Expression of Anxiety-Related Genes, including the Cytoplasmic Polyadenylation Element Binding Protein (CPEB), in the Rat Limbic System.

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Abstract

Anxiety disorders are one of the most prevalent mental disorders in the world. While "normal" anxiety serves as an important protective mechanism, "pathological" anxiety characteristic of an anxiety disorder is both maladaptive and disruptive. The majority of studies have focused on the neurotransmitter systems associated with the actions of known anxiety drugs. This focus may likely limit the exploration of mechanisms underlying anxiety disorders. This project aims to examine changes in gene expression that may underlie higher or lower levels of inherent anxiety.

Using a well-established behavior test for anxiety, the elevated plus maze, we identified male Wistar rats exhibiting inherently high- or low-anxiety levels. Brain regions known to mediate anxiety, the amygdala, hippocampus and nucleus accumbens, were dissected and total mRNA isolated. The mRNA was converted to cDNA via reverse transcription-polymerase chain reaction (RT-PCR). Then, the cDNA was used in suppression subtractive hybridization, a technique used to compare two complete populations of cDNAs and identify cDNAs that are upregulated in one population in relation to the other. In this project suppression subtractive hybridization was used to compare high- and low-anxiety cDNA populations. The upregulated cDNAs were amplified in a PCR reaction that enables rare transcripts to be identified. The PCR products from the suppression subtractive

hybridization were cloned and used to create two cDNA libraries for high- and low-anxiety related genes. These clones were sequenced to show over 1000 genes upregulated in high- and low-anxiety. The gene list was then subjected to bioinformatic analysis to identify one candidate to be studied in further detail.

The prion protein was identified as a potential candidate. Examination of the literature sparked an interest in studying other prion-like proteins, more specifically the cytoplasmic polyadenylation element binding protein (CPEB). The CPEB protein is a potent regulator of mRNA translation in both mature oocytes and the adult brain. While unphosphorylated the CPEB protein keeps specific mRNAs dormant in the cytoplasm. In its phosphorylated form CPEB catalyzes polyadenylation of the mRNA, leading to protein synthesis.

PCR was used to show the presence of CPEB mRNA transcripts in the rat hippocampus. CPEB protein expression was examined in the brain samples isolated from control, high- and low-anxiety rats. It was found that CPEB was significantly upregulated in high- and low-anxiety rats compared to control. The protein expression of an upstream kinase, Aurora A kinase, and a downstream target, Calcium/Calmodulin Dependent Kinase II (CaMKII), was also investigated. The results from Aurora A kinase were inconclusive. CaMKII, on the other hand, was significantly upregulated in high-anxiety over both control and low-anxiety. These results suggest that CPEB may catalyze increased translation of mRNAs in high-anxiety while acting as a repressor of those same mRNAs in low-anxiety.

Recent studies have suggested that CPEB protein plays an important role in synaptic plasticity. The regulation of synaptic plasticity, and its impact on learning and memory, is believed to be a key mechanism behind the maintenance of anxiety disorders. Therefore the results of this study suggest a new molecular mechanism in the development of anxiety disorders.

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3′ UTR	3 prime Untranslated Region
5-HT	Serotonin
ATF4	Activating Transcription Factor 4
CaMKII	Calcium/Calmodulin-dependent Protein Kinase II
CPE	Cytoplasmic Polyadenylation Element
CPEB	Cytoplasmic Polyadenylation Element Binding Protein
CPSF	Cleavage and Polyadenylation Specificity Factor
EPM	Elevated Plus Maze
GABA	γ-aminobutyric acid
G3PDH	Glyceraldehyde-3-phosphate Dehydrogenase
LC	Locus Coeruleus
NE	Norepinephrine
NMDA	*N*-methyl-*D*-aspartate
NP25	Neuronal Protein 25
PAG	Periaqueductal Gray
PAP	PolyA Polymerase
PC12	Phaeochromocytoma Cell Line
PCR	Polymerase Chain Reaction
PTSD	Post Traumatic Stress Disorder
RT-PCR	Reverse Transcription-Polymerase Chain Reaction

siRNA	Small Interfering Ribonucleic Acid
TBST	Tris Buffered Saline with Tween 20

1 Introduction

1.1 Normal Anxiety

Anxiety is a biologically important mechanism conserved across many species. Normal anxiety serves as an adaptive response to potentially threatening situations (Clement *et al*., 2002; Finn *et al*., 2003; Sandford *et al*., 2000). Anxiety allows an organism to protect itself against future danger by responding to threatening stimuli through characteristic responses of fight, flight or freezing (Finn *et al*., 2003; Gordon and Hen, 2004; Sandford *et al*., 2000). In humans anxiety may also be expressed psychologically as worry (Antony and Swinson, 1996; Finn *et al*., 2003). These anxious reactions enable an organism to evaluate a threatening situation and react in an appropriate manner to reduce the risk of harm (Antony and Swinson, 1996).

1.2 Pathological Anxiety

Although anxiety is an important protective mechanism it can become maladaptive and disruptive (Clement *et al*., 2002; Finn *et al*., 2003; Sandford *et al*., 2000). Pathological anxiety, as manifested in anxiety disorders, is an anxious response that occurs out of proportion to the threat, becomes disruptive to daily life and causes suffering (Antony and Swinson, 1996; Clement *et al*., 2002; Finn *et al*.,

2003; Sandford *et al*., 2000). Although many authors see pathological anxiety not as a separate and unique state from normal anxiety but as an extreme expression of it (Finn *et al*., 2003; Lesch, 2001; Sandford *et al*., 2000), anxiety disorders may be defined as a collection of psychological problems that include excessive anxiety, worry, fear and avoidance (Antony and Swinson, 1996). Diagnostically, the difference between normal and pathological anxiety can be said to lie in the fact that the latter is disruptive and causes suffering for an individual.

Anxiety disorders are divided into five major diagnoses according to the Diagnostic and Statistical Manual IV. These five disorders are generalized anxiety disorder, obsessive compulsive disorder, phobias, panic disorder and post traumatic stress disorder (PTSD). While anxiety disorders may be classified into five categories they are not isolated from each other, and many of their behavioral and physiological symptoms overlap (Finn *et al*., 2003; Gross and Hen, 2004). Also, many of the disorders respond to the same treatment, highlighting underlying commonalities between disorders.

Anxiety disorders affect a large proportion of the population worldwide. An estimated 30 million people in the United States alone will experience an anxiety disorder at some point in their lives (Finn *et al*., 2003; Lepine, 2002). Associated with anxiety disorders are large personal and socio-economic costs ranging from medical treatments to reduced workplace productivity to suicide (Antony and Swinson, 1996; Lepine, 2002). As a result of these (and other) factors

there is a great deal of interest in discovering the underlying mechanisms of anxiety disorders in order to improve diagnosis and treatment of these complex disorders.

1.3 Animal Behavior Models

While anxiety is believed to be a uniquely human trait, anxiety-like behaviors have been observed across many other species. As a result, animal models can be used to obtain information about molecular mechanisms involved in anxiety that would be impossible in humans. Animal models allow investigators to test hypotheses under controlled conditions and using methods that would be difficult to manage in humans (Hitzemann, 2000; Kalueff and Tuohimaa, 2004).

Unfortunately many difficulties arise when modeling human psychiatric disorders, such as anxiety disorders, in another species. The major difficulty in modeling anxiety disorders in animals is that they are not capable of verbal communication which is a critical component of diagnosing psychiatric disorders. It is difficult to identify analogous behaviors (Kalueff and Tuohimaa, 2004). It is also difficult to distinguish between fear and anxiety. The behavioral and physiological responses in fear and anxiety are highly similar. The distinction between fear and anxiety lies in the concept that the former is a response to an actual threat while the latter is a response to a potential threat (Belzung and Griebel, 2001; Gordon and Hen, 2004; Gray and McNaughton, 2000). This definition is ambiguous in animals so anxiety in animals can only be implied at best. Lastly, there are structural and

functional differences between the nervous systems of animals and that of humans (Kalueff and Tuohimaa, 2004). Therefore, the accuracy of the data obtained using animal models is dependent on the validity of the model (Kalueff and Tuohimaa, 2004).

1.3.1 Conditioned Tests

In the field of anxiety research there are two main categories of animal models: those that involve conditioned responses and those that involve unconditioned responses (Rodgers and Dalvi, 1997). Conditioned tests combine elements of learning and memory with aversive stimuli and require pre-test training paradigms. They measure a conditioned response, in other words, a specific response that is learned through association with an aversive stimulus (Hitzemann, 2000). Examples of conditioned tests are fear-potentiated startle and Geller-Seifter conflict (Hitzemann, 2000; Rodgers and Dalvi, 1997). Conditioned tests require extensive pre-test training and often use food/water deprivation or electric shock as an aversive stimulus (Rodgers and Dalvi, 1997). The required pre-test training associated with conditioned tests makes them more amenable to experimental manipulation than unconditioned tests (Flint, 2003).

1.3.2 Unconditioned tests

Unconditioned tests on the other hand do not require time consuming pre-test training as they measure un-learned, inherent anxiety. Unconditioned tests include the open field, light-dark transition and elevated plus maze tests (Hitzemann, 2000). Unconditioned tests are believed to be more sensitive to stress compared to conditioned tests as the latter tend to use strong and often painful stressors such as foot shock. It is argued that these stressors may suppress activity and cause complex changes in animal behaviors, making interpretation of results difficult (Kalueff and Tuohimaa, 2004).

1.3.3 The Elevated Plus Maze test

The elevated plus maze (EPM) is a simple and highly validated behavioral test for anxiety in rodents. The history of the EPM goes back to the 1950's when Montgomery observed that rats showed high levels of exploration (and therefore preference) for elevated enclosed alleys over elevated open alleys (Pellow *et al.,* 1985; Rodgers and Dalvi, 1997). He inferred that since both alleys were novel and would therefore produce the same drive to explore, the rat's avoidance of the open arms was the result of a fear response (Rodgers and Dalvi, 1997).

Based on Montgomery's findings Handley and Mithani developed the EPM (Pellow *et al.,* 1985; Rodgers and Dalvi, 1997). The maze consisted of two open

arms set across from each other and two closed arms set at 90° to the open arms (Figure 2.1). The apparatus' ability to measure anxiety-like behavior was established through the ability of anxiolytics and anxiogenics to affect the behavior within the apparatus (Pellow *et al,* 1985).

Since its first description, the EPM test has been validated pharmacologically, physiologically and behaviorally and has become one of the most widely used behavioral tests for anxiety-like behavior (Gordon and Hen, 2004; Hogg, 1996; Pellow *et al.,* 1985; Rodgers and Dalvi, 1997). Montgomery's view that aversion to the open arms reflected fear or anxiety has been validated behaviorally as rats display more anxiety-related behaviors, including freezing and defecation, in the open arms compared to the closed arms. Physiologically, rats confined to the open arms show higher levels of plasma corticosterone than those confined to the closed arms (Cruz *et al*, 1994; Hogg, 1996; Pellow *et al*, 1985). As corticosterone is a stress hormone, its increased release strongly suggests that the open arms create an increased stress response in rats (Pellow *et al.,* 1985). Finally, agents that increase anxiety levels (anxiogenics) decrease the amount of time spent in the open arms while agents that decrease anxiety (anxiolytics) predictably increase the amount of time spent in the open arms (Cruz *et al*, 1994; Hogg, 1996; Pellow *et al*, 1985; Rogers and Dalvi, 1997).

1.3.4 Elevated Plus Maze measurements

As the EPM measures the conflict between the drive exploration and aversion to the open arms, the major behavioral measure is the exploration of the open arms. Exploration of the open arms can be measured by two methods. One is the number of entries into the open arms expressed as a total number or as a percentage of open versus total arm entries. A second measure is the amount of time spent on the open arms (Cruz *et al*., 1994; Rogers and Dalvi, 1997). Behaviors other than exploration, such as freezing, grooming or risk assessment, have been examined as other possible indices of anxiety (Cruz *et al*., 1994; Rogers and Dalvi, 1997). Unfortunately, these ethological measures are time-consuming and tend to require video capture systems and thus are not always practical (Cruz *et al.*, 1994).

As with all behavioral tests, the EPM is sensitive to many outside factors. Therefore the procedure needs to be standardized and great attention needs to be spent on maintaining these standards. Housing conditions, lighting, test duration and prior handling have all been shown to affect the test results. These outside factors are believed to be the root cause of variation in results seen between laboratories (Rogers and Dalvi, 1997).

