, such as muscle tension and insomnia, since serotonergic disfunction has been associated with sleep disorders and the S allele of the 5HTTLPR has been associated with increased muscle tension (Chen et al. 1992; Lesch et al. 1996). The other symptoms may not be associated with serotonin neurons. Similarly, a diagnosis of Post-Traumatic Stress Disorder must contain at least two of: insomnia; irritability; poor concentration; hyper-vigilance and increased startle response. The heterogenous nature of these diseases may lead to population samples, some of whom have serotonin related pathologies and some who do not, even though they are all diagnosed with the same condition. This problem could be one of the causes of the difficulty in replicating the human 5HTTLPR association studies, and may hinder an experimental understanding of the role of serotonin neurons, and genetic differences thereof, in anxiety and anxiety disorders.

Taken together with this study, the data suggests a new approach to 5HTTLPR association studies. The work with animal models, such as this study, argues for examination of anxiety related traits on a symptom or trait basis, as opposed to the disease or personality cluster approach used now. Our results also argue for the importance of context dependence

in the expression of anxiety related traits. Different contexts used in human studies, such as an anxiety provoking stimulus in one study versus a scheduled clinical interview in another, may mask the true contribution of serotonin related genetic polymorphisms, just as in this study fear grimacing is present in one context (Remote Controlled Car) and not another (Human Intruder).

Due to the limited information available on the functional consequences of the 5HTTLPR for neuronal function, the best information to date would suggest that the S allele of the 5HTTLPR mediates a decreased activity of serotonin neurons, either by serotonin content or neuron firing activity (Reist et al. 2001). This has several possible consequences for the state of the anxiety circuit in s/s individuals. A decreased serotonin activity in s/s homozygotes could result in a decrease of inhibitory tone to critical areas of the anxiety circuit, such as the right medial prefrontal cortex, the lateral amygdala or the locus coeruleus. Serotonin neurons have been shown to have an inhibitory effect in the lateral amygdala (Stutzmann and LeDoux 1999), and a decrease in this inhibition could lead to enhanced long term potentiation of associated stimuli. This could explain the abnormal levels of anxiety associated with normal stimuli in sufferers of anxiety disorders, such as a "conditioned fear" to innocuous social interactions.

Similarly, decreased inhibitory tone to the right medial prefrontal cortex (Benes et al. 2000) could result in the hyperactivity observed in macaques and humans with high levels of anxiety and fear (Tomarken et al. 1990; Kalin et al. 2000). Since the medial prefrontal cortex appears to increase the acquisition and extinction of conditioned fear (Morgan and LeDoux 1999), a serotonergic disfunction could manifest as either an abnormally high acquisition or an

abnormally long extinction period.

Changes in serotonin neural function due to the 5HTTLPR can also be examined using the model of stress sensitization (Meaney 2001). Decreased serotonergic neurotransmission could result in several changes to this circuit, such as a decreased inhibition to the PVN. Without serotonergic inhibition in this region, the release of CRF from the median eminence due to input from the locus coeruleus could be potentiated, resulting in a greater stress/anxiety response due to enhanced secretion of cortisol and glucocorticoids. Decreased serotonergic tone could also enhance the inhibitory input of noradrenergic neurons from the locus coeruleus or CRF neurons from the central nucleus of the amygdala, further depressing the capability of serotonin to modulate this circuit. The effects of decreased serotonergic tone could also manifest as chronic changes in the anxiety circuit, since the early stresses described by Francis and Meaney (1999) might be able to sensitize the CRF anxiety circuit to a greater degree, thus potentiating later anxiety and anxiety disorders, without serotonin input to modulate and moderate the process.

One way to address these possibilities is to examine studies already completed, retrospectively if possible, and use 5HTTLPR genotype as the grouping variable. For instance, the imaging study by Davidson et al. (2000) would be a perfect tool in order to examine the possible correlation between genotype and right medial prefrontal cortex hyperactivity if genotype was substituted for anxiety disorder as the grouping variable. Another possibility would be to examine the relationship between 5HTTLPR genotype and blood levels of cortisol, which has been shown to correlate with anxiety as well as right medial prefrontal hyperactivity (Kalin et al. 2000).

Another area of inquiry is how changes in serotonin neuron activity could result in changes in very specific anxious behaviors via the anxiety circuit. One possibility is that specific anxious behaviors may be mostly influenced by neural circuits that don't receive much serotonergic input. For instance, brain regions such as the entorhinal cortex and the hippocampus are most directly tied to the contextual aspects of conditioned fear. Static tests such as the Free Play test may primarily involve these areas, while dynamic stimuli such as the remote controlled car may more heavily involve regions such as the amygdala (it should be noted that propensity to explore in the Free Play Test may have nothing to do with conditioned fear). Such heterogeneity could allow serotonin neurons to mostly or exclusively target one area and thus one type of behavior, such as the amygdala, while excluding others. Other evidence suggests that serotonin neurons may influence anxiety through distinct pathways, such as the periaqueductal gray. 5HTTLPR genotype might only influence serotonin neurons in one such pathway, and thus a certain subset of behavior, while leaving others unaffected, although it should be noted that there is no evidence suggesting such a region specific effect for the 5HTTLPR. For instance, the periaqueductal gray pathway is involved in certain behavioral responses to conditioned fear, such as freezing (Amorapanth et al. 1999), which may be differentially affected by serotonin neurons and only manifest in certain tests. For instance, behavioral freezing mediated by the periaqueductal gray would be unlikely to affect the outcome of the Novel Fruit test.

In future studies, care needs to be taken to resolve the conflict between the 5HTTLPR association studies. As much as it is possible, environmental and experiential influences need to be controlled for, especially in humans. Also, the same types of specific anxious behaviors

need to be examined in several contexts.

Eventually it will be necessary to move beyond association studies. These studies have a variety of limitations, which makes it difficult to fully understand the role of any polymorphism in any psychiatric disorder. In the future, direct manipulation and examination of serotonin neurons will be necessary. Conditional knockouts, knock-ins, pharmacological mimicry of the 5HTTLPR and other technologies will help establish a cause and effect relationship between the 5HTTLPR and specific anxious behaviors. The goal of understanding anxiogenesis and how to address the human cost of this problem is still ahead.

Chapter 5: Summary and Conclusions

128 rhesus macaques were genotyped for this study, of which 90 were used for behavioral analysis. The allele frequencies were not different from expected values for a normal population. The monkeys were subjected to four specific behavioral tests, a free play, remote controlled car, human intruder and novel fruit test. The s/s monkeys were found to be more "anxious" in regards to the free play, remote controlled car, and human intruder test, in that they spent less time exploring in the free play, engaged in more fear and lipsmacking displays in the remote controlled car test, and spent more time threatening during the stare portion of the human intruder test. The s/s monkeys were not significantly different from the l/l and l/s monkeys in the other variables recorded for the three tests, as well as the novel fruit test.

We conclude that monkeys which are homozygous for the short allele of the 5HTTLPR gene show a specific set of increased anxious behaviors that include increased

behavioral inhibition, increased fear and uncertainty, and increased propensity to show aggression to a threatening social stimulus. Importantly, s/s monkeys do not show increased levels of all forms of anxious behavior, rather they show increases in very specific anxious behaviors.

We suggest that the experimental approach that we have taken in this study, carefully assessing different forms of anxious behaviors, will play a key role in our eventual ability to understand which aspects of behavior are influenced by the 5-HTTLPR polymorphism.

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