1.4 Brain Regions involved in Anxiety

The complexity of anxiety disorders suggests numerous areas of the brain are involved. The brain regions associated with anxiety have been identified through numerous lesion and microinjection studies in animals and human neuroimaging studies (Gordon and Hen, 2004). A few of these regions will be briefly discussed here.

The brain regions involved in anxiety are said to be organized into a hierarchy of responses. The physiological responses are mediated by so called "lower areas" which include the locus coeruleus (LC), the periaqueductal grey matter (PAG) and the hypothalamus (Sandford *et al.*, 2000). The intermediate level (our area of interest) mediates the "practiced response" to anxiogenic stimuli and includes the amygdala and septo-hippocampal regions (Sandford *et al.*, 2000). The final level of the hierarchy involves the higher cortical regions and directs complex, cognitive processing (Sandford *et al.*, 2000). Each of these levels has the ability to feedback into, and directs the response of, the level(s) below it (Sandford *et al.*, 2000).

The LC is believed to be an important modulator of anxiety. Activation of the LC, either by electrical stimulation or by drugs, elicits an anxiety response. Lesions that inactivate the LC reduce anxiety (Sandford *et al.*, 2000). The LC contains the major norepinephrine (NE) producing neurons in the brain (Ressler and Nemeroff, 2000). It projects to the cerebellum, medulla and spinal cord along with

cortical, sub-cortical and limbic structures (Ressler and Nemeroff, 2000). It receives input from numerous brain stem nuclei including the hypothalamus, PAG and the amygdala (Ressler and Nemeroff, 2000). The LC and its associated NE release are believed to control arousal, vigilance, activation of the stress response and modulation of memory (Ressler and Nemeroff, 2000). Both novelty and stress have been shown to increases LC firing which results in increased vigilance and arousal (Ressler and Nemeroff, 2000).

The PAG is believed to control the responses associated with fight and flight as stimulation of this area induces responses associated with fight and flight, such as changes in heart rate and decreased sensitivity to pain (Sandford *et al.*, 2000). The PAG receives inputs from the limbic system and various sensory structures (Sandford *et al.*, 2000). It has been hypothesized that the PAG is responsible for selecting, organizing, and executing the appropriate behavioral responses in reaction to information it obtains from the amygdala (Graeff *et al.*, 1993). This direct control over the behavioral response has resulted in the hypothesis that the PAG is responsible for panic disorder (Graeff *et al.*, 1993).

The hypothalamus is an important coordinator of the neuroendocrine response through activation of the hypothalamic-pituitary-adrenal axis. The neuroendocrine response provides the metabolic and cardiovascular support for behavioral response (Chaouloff *et al.*, 1999). Signaling from the amygdala to the hypothalamus has been shown to mediate the neuroendocrine response to stressful and fearful stimuli, including plasma corticosterone release (Davis, 1992).

1.4.1 The Amygdala

The amygdala has long been thought to be the center of a defense system involved in both the expression and acquisition of fear and anxiety (Lang *et al*., 2000). Expression of anxiety has been shown to require the transmission of information to and from the amygdala. The amygdala receives information from the thalamus and the cortex, and that information is processed within the various amygdaloid nuclei. The processed information is then sent out to targets in the hippocampus, brain stem, hypothalamus and other regions that direct behavioral, autonomic and stress hormone responses (Gordon and Hen, 2004).

Not all of the 13 amygdaloid nuclei and numerous sub-nuclei are involved in anxiety. In fact only the central nucleus and basolateral complex (consisting of the lateral, basal and accessory basal nuclei) are believed to be essential. These nuclei function as an interface between sensory input and motor output important for both the behavioral response to and the learning involved in anxiety (Rosen, 2004). The central nucleus of the amygdala receives input from the prefrontal cortex and, in turn, projects to numerous nuclei in the midbrain and brain stem. These projections make significant contributions to the behavioral, autonomic and endocrine responses to danger (Rosen, 2004).

The basolateral nucleus receives the majority of the sensory information from the thalamus and the cortex. The basolateral complex also receives innervation from the hippocampus and the prefrontal cortex. The role of the basolateral complex in anxiety is believed to be in the "emotional" evaluation of

sensory information. This evaluation then influences other regions of the amygdala and brain to ensure an integrated response. Efferents from the basolateral complex are believed to orchestrate active avoidance behaviors most likely through the nucleus accumbens, striatum and thalamus (Rosen, 2004).

1.4.2 The Hippocampus

A host of information has been building to suggest a major role of the hippocampus in fear and anxiety (McHugh *et al*., 2004). The ventral region of the hippocampus is closely associated with the amygdala. It has connections to and from the basal and lateral nuclei of the amygdala (McHugh *et al*., 2004). Lesions of the ventral hippocampus have been shown to produce anxiolytic effects in various behavioral tests for anxiety. Hippocampal lesions also decrease plasma corticosterone levels in animals exposed to stressful situations. As corticosterone levels reflect stress levels these results suggest the hippocampus has an important role in mediating the stress response. Lesions to the septo-hippocampal region, therefore, appear to produce the same effect as classical anxiolytics, like the benzodiazepines (Gray and McNaughton, 2000).

In their text, "The neuropsychology of anxiety: an enquiry into the functions of the septo-hippocampal system", Gray and McNaughton (2000) have put forward a septo-hippocampal-centric theory of anxiety. They believe the hippocampus, not the popular amygdala, plays a central role in anxiety. In their view, the

hippocampus can add positive or negative values to memories. A hyperactive or hypersensitive hippocampus may be biased towards negative associations. Thus, it would increase the negative value of experiences or preferentially store highly threatening associations (Gray and McNaughton, 2000). This would prime one of the major centers of the brain to perceive incoming information as more threatening, triggering a reaction that is inappropriate to the level of threat, a hallmark of anxiety disorders.

1.4.3 The Nucleus Accumbens

The nucleus accumbens is divided into two regions, the central core and the peripheral shell, although the division is poorly characterized in humans and primates (Sturm *et al*., 2003). The central core is associated with extrapyramidal motor functions, while the peripheral shell is associated with the limbic system. The shell is both biochemically and histologically similar to the central nucleus of the amygdala (Sturm *et al*., 2003). In humans, the nucleus accumbens receives input from the basolateral, medial and central nuclei of the amygdala. It projects to the pallidum, striatum, mediodorsal thalamus, prefrontal cortex and mesolimbic dopaminergic areas. Thus, the nucleus accumbens occupies a central position between many of the brain regions involved in the development of anxiety disorders (Sturm *et al*., 2003).

1.5 Molecular Mechanisms of Anxiety

Several environmental risk factors have been identified for anxiety disorders, such as severe abuse or overprotective parents (Antony and Swinson, 1996). Yet these risk factors are not sufficient in themselves, as not everyone who experiences known risk factors will develop an anxiety disorder. For example, a traumatic event, such as participation in war, can cause PTSD, but not all individuals who experience a traumatic event will develop PTSD (Antony and Swinson, 1996; Gross and Hen, 2004). This variance has been attributed to genetic influence. A genetic predisposition plus environmental factors combine to result in the expression of an anxiety disorder. Family, twin and adoptee studies have calculated the genetic contribution of anxiety disorders to be 25-65% (Finn *et al*., 2003).

Early studies into the genetics of anxiety have investigated the role of various neurotransmitter systems including the adrenergic, dopaminergic, adenosinergic and cholecystokinin systems, although most studies have focused on γ-aminobutyric acid (GABA) and serotonin (5-HT) as they are the targets of known anxiolytics (Antony and Swinson, 1996; Finn *et al*., 2003; Gordon and Hen, 2004; Gross and Hen, 2004; Sandford *et al*., 2000).

GABA is the primary inhibitory neurotransmitter in the brain (Kalueff and Nutt, 1996; Lydiard, 2003). GABA counteracts the activity of the excitatory neurotransmitter glutamate to maintain homeostasis. It is hypothesized that in anxiety the GABA system is down-regulated, possibly resulting in a state of

excessive neuronal hyper-excitability (Lydiard, 2003). The inhibitory function of GABA is mediated mainly thru the GABA-A receptor. The GABA-A receptor is composed of five receptor subunits arranged around a central chloride channel and is highly expressed in many anxiety-related brain regions including the amygdala and hippocampus. When GABA binds and activates the GABA-A receptor the chloride channel opens and chloride ions move into the neuron. This influx inhibits neuronal activity by hyperpolarizing the cell, blocking neuronal depolarization (Kalueff and Nutt, 1996).

Many anxiolytics impact GABA transmission by activating the GABA-A receptor. The benzodiazepines exert their effects by making the GABA-A receptor more sensitive to the effects of GABA, enhancing the inhibitory action of GABA itself (Kalueff and Nutt, 1996). Other known, but obviously less popular, anxiolytics such as ethanol and barbiturates, affect the GABA-A receptor by directly opening its chloride channel (Kalueff and Nutt, 1996). On the opposite end of the spectrum compounds that act as inverse agonists, which inhibit GABA-A activity, have been shown to produce severe anxiety in both human and animal studies (Kalueff and Nutt, 1996).

Studies attempting to understand the finer details of the role of GABA in anxiety have examining the effects of inactivation of genes involved in GABA neurotransmission in animal models. For example, inactivation of various subunits of the GABA-A receptor has been investigated along with various enzymes involved in the synthesis and metabolism of GABA. Many of the GABA-A subunit

mutations had no measurable effect on the expression of anxiety although inactivation the γ2 subunit increased anxiety (Belzung and Griebel, 2001; Clement *et al.*, 2002; Finn *et al.*, 2003). Inactivation of the GABA synthesis enzyme GAD65, resulted in increased anxiety, probably as the result of reduced amount of GABA available (Belzung and Griebel, 2001; Clement *et al.*, 2002; Finn *et al.* 2003).

The therapeutic benefits of some antidepressants, such as the selective serotonin reuptake inhibitors, have implicated 5-HT in anxiety. The activity of 5-HT in anxiety is complex as there are at least 14 subtypes of 5-HT receptors and some are inhibitory and others excitatory (Ressler and Nemeroff, 2000). 5-HT is believed to play a modulatory role in memory, behavior and mood (Ressler and Nemeroff, 2000). There is extensive 5-HT innervation in brain regions associated with anxiety including the amygdala, hippocampus, PAG and hypothalamus (Chaouloff *et al.*, 1999). Activation of the 5-HT_{1A} receptors in the hippocampus is believed to reduce anxiety by increasing the resilience of the hippocampus to aversive stimuli (Finn *et al.*, 2003; Ressler and Nemeroff, 2000). On the other hand, activation of the 5-HT_2 receptors in the amygdala and hippocampus has been shown to increase anxiety (Finn *et al.*, 2003). While the therapeutic benefit of selective serotonin reuptake inhibitors suggest anxiety may be caused by a decrease in 5-HT transmission this is not conclusive as 5-HT activity can either increase or decrease anxiety on which receptor is being activated (Finn *et al*, 2003).

Like the GABAergic system, the 5-HT system has been studied using gene inactivation experiments. Null mutation of the 5-HT_{1A} receptor increased anxiety (Clement, *et al.*, 2002; Finn *et al.*, 2003, Gross and Hen, 2004), while a mutated 5-HT_{1B} mutation had no effect in two anxiety tests but decreased anxiety in a third test and mutation of the 5-HT_{5A} receptor had no effect (Clement *et al.,* 2002).

The majority of genetic or molecular studies into anxiety have been directed by the actions of known anxiolytics. Unfortunately this reliance on anxiolytics may be limiting the field of study. New molecular techniques are now available that allow the entire populations of expressed genes to be screened and identified.

Recently, Wang *et al.* (2003) used cDNA microarray to examine the differences in gene expression in the cortex of rats exposed to a cat. They used two different strains of rats that expressed different levels of anxiety when exposed to a cat. Their results identified changes in expression levels of genes known to be involved in anxiety, such as GABA and 5-HT receptor subtypes, as well as genes so far unrelated to anxiety, such as fibroblast growth factor and the microtubule-associated protein (Wang *et al.*, 2003).

In another study, Koks *et al.* (2004) used suppression subtractive hybridization to study the gene expression changes in the amygdala rats exposed to cat odor compared to untreated rats. They also showed upregulation of genes known to be associated with anxiety, carboxypeptidase E which is involved in the synthesis of neuropeptides, and unique genes, such as melanocyte proliferating gene 1 whose function is unknown (Koks *et al.*, 2004). While still in their early stages

new molecular techniques are enabling researchers to probe into the molecular workings of cells and systems with greater ease than ever before. This has allowed research to go in new directions, independent of known drug mechanisms.

1.6 Prion protein

The term prion was first applied to a proteinacious factor associated with mammalian neurodegenerative disorders such as transmissible spongiform encephalopathies (Si *et al.,* 2003). A distinctive characteristic of these disorders is the presence of aggregates of protease-resistant proteins. These proteins were discovered to be misfolded, protease-resistant, β-sheet–rich versions of the normal prion protein found in the host cell (Glatzel *et al*., 2005). The infectious, misfolded prion proteins recruit normal cellular prion proteins and cause them to switch from their normal conformation to the misfolded conformation (Glatzel *et al*., 2005; Si *et al*., 2003).

In the beginning prion was a term only applied to the infectious mammalian prion protein. Later, a handful of proteins were identified in yeast that exhibited numerous characteristics similar to the infectious prion protein although they had no significant sequence homology between them. After the identification of the yeast prion proteins the definition of a prion protein changed (Wickner *et al*., 2004). It no longer only applied to the proteinacious factor associated with mammalian neurodegenerative disorders. The term prion protein now refers to any protein that

can change its conformation and in turn impose the same conformational change on other proteins (Wickner *et al.,* 2004).

The discovery that prion proteins are not purely pathological fueled the search to determine the cellular role of prions. The "benign" cellular form of the prion protein is expressed in neurons as a glycoprotein anchored to the outer cell surface of neurons (Glatzel *et al*., 2005; Nico *et al.,* 2005; Roesler *et al.,* 1999). While it has been found to be non-pathogenic in neurons its function in the cell is unclear. Some of its hypothesized roles include a signal transduction molecule, a protease, a superoxide dismutase or a component in a signal cascade (Glatzel *et al*., 2005). While many roles have been suggested none have been adequately supported.

There is some evidence that the cellular prion protein may modulate anxiety. It is highly expressed in the hippocampus (Roesler *et al.,* 1999) and has been shown to influence excitatory neurotransmission in the hippocampus (Carleton *et al.,* 2001). Excitatory synaptic transmission increases in relation to the amount of prion protein expressed; the more protein the stronger the transmission (Carleton *et al.,* 2001). Another study used prion protein knockout mice in an investigation of anxiety. The mice lacking the prion protein showed normal short- and long-term memory and had anxiety levels identical to controls (Roesler *et al.*, 1999). A later study showed that mice lacking the prion protein did have a stronger anxiety reaction, but only after acute stress (Nico *et al.,* 2005). Thus, the role of the prion

protein in anxiety is believed to be in modulating behavior in response to acute stress (Nico *et al.,* 2005).

1.7 Cytoplasmic Polyadenylation Element Binding Protein

While investigating the relationship of the prion with anxiety, we became aware of other prion-like proteins and their possible roles in anxiety. The cytoplasmic polyadenylation protein was one such case.

Yeast prions contain a glutamine or asparagine rich region in the N-terminal that has been shown to be critical to its prion behavior (Si *et al.,* 2003). A neuronal protein in *Aplysia californica*, the cytoplasmic polyadenylation element binding protein (CPEB), was also found to contain this unique N-terminal region (Si *et al.,* 2003). Further investigation revealed other prion-like characteristics. In its prion conformation or "state" the CPEB protein is able to convert other CPEB proteins to the prion state without the assistance of any other outside factors (Si *et al.,* 2003). CPEB was also shown to be more active, resulting in increased polyadenylation, while in its active prion state. This self-perpetuating, highly active characteristic of *Aplysia* CPEB has been hypothesized to play an important role in synapse-specific plasticity, a mechanism important for learning, memory (Si *et al.,* 2003).

Learning and memory are key components of anxiety disorders. Modulation of synaptic plasticity is believed to be the neural basis of learning and memory. Therefore the cellular control of synaptic plasticity could be an important area of

study into anxiogenesis. The long-term maintenance of synaptic plasticity has been shown to require the synthesis of new proteins at an activated synapse (Huang *et al.*, 2002; Wells *et al.*, 2001; Wu *et al.*, 1998). But how the translation of specific mRNAs within an activated synapse was controlled was unknown. The search to find a mechanism that could guide specific protein translation turned to developing *Xenopus* eggs.

Oocytes inherit a large amount of maternal mRNA that remains dormant in the cytoplasm until a signal triggers their translation. The maturation of oocytes and the early embryonic stages after fertilization are dependent on the translation of these dormant maternal mRNAs (Richter, 1999; Welk *et al.*, 2001). Somehow these mRNAs were translated in not only a time-specific manner but also a location-specific manner. This triggered translation is not a global event, but occurs with different mRNAs at different times.

Nearly all mRNAs receive a polyA tail in the nucleus as part of mRNA processing. PolyA tails are hypothesized to stabilize mRNAs and protect them from degradation after they enter the cytoplasm. Within the nucleus the mRNAs are cleaved and then a polyA tail is added. These two reactions require the cleavage and polyadenylation specificity factor (CPSF) to bind to a *cis*-acting element (a short, specific sequence of nucleotides within the mRNA itself) called the hexanucleotide. The hexanucleotide is located in the 3-prime untranslated region (3′-UTR) and has the sequence AAUAAA. The bound CPSF interacts with a polyA

polymerase (PAP) to add the polyA tail. The mature mRNAs are transported to the cytoplasm where they may be translated into proteins.

Within the cytoplasm not all mRNAs are immediately translated. In the oocytes certain mRNAs were found to have their polyA tails shortened and held dormant in the cytoplasm. Later, just prior to translation, these same mRNAs had their polyA tails lengthened (Dickson *et al*., 2001; Richter, 1999). These unique mRNAs were found to have two sequences in their 3´-UTR that targeted them for this polyadenylation event within the cytoplasm. The first is the same *cis*-acting element required for nuclear polyadenylation, the hexanucleotide. The second *cis*-acting element resides approximately 20 nucleotides upstream of the hexanucleotide and is called the cytoplasmic polyadenylation element (CPE). The CPE has the sequence UUUUUAU. These two sequences allow the mRNA to be bound by numerous *trans*-acting factors that are responsible for the cytoplasmic polyadenylation. *Trans*-acting factors are regulatory elements, usually proteins, which bind *cis*-acting elements in a sequence specific manner.

Within the cytoplasm the CPE sequence is bound by the cytoplasmic polyadenylation element binding protein (CPEB). It recruits the CPSF to the hexanucleotide sequence (Figure 1.1). CPSF then brings in the PAP to catalyze the polyadenylation (Huang *et al*., 2002).

As previously mentioned, not all mRNAs targeted for polyadenylation do so at the same time. The mRNAs must be held dormant in the cytoplasm. Instead of requiring an entirely separate mechanism, CPEB also acts as a repressor of

translation. In its dormant state CPEB bound to the CPE associates with another protein, maskin. Maskin binds the cap binding factor eIF4E. This prevents the interaction of eIF4E with eIF4G and the docking of the 40S ribosomal subunit (Huang *et al*., 2002). The maskin-CPEB interaction prevents the assembly of the ribosome, preventing the translation of mRNA. Thus the dormant form CPEB functions as a translational repressor.

A phosphorylation event triggers the conversion of CPEB from its repressive form to the form that catalyzes polyadenylation. CPEB becomes phosphorylated by the serine/threonine kinase, Aurora A kinase. The phosphorylation event allows the association of CPEB with CPSF. It is believed that the polyadenylation causes maskin to dissociate from eIF4E. This allows the eIF4E-eIF4G interaction and the docking of the 40S ribosomal subunit. The mRNA is then translated (Huang *et al*., 2002). Cytoplasmic polyadenylation is a tight and elegant mechanism for translational regulation. It allows specific mRNAs to be translated in a temporal- and spatial-specific manner based on the modification of the polyA tail.

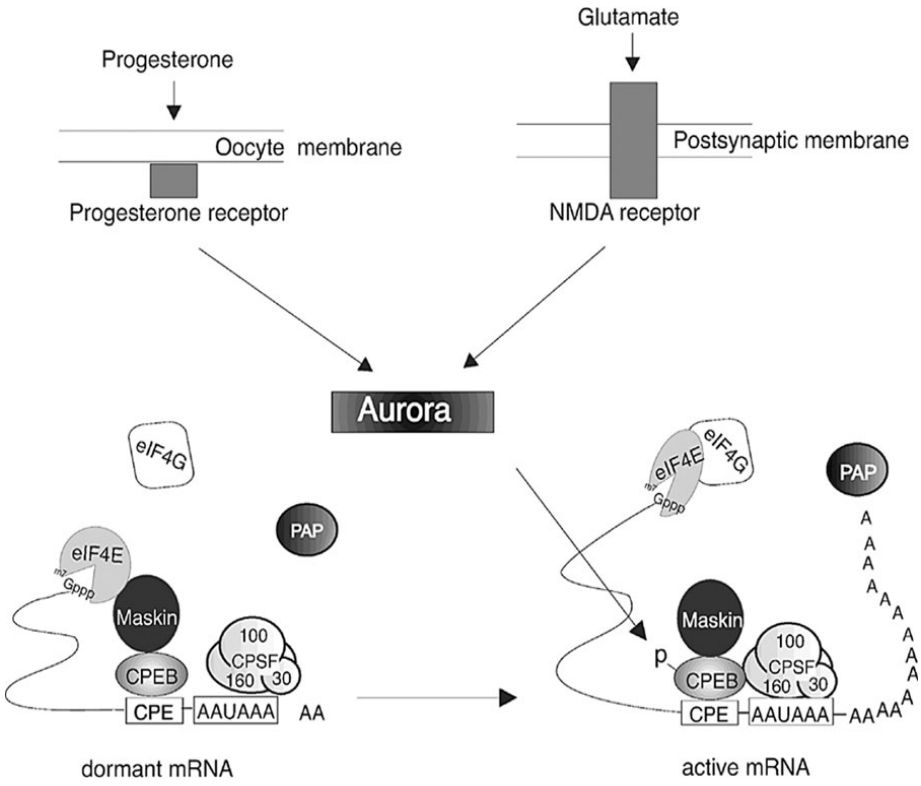

Figure 1.1: Diagram of cytoplasmic polyadenylation, a mechanism conserved in both oocytes and neurons. Signaling by either progesterone or NMDA receptor triggers phosphorylation of CPEB by Aurora Kinase, triggering polyadenylation (Huang *et al*., 2002)

1.7.1 CPEB in the Rodent Brain

The adult rodent has borrowed a mechanism from development. CPEB is indeed found in the adult brain of rodents. It has been identified in the hippocampus, the cerebellum and the cerebral cortex. More specifically, CPEB has been located in the dendritic layers of the hippocampus and synapses of cultured hippocampal neurons (Richter, 1999; Wu *et al*., 1998).

Many components required for polyadenylation in *Xenopus* oocytes have been identified in the rodent brain. CPEB, Aurora A kinase and maskin all have been found to be enriched in postsynaptic density compartments (Richter, 1999; Wu *et al*., 1998). PAP, CPSF and eIF4E have also been found not only in the postsynaptic compartments but also in the cell body (Huang *et al.*, 2002).

This evidence suggests that CPEB-mediated cytoplasmic polyadenylation and translation can occur within dendrites (Huang, *et al*., 2002). Experiments using dark-reared rats have confirmed CPEB-mediated polyadenylation and translation occurs in the synapses. In response to exposure to light CPEB was found to be phosphorylated by a kinase downstream of the NMDA receptor. This event resulted in the polyadenylation of the CPE containing Calcium/Calmodulin-dependent protein kinase II (CaMKII) mRNA, leading to its translation (Huang *et al*,. 2002; Wu *et al.,* 1998). These experiments have shown CPEB to function in the rodent

brain as a regulator of translation in a similar manner to that found in *Xenopus* oocytes.

1.8 Project

Deborah Finn has written that "genetic research on anxiety disorders will benefit from the continued investigation of the neural circuits involved in anxiety, allowing for the identification of candidate genes and proteins that is not just based on clinical observations of a therapeutic response" (Finn *et al.*, 2003). This statement summarizes the aim of this project. This project seeks to uncover some molecular mechanisms underlying anxiety that have been overlooked due to the focus of research on the mechanisms of known anxiolytics drugs.

Pathological anxiety may be seen as an extreme expression of normal anxiety. It is likely that an alteration of a normal mechanism may be at the root of anxiety disorders. Perhaps within the brain certain molecular changes cause normal anxiety to become excessive and pathological. We hypothesize inherent high- and low-anxiety are caused by or accompanied with overexpression of a variety of genes in the amygdala, hippocampus, nucleus accumbens. The purpose of this study is to examine changes in gene expression that may underlie inherent anxiety.

Specifically, five objectives will be addressed in this project:

1. To identify rats with inherently high or low levels of anxiety using the elevated plus maze;

2. To prepare two cDNA populations using the amygdala, hippocampus and nucleus accumbens isolated from high- and low-anxiety-like behavior rats;
3. To identify genes upregulated in the two cDNA populations using the suppression subtractive hybridization technique;
4. To manipulate CPEB protein expression by construction of vectors to over-express or knock down the CPEB protein;
5. To examine the expression of the CPEB, its upstream effector, Aurora A kinase, and its downstream target, CaMKII, in the amygdala, hippocampus and nucleus accumbens of high- and low-anxiety rats.

2 Methods

2.1 Animal Model of Anxiety

Male Wistar rats (250-280g) were used to model anxiety, as it has been reported that while identical in strain, sex and age, the anxiety-like behavior of individual Wistar rats can be different as measured by the EPM (Ho *et al*., 2002). These differences in anxiety measured in individual Wistar rats are repeated in other anxiety models and the anxiety levels measured by the EPM can also predict the behavior in other anxiety models (Ho *et al.,* 2002). Thus, they were the ideal rat strain to study inherent levels of anxiety.

To establish the high- and low-anxiety experimental groups, the rats were tested using the standard EPM (Figure 2.1). The maze dimensions were: arms 4.25" (10.80 cm) wide and 19.75" (50.17 cm) long, intersection 4.25" (10.80 cm) x 4.25" (10.80 cm), closed arm walls 15.75" (40.01 cm) high. The maze was elevated 19" (50 cm) off the ground.

Provisions were made to ensure consistency during behavioral testing. The maze was set in a closed room with fluorescent lighting. It was centered so that no shadows were cast on the maze apparatus. Each rat was handled 1 hour a day for 5 days prior to testing to ensure any measured anxiety levels were not due to handling stress. The rats were allowed to acclimatize to the testing room for half an hour

before testing. The testing room was set up so there were no disruptions during testing.

During the test a rat was placed in the intersection facing an open arm. Timers were started when the rat was released. It was left undisturbed for 5 minutes. An arm entry was defined as all 4 feet having crossed the threshold of the arm. Time spent in the center intersection was not measured. Total amount of time spent in either the open or closed arms was recorded by two people in the room; each was responsible for either the open or closed arms. The maze apparatus was cleaned after every test with 95% ethanol and allowed to dry before a new rat was placed in the maze.

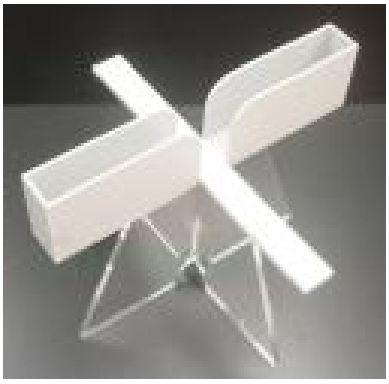

Figure 2.1: Example of Elevated Plus Maze Apparatus

2.2 Dissection of Rat Brain

In accordance with a protocol approved by the University of Saskatchewan Animal Care and Use Committee, trained personnel anaesthetized rats with sodium pentobarbital (65mg/kg body weight) 1 week after behavioral testing. The rats were then decapitated and the brain removed and the amygdala, hippocampus and nucleus accumbens immediately dissected. Half of each brain sample was used immediately for poly[A+] RNA isolation. The other half was frozen in liquid nitrogen and then stored at -80°C for later use.

2.3 Isolation of poly[A+] RNA

Total poly[A+] RNA was isolated using Oligotex mRNA kit (Qiagen Inc, Mississauga, On.). The kit isolated mRNAs using a hybridization reaction between the polyA+ tails of the mRNAs and a dT oligomer attached to a solid particle. The mRNAs were then bound in the solid phase while other cellular molecules, including rRNAs and tRNAs, remained in the liquid phase and were removed by discarding the supernatant. In this case the brain samples were homogenized and lysed in the presence of a highly denaturing guanidine-isothiocyanate (GITC) buffer to inactivate any RNases. The Oligotex suspension was added to bind the mRNA. After the supernatant was washed away the pure mRNA content was eluted.

2.4 Conversion of mRNA to cDNA

The isolated mRNA was converted to cDNA using the reverse transcription-polymerase chain reaction (RT-PCR) outlined in the BD PCR-Select™ cDNA Subtraction Kit User Manual (BD Biosciences Canada, Mississauga, ON). The mRNA was converted to cDNA using AMV Reverse Transcriptase enzyme as described by the manual. The double stranded cDNA was used directly in the suppression subtractive hybridization experiment.

2.5 Confirmation of cDNA

The cDNA samples were subjected to a test PCR to confirm the success of the RT-PCR to produce double stranded cDNA. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a control for all the samples. G3PDH was amplified via PCR using *Pfu* polymerase (Fermentas, Burlington, ON). The G3PDH primers synthesized by Invitrogen (Carlsbad, CA) had the following sequences: sense, 5´-ACCACAGTCCATGCCATCAC; antisense, 5´-TCCACCACCCTGTTGCTGTA. *Pfu* polymerase was added after the reaction had reached 94°C. The PCR cycle was as follows: 94°C for 40 seconds, 55°C for 30 seconds, 72°C for 1 minute. This cycle was repeated 35 times. The PCR finished with an elongation period of 10 minutes at 72°C. The PCR products were visualized via electrophoresis in a 1% agarose gel.

2.6 Suppression Subtractive Hybridization

Suppression subtractive hybridization has become a popular method for studying differential gene expression in cells and tissues. Through a series of hybridizations sequences only expressed in one population are isolated and identified. In the past the traditional method of subtractive hybridization was inefficient at obtaining rare transcripts thus allowing many genes expressed at low levels go unidentified (Diatchenko *et al.*, 1996; Gurskaya *et al.*, 1996). Recently a PCR based subtractive hybridization method was developed. The PCR suppression step allows the amplification of differentially expressed target sequences while suppressing the non-target DNA (Diatchenko *et al.,* 1996).

In suppression subtractive hybridization two populations of cDNA are compared against each other and genes expressed in the one population are isolated though two hybridization steps (Figure 2.2). In the first step the tester cDNA population is divided into two populations. Each population is ligated to one of two double stranded adaptors at their 5′ end. The result is two unique tester sub-populations: one sub-population with adaptor A and another with adaptor B. Excess driver cDNA is added to each sample and allowed to hybridize. Any common sequences between the tester and driver will form hetero-hybrids. In the second step

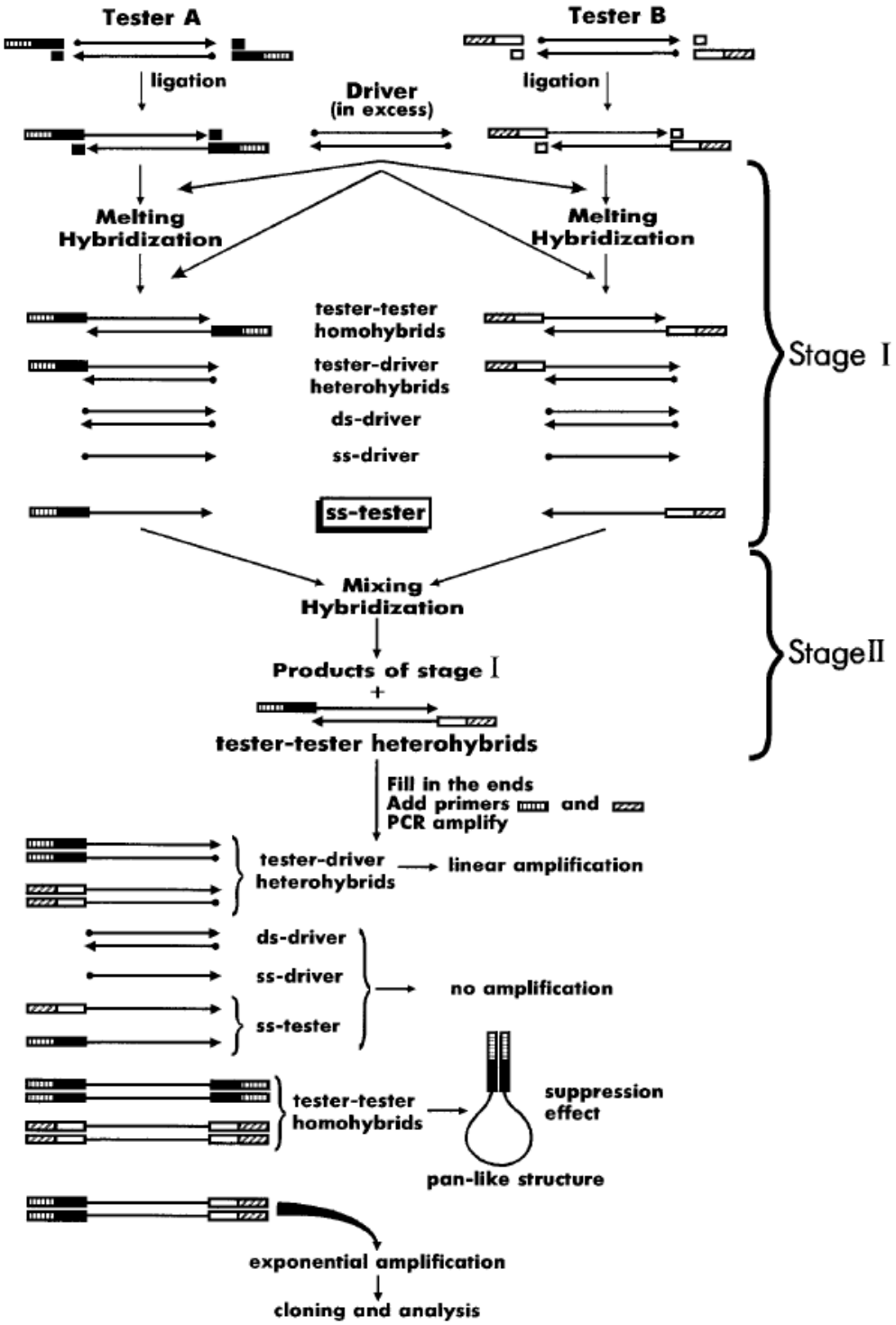

Figure 2.2: Schematic of Suppression Subtractive Hybridization (Gurskaya *et al.*, 1996). Tester A and Tester B represent the tester population split into two subpopulations and each ligated to two unique adaptors. Stage one represents the first hybridization, stage two, the second. The last section shows the possible combinations of tester and driver hybrids and their response to the PCR step.

the two subpopulations are added together and allowed to hybridize. In this step the sequences unique to the tester will form homo-hybrids with a unique adaptor on each end. At this stage PCR is used in a suppression step. PCR is performed using primers complementary to the two adaptors. This allows only sequences with both adaptors A and B to be exponentially amplified (Diatchenko *et al.*, 1996; Gurskaya *et al.*, 1996). The result is a collection of sequences that are upregulated in only one population, the tester.

Here the high- and low- anxiety cDNA samples were compared using BD PCR-Select™ cDNA Subtraction Kit (BD Biosciences Canada, Mississauga, ON). Each library was used as both tester and driver in separate hybridizations to identify genes upregulated in each population.

The cDNAs were digested with *RsaI*, as described in the protocol, to create blunt ended cDNAs. Adaptors were ligated to the tester cDNAs. In this case adaptors were ligated to both high- and low-anxiety in separated, parallel reactions. The hybridizations were also done in parallel with the high- and low-anxiety cDNAs acting as tester in separate reactions. After the second hybridization the samples were amplified using the PCR protocol outline in the manual. The differentially expressed genes were subjected to T/A cloning using the T-Easy vector of the p-GEM T-easy vector system (Promega, Madison, WI) and used to transform competent cells as described in detail below.

2.7 Sequencing and Bioinformatics

Successfully transformed bacterial clones, which were the result of the suppression subtractive hybridization experiment, were grown in 96-well plates in 100μL Luria Broth for 12 hours. The plasmids containing the differentially expressed cDNAs were both isolated and sequenced by the National Research Council's Plant Biotechnology Institute (Saskatoon, SK). When the sequences were returned the sequence information was submitted to WorkBench (http://workbench.sdsc.edu/) to remove any remaining common sequences. Open Reading Frame analysis was used to remove all non-coding fragments. Finally, BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) was used to identify gene names. All structural and mitochondrial genes were removed. The final list of genes was subjected to a literature search to identify any known relationship to anxiety.

2.8 PCR for CPEB

CPEB open reading frame was amplified from cDNA obtained from either PC12 cell or rat hippocampal cDNA using the polymerase chain reaction (PCR). The primers were synthesized by Invitrogen (Carlsbad, CA) and had the following sequences: Sense, 5′-AGATCTATGCTTTTCCCCACCTCTG;

anti-sense, 5′-GAATTCTTCTCCCTTGTCTGCAGAAAG. The sense primer was designed to include a *BglII* restriction enzyme site, whereas the anti-sense primer included an *EcoRI* restriction enzyme site (underlined in sequence above) to be used for future sub-cloning into the pIRES2-EGFP vector.

The CPEB sequence was amplified from PC12 cell cDNA using the primers described above and *Pfu* DNA polymerase. *Pfu* polymerase was added after the reaction had reached 94°C. The PCR cycle was as follows: 94°C for 40 seconds, 55°C for 40 seconds, 72°C for 3 minutes. This cycle was repeated 35 times. The PCR finished with an elongation period of 10 minutes at 72°C.

The full length CPEB sequence could not be obtained from rat hippocampal cDNA using the traditional method of using primers complimentary to the start and end of the gene. Using a modified protocol the gene was amplified in two halves, which were then used to create the full length sequence. In the first reaction the sense primer was paired with the intermediate anti-sense primer complementary to the middle section of the CPEB sequence. In a second reaction the anti-sense primer was paired with the intermediate sense primer complementary to the middle of the CPEB sequence. The intermediate primer sequences were as follows: Intermediate sense, 5′- GTGTTAACCAATCCAAGCTTCTG; Intermediate anti-sense, 5′-CAGAAGCTGGATTGGTTAACAC.

The halves of the full CPEB sequence were amplified using *Pfu* polymerase to minimize the chance of mutations. *Pfu* polymerase was added after the reaction had reached 94°C. The PCR cycle was as follows: 94°C for 40 seconds, 55°C for 1

minute, 72°C for 3 minutes. This cycle was repeated 40 times. The PCR finished with an elongation period of 10 minutes at 72°C.

The two PCR reactions were then combined together and amplified using the sense and anti-sense primers (primers for the beginning and end of the cDNA) to obtain the full length sequence from the two halves. The cycle conditions for the second round of PCR were identical to the above description except the cycle was repeated 30 times. To allow cloning into the T-Easy vector the full length hippocampal CPEB sequence was incubated in the presence of dATP and *Taq* polymerase (Invitrogen, Burlington, ON) at 72°C for two hours to add terminal 5′ adenine overhangs to the sequence. The PCR products were then isolated on a 1% agarose gel and purified using the Wizard SV Gel and PCR clean-up system (Promega, Madison, WI).

2.9 Ligation into T-Easy vector

T/A cloning was used in the creation of the cDNA libraries (post-suppression subtractive hybridization) and the initial cloning of the CPEB fragment. T/A cloning was performed using the T-Easy vector of the p-GEM T-easy vector system. Adenine bases (A) were added to the blunt ended cDNA to enable base pairing to the free thymine bases (T) in the T-easy vector. The DNA fragments were incubated with the T-easy vector in the presence of T4 DNA ligase. The ligation reaction was incubated at 22°C for 2 hours and then incubated for another 2

hours at 16°C. The reaction was then placed in an 80°C water bath for 2 minutes to kill the ligase. The T-Easy vector containing the insert of choice was then used to transform the DH-5α strain of competent *E. coli* cells as described in a later section.

2.10 Selection of cells transformed with T-Easy vector

The cells transformed with the T-easy vector were plated in the presence of ampicillin, isopropyl-beta-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-GAL) (Fermentas, Burlington, ON) to identify colonies that were successfully transformed with the vector. The T-easy vector not only confers ampicillin resistance but is also built to allow colorimetric identification via β-galactosidase activity. The successfully transformed white colonies were then cultured in Luria Broth containing ampicillin (to maintain selection of the clone) at 37°C and shaking overnight. The T-Easy vector was recovered from the cultures using the Promega Mini-prep kit (Madison, WI). The vectors containing CPEB were digested with *EcoRI* and *BglII* (Fermentas, Burlington, ON) and run on a 1% agarose gel to confirm the presence of CPEB in the vector. The CPEB fragment from one of the successful vectors was isolated, purified using the Wizard SV Gel and PCR clean up system (Promega, Madison, WI) and was sequenced for confirmation and to ensure no mutations were present.

2.11 Sub-cloning into pIRES2-EGFP

T-Easy vector containing CPEB was digested with *EcoRI* and *BglII*. CPEB sequence was isolated on a 1% agarose gel and purified as described above. The mammalian expression vector pIRES2-EGFP was also digested with *EcoRI* and *BglII*. CPEB was ligated into pIRES2-EGFP using the following reaction: 1uL 10X ligation buffer, 7uL CPEB, 1uL pIRES2-EGFP, 1ul T4 DNA ligase. The reaction was allowed to proceed at 25°C for 4 hours. The DH-5α strain of *E. coli* was transformed as described below. In this instance colonies were plated with kanamycin as pIRES2-EGFP confers kanamycin resistance. The successful clones were confirmed through plasmid isolation and digestion as described above for the T-Easy vector.

2.12 siRNA Design and pSilencer Construction

Small interfering RNAs (siRNAs) are short RNA sequences that target specific mRNAs and prevent their translation. The siRNAs are constructed to be complementary to the mRNA sequence. The siRNA forms a double stranded molecule with the mRNA through hybridization. The double-stranded RNA is then targeted by the cell's own defense mechanism. The specific mRNA is degraded and its protein product reduced or eliminated completely (Elbashir *et al.*, 2001).

Three unique siRNA sequences were designed against CPEB. The sequences were built using siRNA target finder from GenScript (www.genscript.com/ssl-bin/app/rnai). Ten sequences were generated against CPEB. Three out of the original ten sequences were chosen using the siRNA design features outlined in Elbashir *et al.* (2001). The sequences were as follows (all sequences 5′-3′):

1. CPEB-S1 sense:
   GATCCAACTGCAGATAAGACACAGAGTTGATATCCGCTCTCTGTG
   TCTTATCTGCAGTTTTTCCAAA;
2. CPEB-S1 anti-sense:
   AGCTTTTCCAAAAACTGCAGATAAGACACAGAGAGCGGATATCA
   ACTCTGTGTCTTATCTGCAGTTG;
3. CPEB-S2 sense:
   GATCCAAGCAGACCTGATCTCGGCTGTTGATATCCGCAGCCGAGA
   TCAGGTCTGCTTTTTTTTCCAAA;
4. CPEB-S2 anti-sense:
   AGCTTTTGGAAAAAAAGCAGACCTGATCTCGGCTGCGGATATCAA
   CAGCCGAGATCAGGTCTGCTTG;
5. CPEB-S3 sense:
   GATCCAAGTCACACGACCAGACCCAACCACACCTTGGGTCTGGTC
   GTGTGACTTTTTTTCCAAA;

6. CPEB-S3 anti-sense:

   AGCTTTTGGAAAAAAAGTCACACGACCAGACCCAAGGTGTGGTT

   GGGTCTGGTCGTGTGACTTG.

The sequences were synthesized by Invitrogen (Carlsbad, CA). Each siRNA sequence is complementary to a unique sequence within the open reading frame of CPEB. Each sequence was designed with a *BamHI* and *HindIII* restriction site at either end to allow insertion into the pSilencer neo vector (Ambion Inc., Austin, TX). The sequences were annealed to their compliment and ligated into the pSilencer neo Vector according to the protocol described by Ambion Inc. The stock pSilencer was previously modified with the insertion of two *PstI* sites to allow ligation of the siRNA inserts to be confirmed. The ligated vector was used to transform the DH-5α strain of competent *E. coli* cells as described below. The cells were plated in the presence of ampicillin overnight to screen for successfully transformed colonies. The plates were incubated at 37°C overnight. The four randomly selected colonies were then cultured in Luria Broth in the presence of ampicillin (to maintain selection) at 37°C overnight. The pSilencer vectors were recovered from the cultures using the Promega Mini-prep kit (Madison, WI). The isolated vectors were digested with *PstI* and run on a 1% agarose gel to confirm if the siRNA inserts were present in the vectors.

2.13 Transformation of DH-5α strain of competent E. coli Cells

DH-5α strain of competent *E. coli* cells were removed from -70°C freezer and placed directly on ice. Twenty µL of selected plasmid was added to the cells and allowed to incubate on ice for 30 minutes. The cells were then transferred to a 42°C water bath for 1 minute and 30 seconds. They were immediately returned on ice for 5 minutes and 400 µL SOC medium was added. After the 5 minutes on ice the cells were incubated at 37°C for 30 minutes. After the 30 minute incubation the cells were centrifuged at 5000 g for 5 minutes. Most of the supernatant was removed leaving the cells to be resuspended in approximately 50 µL of media. The cells were then spread on agar plates containing the appropriate selection components. The plates were allowed to sit for 5 minutes prior to being placed in a 37°C incubator overnight.

2.14 Stable Transfection of PC12 cells

Rat phaeochromocytoma (PC12) cells were grown in DMEM media supplemented with 10% horse serum, 5% FBS, 25ug/mL penicillin, 25ug/mL streptomycin and 2.5 µg/mL Fungizone® Antimycotic (Invitrogen, Burlington, ON). Flasks and plates were coated with rat-tail collagen. Cells were incubated at 37°C in 5% CO_2. Media was changed every 3 days. For transfection cells were

plated at $2x10^5$ on a 24-well plate in DMEM with serum 24 hours prior to transfection. Plasmids to be transfected were diluted to 0.8 μg in DMEM. Lipofectamine 2000 (Invitrogen, Burlington, ON) was mixed with DMEM in a separate tube. The plasmids were incubated in DMEM for 5 minutes at room temperature prior to combining with the Lipofectamine solution. The DNA was incubated with Lipofectamine for 20 minutes at room temperature. One hundred μL of the DNA/Lipofectamine complex were added to each well. After 48 hours of incubation the media was changed to the selection media that contained 400 μg/mL of G418, the selection agent. The selection media was completely replaced every 3 days for 3 weeks. At this point the control cells had died and colonies began to appear only in transfected wells. The selection media was then changed once a week for two weeks. The cells were then transferred to P25 flasks and grown in the selection media until confluent.

2.15 Preparation of Total Cell Lysate

Frozen brain tissues were allowed to thaw on ice. The samples were manually homogenized in RIPA buffer with protease inhibitor cocktail (1:100) (Sigma-Aldrich, Oakville, On.) on ice. Cell culture samples were centrifuged at 4000 g and the media removed. The pellet was then washed 3 times in PBS and RIPA lysis buffer with protease inhibitor added after the last wash. The samples

were incubated on ice for 30 minutes and then centrifuged at 14,300 g for 10 minutes at 4°C. The supernatant was collected and the pellet discarded.

Total protein concentration was determined using the BCA Protein Assay kit (Pierce, Rockford, IL) before samples were used for immunoprecipitation or western blot. The samples were mixed with the working reagent in a 96-well plate and incubated at 37°C for 30 minutes. The samples were then cooled to room temperature prior to spectrophotometric analysis as described by the kit. The samples were read at 562 nm. The concentrations of the brain samples were established by comparing their absorbances to a standard curve of known protein concentrations.

2.16 Immunoprecipitation of Proteins

The total protein of brain samples was diluted to 1µg per µL in RIPA buffer containing protease inhibitor. One µg of specific antibody was added to every 100µg of total protein and the samples were incubated at 4°C on a rocker for 2 hours or overnight. Sepharose Protein A (Rockland Inc., Philadelphia, PA) was added and the samples incubated for another hour rocking at 4°C. The samples were centrifuged and the supernatant discarded. The pellet was washed 3 times in cold PBS. After the last wash the pellet was resuspended in SDS loading buffer and incubated in 95°C water bath for 5 minutes. The samples were then centrifuged at

14,300 g for one minute. Each immunoprecipitation experiment was repeated 3 times to ensure accuracy of the results

2.17 Western Blot

Equal total concentrations of protein sample were resolved by SDS-PAGE gel with a 4% stacking gel and 10% resolving gel at 120 V for 1 ½ hours. The proteins were then transferred to an Immun-Blot PVDF membrane (Bio-Rad, Mississauga, ON) at 0.22 A for 1 ½ hours. After transfer the membrane was briefly washed for 30 seconds in TBST (1:1000 Tween 20 in TBS) to remove any transfer buffer. The membrane was then incubated in a blocking solution containing 5% instant skim milk powder in TBST for one hour. The membrane was incubated in primary antibody (1:1000 anti-CPEB, anti-CaMKII or anti-Aurora A kinase; AbCam, Cambridge, MA) or 1:2000 anti-β-actin (Sigma-Aldrich, Oakville, ON) for 2 hours at room temperature or overnight at 4°C. The membrane was then washed for 3x5 minutes in TBST. The membrane was incubated with HRP-conjugated secondary antibody (1:2000) at room temperature for 2 hours. The membrane was washed in TBST as described above and then incubated for 1 minute in ECL™ Western Blotting Detection Reagent (Amersham Biosciences, Piscataway, NJ). The membrane was exposed to X-Ray film (KODAK, New Haven, Conn.) for variable exposure times. Membranes were stripped, if required, in stripping buffer (2% SDS,

62.5 mM Tris-HCl, 100 mM –mercaptoethanol) for 1 hour at 60°C. The membrane was washed for 3x10 minutes in TBST prior to blocking in milk solution.

2.18 Analysis of Western Blot

Western blot images were scanned into a computer. Adobe Acrobat Photoshop® CS was used to analyze the images. The images were inverted and a box drawn around the most intense band. The mean intensity was measured using the histogram function. This was repeated for all bands. The area measured for all bands was fixed. The background was measured and subtracted from the band means. The band corresponding to the control sample was set to 100% and the intensity of the remaining bands was calculated relative to the control band. The procedure was repeated for each replicate experiment and the mean of the relative intensities calculated and graphed using Microsoft Excel. One-way AVOVA was used to determine any significant difference among control, high- and low-anxiety groups.

3 Results

3.1 Elevated Plus Maze

Rats were classified as high- or low- anxiety by the total amount of time spent in the closed or open arms respectively. Fifty one rats in total were tested (Table 3.1). The letters assigned to the groups indicate the day the group was tested together. Out of the original 51 rats 2 showed high-anxiety. Rat C1 spent 4 minutes 29 seconds exploring the closed arms and only 8 seconds in the open arms. Rat C4 spent 4 minutes and 23 seconds in the closed arms and 15 seconds in the open arms. Three rats showed low-anxiety. Two were chosen for further experiments to match the number of high-anxiety rats. Rat A9 spent 4 minutes 14 seconds in the open arms and no time in the closed arms. Rat A12 spent 3 minutes 46 seconds in the open arms and 24 seconds exploring the closed arms. Two rats labeled by the # symbol were eliminated from the experiments. Rat D4 was deemed unsuitable as it froze in the middle of an open arm and remained immobile for the entire 5 minute testing period. Another rat (number C11) was deemed unsuitable because it fell off the open arm during testing. Four controls were chosen out of the remaining rats (numbers A2, A5, A10 and D3). The 4 controls spent equal amounts of time in both the open and closed arms.

The rats spent a higher amount of time exploring the open arms than expected. A possible explanation for this is that the two people observing the test

were seated in the same testing room and were a few feet away but in line with the open arms. As the rats were previously handled by these two observers their presence may have inadvertently decreased the anxiogenic properties of the open arm. Also, the high-anxiety rats were identified on the same day while the low-anxiety rats were also identified on the same day (although not the same day as the high-anxiety rats). This clustering may indicate that there was an unknown factor influencing the behavior of the rats on each day. To ensure the test days did not influence the overall "emotionality" of the entire group of rats and introduce a bias a statistical analysis was performed on the results from groups A and C. The control rats from days A and C were compared to each other using Student's *t*-test. The statistic analysis showed no significant difference between the control times between the two days ($P < 0.05$, $df = 10$, $t = 1.7046$). Thus, there appears to be no underlying factor which caused group A to have lower anxiety and group C to have higher anxiety in general. The difference in anxiety levels of rats A9, A12, C1 and C4 were specific to the individuals not the group.

Table 3.1: Total Closed (C) and Open arms (O) times for Elevated Plus Maze.
(*denotes rats chosen for further experiments. # denotes rats rejected from study due to their unconventional behavior during testing)

Rat	C	O	Rat	C	O	Rat	C	O	Rat	C	O
A1	2:22	2:02	B1	2:52	1:01	C1*	4:29	0:08	D1	2:56	1:31
A2	2:06	2:04	B2	1:27	2:45	C2	3:10	0:57	D2	3:38	0:49
A3	3:11	1:09	B3	2:28	1:39	C3	2:37	1:20	D3	2:06	2:15
A4	1:31	2:36	B4	1:13	3:04	C4*	4:23	0:15	D4#	0	5:00
A5	2:05	2:20	B5	1:16	2:44	C5	3:01	1:05	D5	3:29	0:45
A6	0:09	3:33	B6	1:25	2:22	C6	3:26	1:09	D6	2:35	1:23
A7	1:59	2:28	B7	1:09	2:58	C7	1:37	2:42	D7	3:19	0:07
A8	1:17	2:46	B8	1:14	2:35	C8	3:01	0:56	D8	3:11	1:13
A9*	0	4:14	B9	1:56	2:21	C9	2:25	1:33	D9	2:15	1:24
A10	2:06	2:03	B10	1:53	2:18	C10	2:47	0:48	D10	2:55	0:53
A11	2:19	1:33	B11	3:03	1:01	C11#	3:29	1:10	D11	3:55	0:30
A12*	0:24	3:46	B12	1:09	2:47	C12	3:36	0:38	D12	3:29	0:51
A13	1:24	2:40	B13	2:38	1:02	C13	2:32	1:54			

3.2 Anxiety Related Genes

Total polyA+ RNA was isolated from the brain samples containing the amygdala, hippocampus and nucleus accumbens dissected from the high- and low-anxiety rats. The polyA+ RNA was converted to cDNA via RT-PCR as outlined in the methods. To ensure the RNA samples were successfully converted to cDNA the control, high- and low-anxiety cDNA samples were subjected to PCR of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as a control (Figure 3.1). All of the samples showed a band indicative of G3PDH and were all of equal intensity. This showed consistency and completeness of the polyA+ RNA samples and their successful conversion to cDNA. The samples, therefore, were suitable for further experimentation.

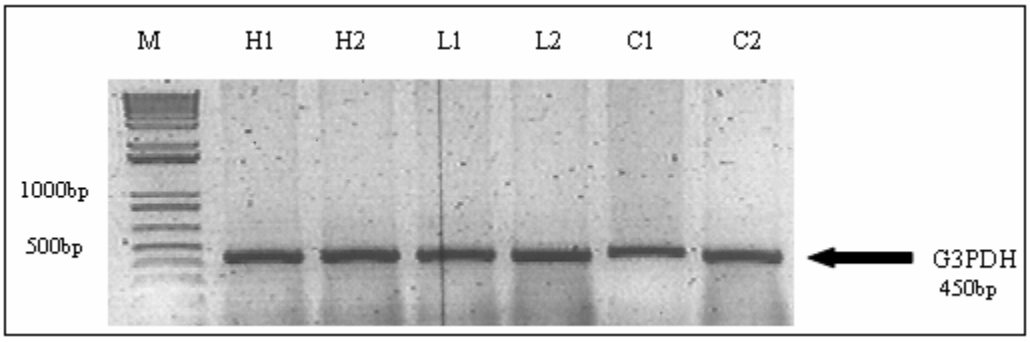

Figure 3.1: PCR of G3DPH as a control for cDNA samples.
M: 1 Kb DNA ladder
H1 & H2: High-anxiety cDNA.
L1 & L2: Low-anxiety cDNA.
C1 & C2: Control cDNA

Once the cDNA populations from the high- and low-anxiety samples were created and confirmed, they were used in suppression subtractive hybridization. The goal was to identify genes upregulated in high-anxiety compared to low-anxiety and low compared to high.

The final step of suppression subtractive hybridization is a PCR amplification of the remaining, and therefore upregulated, cDNAs. These cDNAs were cloned as described above and then sent for sequencing. The sequencing data returned 1000 genes in total that were found to be upregulated in either library. An exampled of the sequence data obtained is presented in Figure 3.2.

To obtain a more manageable list of genes, bioinformatic analysis was employed. First, all common and non-coding sequences were removed. Next, the gene names were identified using BLAST analysis. Lastly, all structural and mitochondrial genes were manually removed to condense the list. After all bioinformatic analysis the candidate gene list was comprised of 104 low-anxiety genes and 67 high-anxiety genes. The final list is presented in Table 3.2.

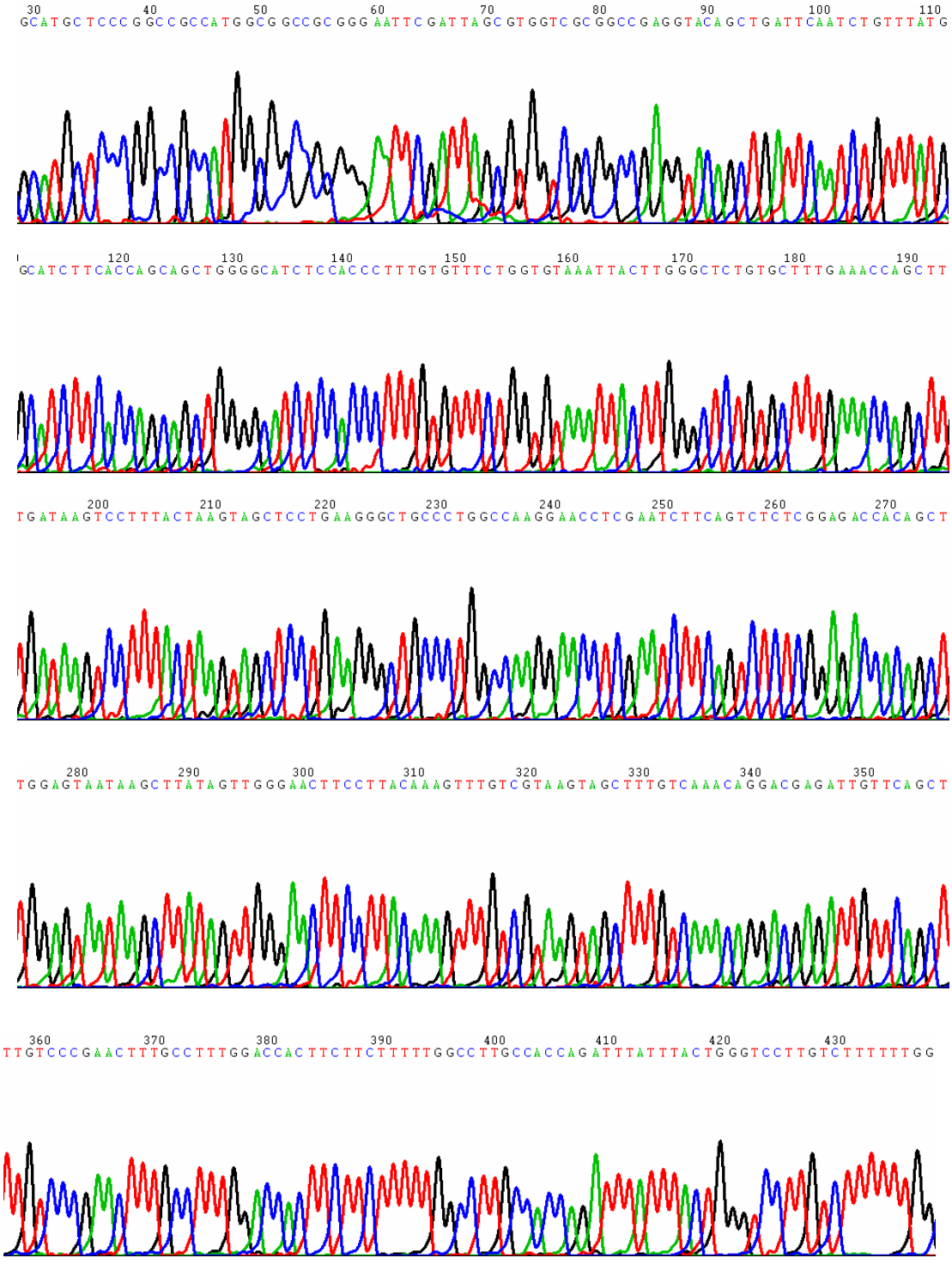

Figure 3.2: Representation of Sequencing Data: Partial sequence of *Rattus norvegicus* ribosomal protein s25 (upregulated in high-anxiety).

Table 3.2: List of Low- and High-Anxiety genes identified using Suppression Subtractive Hybridization. All sequences from *Rattus norvegicus* unless otherwise noted.

Low-Anxiety Genes

1. **AJ011971.1|MMU011971** M. musculus mRNA for wolframin
2. **NM_007459.2|** M. musculus adaptor protein complex AP-2, alpha 2 subunit (Ap2a2)
3. **AY004289.1|** profilin II
4. **BC018374.1|**M. musculus cysteine and histidine-rich domain (CHORD)-containing,zinc-binding protein 1
5. **BC061534.1|** clusterin
6. **M34043.1|RATROSB4T** thymosin beta-4
7. **NM_009871.2|** M. musculus cyclin-dependent kinase 5, regulatory subunit (p35) 1
8. **AF439750.1|** myelin basic protein (Mbp)
9. **XM_233556.3** serine/arginine repetitive matrix 1 (predicted)
10. **AB045983.1|** M. musculus gene for gliacolin, complete cds
11. **AY255791.1|** NDRG4-A1
12. **X13933.1|RNRCM1** calmodulin (pRCM1)
13. **BC061962.1|** reticulocalbin 2
14. **NM_019299.1|** clathrin heavy polypeptide (Hc) (Cltc)
15. **M88469.1|RATFSAA** f-spondin
16. **NM_014991.3|** H. sapiens WD repeat and FYVE domain containing 3 (WDFY3), transcriptvariant 1
17. **BC020359.1|** M. musculus Nedd4 family interacting protein 1
18. **BC061721.1|** vacuole Membrane Protein 1 (VMP1)
19. **NM_020610.1|** M. musculus nuclear receptor interacting protein 3 (Nrip3)
20. **BC048929.1|** M. musculus dystonin
21. **BC051641.1|** M. musculus tousled-like kinase 1
22. **NM_012505.1|** ATPase, Na+K+ transporting, alpha 2 polypeptide (Atp1a2)
23. **AF332142.1|AF332142** chloride ion pump-associated 55 kDa protein (Clp55)
24. **BC058492.1|** heat shock 10 kDa protein 1
25. **BC060951.1|**M. musculus NADH dehydrogenase (ubiquinone)1,alpha/beta subcomplex,1
26. **NM_004430.2|** H. sapiens early growth response 3 (EGR3)
27. **AF234179.1|AF234179** M. musculus RNA-binding protein (Tbrbp)
28. **NM_057119.1|** splicing factor, arginine/serine-rich 10 (transformer 2 Drosophila homolog)
29. **BC061536.1|** serine (or cysteine) peptidase inhibitor, clade I, member 1
30. **AF254801.1|AF254801** brain-enriched SH3-domain protein
31. **BC060587.1|** lactate dehydrogenase A
32. **XM_341249|** 4-nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog 1 (C. elegans)(predicted)
33. **BC022954.1|** M. musculus synapsin I
34. **XM_215227.3** similar to Eso3 protein
35. **NM_001005554** transmembrane 9 superfamily member 2 (Tm9sf2)
36. **BC024759.1|** M. musculus glycoprotein m6a
37. **AY324140.1|** gap junction membrane channel protein alpha1 (Gja1)
38. **BC034586.1|** M. musculus ATPase, Na+/K+ transporting, beta 2 polypeptide

39. **BC054811.1**| M. musculus myotrophin
40. **BC063081.1**| M. musculus copine IV
41. **BC061760.1**| ras homolog gene family, member Q
42. **M11942.1|RATHSPA** 70 kd heat-shock-like protein
43. **NM_001006963** integral membrane protein 2B (Itm2b),
44. **U30938.1|RNU30938** microtubule-associated protein 2 (MAP2)
45. **BC060177.1**| M. musculus putative homeodomain transcription factor 1
46. **NM_004028.3** H. sapiens aquaporin 4 (AQP4), transcript variant b
47. **L16532.1|RATCNPII** 2',3'-cyclic nucleotide 3'-phosphodiesterase (CnpII)
48. **J04828.1|RATASPEC** nonerythroid alpha-spectrin gene, 3' ennd
49. **AF151813.1|AF151813** H. sapiens CGI-55 protein
50. **NM_001008519**| leucine-rich PPR-motif containing (predicted)
51. **XM_238280.3** START domain containing 7 (predicted)
52. **BC059146.1**| transforming growth factor beta 1 induced transcript 4
53. **BC052427.1**| M. musculus GPI-anchored membrane protein 1
54. **BC003894.1**| M. musculus mortality factor 4 like 1
55. **BC061755.1**| SPARC-like 1 (mast9, hevin)
56. **NM_001004224** similar to B-cell receptor-associated protein 31
57. **BC057390.1**| H. sapiens Ras-induced senescence 1
58. **AE008684.1|AE008684** M. musculus T-cell receptor alpha/delta locus section 2 of 4 of the complete region
59. **AJ428213.1|RNO428213** heat shock protein 86
60. **BC008129.1**|M. musculus 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide
61. **AJ001320.1|RNMUPP1** multi PDZ domain protein
62. **BC061877.1**| glutamate oxaloacetate transaminase 1
63. **NM_009157.2**| M. musculus mitogen activated protein kinase kinase 4 (Map2k4)
64. **NM_008974.3** M. musculus protein tyrosine phosphatase 4a2 (Ptp4a2)
65. **AB113399.1**| rCNR gene for cadherin-related neuronal receptor
66. **M38061.1|RATAMPASGB** glutamate receptor (GluR-B)
67. **BC063164.1**| voltage-dependent anion channel 2
68. **X59993.1|RNZFP** putative zinc finger protein
69. **U67874.1|MMU67874** M. musculus fat facets homolog (Fam)
70. **AB091532.1**| M musculus Sema6D-1 mRNA for semaphorin 6D-1
71. **BC004671.1**| M. musculus FK506 binding protein 1a
72. **XM_214996.3** embryonic ectoderm development (predicted)
73. **BC023864.1**| M. musculus ARP2 actin-related protein 2 homolog (yeast)
74. **BC019118.1**| M. musculus RAB6, member RAS oncogene family
75. **XM_214099.3**| similar to small unique nuclear receptor co-repressor (predicted)
76. **U35246.1|HSU35246** H. sapiens vacuolar protein sorting homolog h-vps4
77. **M63485.1|RATMATRIN3** matrin 3
78. **X15635.1|RNSR2CA** sarcoplasmic reticulum 2+-Ca-ATPase
79. **XM_216717.3** FK506 binding protein 3 (predicted)
80. **S63233.1|S63233** phosphoglycerate mutase type B subunit
81. **BC056345.1**| M. musculus abl-interactor 2
82. **X87157.1|RNRNANE** neurotensin endopeptidase
83. **BC063085.1**| M. musculus DIX domain containing 1
84. **BC042568.1**| M. musculus WD repeat domain containing 2

85. **AF271786.1|AF271786** fibroblast growth factor 13 (Fgf13)
86. **NM_022695.1|** neurotensin receptor 2 (Ntsr2)
87. **NM_177420** M. musculus phosphoserine aminotransferase 1 (Psat1)
88. **BC058083.1|** M. musculus sodium channel, voltage-gated, type III, beta
89. **NM_001009264** T-cell activation protein (Pgr1)
90. **XM_343513.2|** amyloid beta (A4) precursor-like protein 2 (Aplp2) (predicted)
91. **M31178.1|RATCALBD28** calbindin D28
92. **BC058132.1|** epididymal secretory protein 1
93. **U21955.1|RNU21955** tyrosine kinase receptor Ehk-3, truncated form
94. **NM_033566.1|** M. musculus AT rich interactive domain 1A (Swi1 like) (Arid1a)
95. **AK078541.1|** KARP-1-Binding protein 2 (KAB2) homolog [H. sapiens]
96. **AY358097.1|** H. sapiens clone DNA21624 phosphodiesterase Hl
97. **AF106944.1|AF106944** PRx III (PRx III)
98. **BC021374.1|** M. musculus heterogeneous nuclear ribonucleoprotein D-like
99. **NM_021518.2|** M. musculus RAB2, member RAS oncogene family (Rab2), mRNA
100. **XM_226278.3|** similar to HBxAg transactivated protein 2 (predicted)
101. **BC011279.1|** M. musculus RAP1, GTP-GDP dissociation stimulator 1
102. **XM_235480.3** DEAD (Asp-Glu-Ala-Asp) box polypeptide 17 (predicted)

High- Anxiety genes

1. **NM_001024864** leucine zipper domain protein
2. **NM_012628.1|** protein kinase C, gamma, (Prkcc)
3. **NM_001005528|** ribosomal protein s25 (Rps25)
4. **X80029.1|RNHEM2** Hem-2 mRNA
5. **X93208.1|RSPNRD2** NRD2 convertase
6. **AF372834** synaptic vesicle protein 2B (Sv2b)
7. **X07729.1|RNEN4** gene encoding neuron-specific enolase
8. **BC012862.1|** M. musculus DEAD (Asp-Glu-Ala-Asp) box polypeptide 48
9. **BC024706.1|** M. musculus gamma-aminobutyric acid (A)receptor-associated protein-like 1
10. **Y13380.1|RNAMPH2** amphiphysin (amph2)
11. **AJ278701.1|RNO278701** cytosolic branched chain aminotransferase (Bcatc gene)
12. **AY040224.1|** H. sapiens AngRem46
13. **BC063162.1|** eukaryotic translation elongation factor 1 alpha 1
14. **NM_001004212|** similar to N33 protein
15. **L05435.1|RATSV2AA** synaptic vesicle glycoprotein 2a (Sv2a)
16. **AF007758.1|AF007758** synuclein 1
17. **XM_225856.3|** similar to Da1-6 (predicted)
18. **AY241868.1|** M. musculus chemokine-like factor superfamily 6
19. **BC061546.1|** activating transcription factor ATF-4
20. **X66531.1|RNACHRA** nicotinic acetylcholine receptor delta-subunit
21. **XM_213650.2|** Down syndrome critical region gene 5 (predicted)
22. **AF190991.1|AF190991** M. musculus clone MNCb-1314 chimeric Ttyh1 protein
23. **AF212861.1|AF212861** membrane interacting protein of RGS16 (Mir16)
24. **BC059130.1|** glutathione S-transferase, mu type 3
25. **NM_013113.1|** ATPase Na+/K+ transporting beta 1 polypeptide (Atp1b1)
26. **U03414.1|RNU03414** neuronal olfactomedin-related ER localizedprotein (D2Sut1e)

27. **AY011335.1|** amyloid beta precursor protein (App)
28. **XM_215251.3**| zinc finger protein 216 (predicted)
29. **BC036717.1**| M. musculus, Similar to reticulon 3, transcript variant 4
30. **BC031192.1**| M. musculus BTB (POZ) domain containing 1
31. **X14159.1|RN2APHOS** protein phosphatase-2A catalytic subunit
32. **X52817.1|RSC113** C1-13 gene product
33. **XM_214911.2**| programmed cell death 5 (predicted)
34. **U58829.1|RNU58829** ferritin-H subunit
35. **NM_012495.1**| aldolase A (Aldoa)
36. **BC061737.1**| stearoyl-Coenzyme A desaturase 2
37. **M20246.1|RATALBA3** transthyretin gene, exon 4
38. **NM_001009622|** SAR1a gene homolog 2 (S. cerevisiae) (predicted)
39. **BC005490.1**| M. musculus amyloid beta (A4) precursor protein
40. **BC003421.1**| M. musculus ATPase, H+ transporting, V1 subunit E isoform 1
41. **U60579.1|RNCAII9** carbonic anhydrase II gene, 3' flanking sequence
42. **NM_001005547** transmembrane 4 superfamily member 8 (Tm4sf8)
43. **BC062013.1**| pleiotrophin
44. **AF452728.1**| synaptogenesis-related mRNA sequence 7, 3'untranslated region
45. **AY035551.1**| brain Ntab
46. **BC027769.1**| M. musculus RAB15, member RAS oncogene family
47. **BC025597.1**| M. musculus calcium/calmodulin -dependent protein kinase II gamma
48. **BC026538.1**| M. musculus stathmin-like 2
49. **BC051053.1**| M. musculus RAS-related C3 botulinum substrate 1
50. **BC026550.1**| M. musculus protein kinase inhibitor, gamma
51. **NM_001004080** gelsolin (Gsn)
52. **RAT1433PZI** 14-3-3 protein zeta isoform mRNA, 3' end
53. **NM_001008279** flightless I homolog (Drosophila) (predicted)
54. **M84725.1|RATNP25GN** neuronal protein (NP25)
55. **NM_001033680** synaptotagmin 1 (Syt1)
56. **AB095364.1|** ank-s mRNA for ankyrin repeat small protein
57. **D37951.1|RATMIBP1** MIBP1 (c-myc intron binding protein 1)
58. **NM_012631.2**| prion protein (Prnp)
59. **NM_024287.2**| M. musculus RAB6, member RAS oncogene family (Rab6)
60. **AF347688.1|AF347681S9** M. musculus chloride channel isoforms (Clcn3) gene
61. **BC058485.1**| calmodulin 2
62. **AY180177.1**|M. musculus KIS kinase (Kis) gene
63. **X01964.1|RNLADEH1** lactate dehydrogenase
64. **U49062.1|RNU49062** heat stable antigen CD24
65. **NM_013053.1**| tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide (Ywhaq)
66. **L17127.1|RATRN3** proteasome RN3 subunit

3.3 Prion Protein

After the final candidate list was completed a detailed literature search was started to link any of the candidate genes to anxiety. The gene for the prion protein (accession number NM_012631.2, #58 Table 3.2) was found to be upregulated in high-anxiety when compared to low-anxiety. This result is in agreement with the previous findings of the modulating role of prion protein in anxiety in response to acute stress (Nico *et al.,* 2005) as described in detail in section 1.6.

3.4 Selection of Candidate Gene

One gene from the list generated by the suppression subtractive hybridization was to be selected for further, more detailed examination. Learning of the relationship of the prion protein with anxiety, we became interested in exploring other prion-like proteins even without direct evidence in supporting their role in anxiety. After a search of the available literature we were primarily interested in the prion-like protein CPEB for reasons described in section 1.7.

3.5 PCR of CPEB

To confirm the presence of CPEB in the rat hippocampus and PC12 cells, the full sequence of CPEB was amplified from cDNA from both samples. In PC12 cells the full-length sequence of CPEB was successfully amplified in one step

(Figure 3.3). One-step PCR, however, could not amplify the full-length sequence of CPEB from the rat hippocampal cDNA. To obtain the complete sequence of the hippocampal CPEB, the two halves of the sequence were amplified separately and then the complete sequence was created by amplifying the two halves together (Figure 3.3). The PCR for the full sequence resulted in two fragments of similar size. The larger band of approximately 1400 bp (Figure 3.3) was isolated and sequenced (Figure 3.4). BLAST analysis found it to be 100% identical to the 1442 bp rat CPEB sequence. Thus, CPEB transcripts were confirmed to be present in the adult rat hippocampus and PC12 cells.

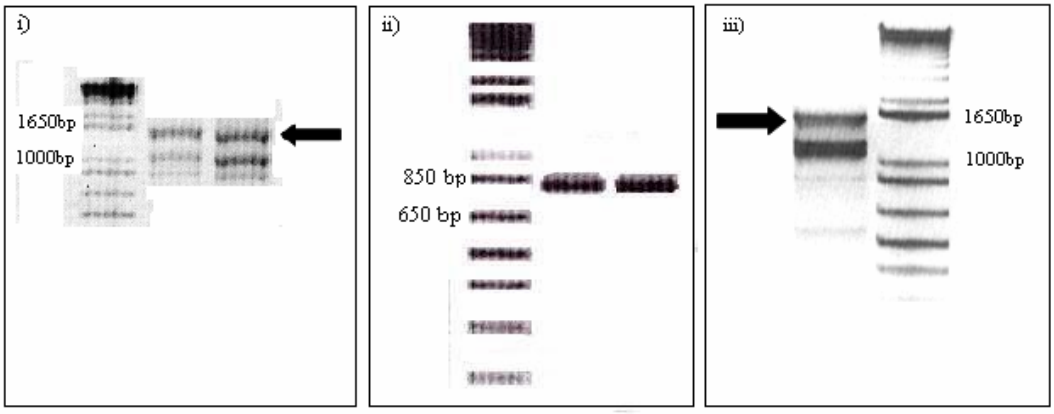

Figure 3.3: Agarose gel of PCR for CPEB using 1 Kb DNA ladder.
i) PCR of full length CPEB from PC12 cDNA
ii) PCR of CPEB halves from hippocampal cDNA (left band represents the upstream half)
iii) PCR of full length CPEB from hippocampal cDNA (arrow denotes fragment isolated for sequencing).

Figure 3.4: Nucleotide sequence obtained using PCR corresponding to the CPEB open reading frame. The sequence is 1442 bp long. Shaded areas denote PCR primer locations: 1, sense primer; 2 and 3, Intermediate sense and anti sense primers; 4, anti-sense primer.

3.6 Expression of CPEB in Mammalian Cells

3.6.1 Creation of pIRES2-EGFP-CPEB vector

The full length CPEB sequence was inserted into the T-easy vector. The vector was used to transform competent *E. coli* cells. CPEB was confirmed to be inserted into both of the isolated T-easy vectors by restriction digest (Figure 3.5). The isolated CPEB fragment was then digested to create stick ends compatible with the pIRES2-EGFP vector and ligated into the digested vector. The pIRES2-EGFP-CPEB plasmid was designed to over-express the CPEB protein in mammalian cells. The pIRES2-EGFP-CPEB plasmid was isolated and digested to confirm CPEB insertion. CPEB was found to be successfully inserted into only one of two clones (Lane 5, Figure 3.6).

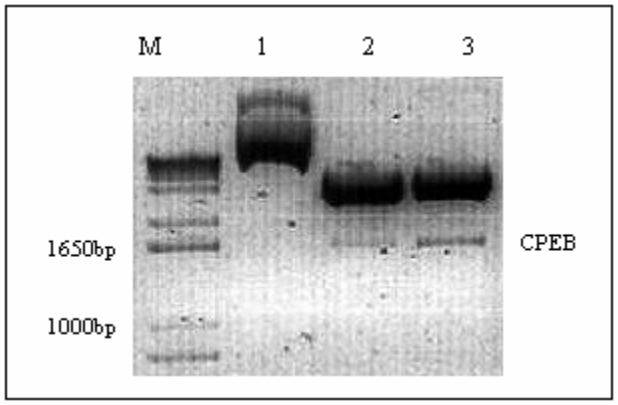

Figure 3.5: Digest of T-Easy containing CPEB.
M: 1 Kb Plus DNA ladder
Lane 1: Undigested Plasmid.
Lane 2: *EcoRI* and *BglII* digested plasmid (clone 1).
Lane 3: *EcoRI* and *BglII* digested plasmid (clone 2).

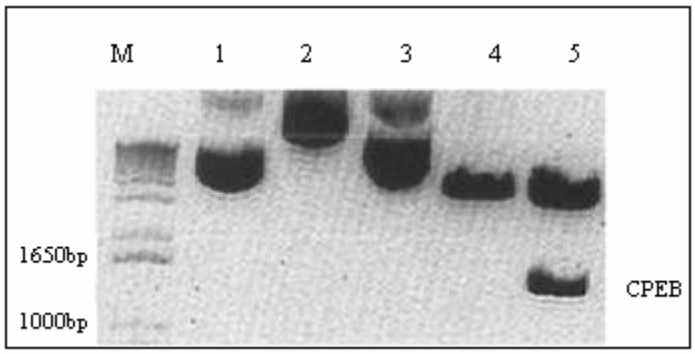

Figure 3.6: Digest of pIRES2-EGFP containing CPEB.
M: 1 Kb Plus DNA Ladder
Lane 1: Empty pIRES2-EGFP undigested.
Lane 2: Undigested pIRES2-EGFP-CPEB (clone 1).
Lane 3: Undigested pIRES2-EGFP-CPEB (clone 2).
Lane 4: *EcoRI* and *BglII* digested pIRES2-EGFP-CPEB (clone 1).
Lane 5: *EcoRI* and *BglII* digested pIRES2-EGFP-CPEB (clone 2).

3.6.2 siRNA Development

Three siRNAs were synthesized to knock down CPEB protein expression. Each was inserted into a modified pSilencer vector and used to transform competent *E. coli* cells. The pSilencer vector was isolated from four bacterial cultures per siRNA sequence. The vector was digested with *PstI*. Insertion of the CPEB fragment eliminates a second *PstI* site on the vector so that a successful ligation should result in only one band. siRNA sequences S2 and S3 yielded single bands but siRNA sequence S1 yielded two bands (Figure 3.7). This confirms the proper ligation of the siRNA S2 and S3 sequences into the pSilencer vector but not siRNA sequence S1. This has been attributed to a base mismatch in the S1 siRNA sequence itself. Therefore, siRNA sequences S2 and S3 were used for further experiments while S1 was discarded.

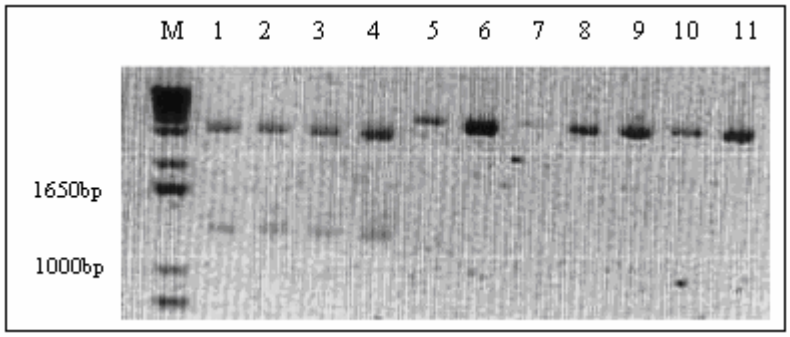

Figure 3.7: Digest of pSilencer vector containing siRNA sequences.
M: 1 Kb Plus DNA ladder.
Lanes 1-4: siRNA sequence S1.
Lanes 5-8: siRNA sequence S2.
Lanes 9-11: siRNA sequence S3.

3.6.3 Transfection of PC12 cells

PC12 cells were transfected with the pIRES2-EGFP-CPEB plasmid and pSilencer plasmid carrying either the siRNA S2 or S3 sequences. Stable cell lines were established for all three plasmids. Samples of the transfected cells were subjected to a western blot against CPEB to determine their efficiency. The pIRES2-EGFP-CPEB plasmid was expected to over-express CPEB while the siRNAs were expected to knockdown CPEB expression, relative to control cells. As can be seen in Figure 3.8 the pIRES2-EGFP-CPEB vector did not over-express CPEB, nor did the siRNAs knockdown CPEB over control cells. These preliminary results were not promising.

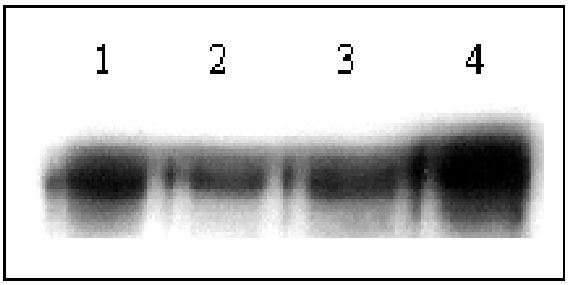

Figure 3.8: Western Blot of Transfected PC12 cells.
Lane1: Control PC12 Cells
Lane 2: PC12 cells transfected with pIRES2-EGFP-CPEB
Lane 3: PC12 cells transfected with siRNA S2 sequence
Lane 4: PC12 cells transfected with siRNA S3 sequence

The goal of building the pIRES2-EGFP-CPEB plasmid and the siRNA plasmids was to obtain a method of manipulating CPEB expression in the rat *in vivo.* The cell culture transformation was to confirm the desired activity of the plasmids before they were to be used *in vivo*. As CPEB was found to be in low concentrations in our brain samples (see below) it is believed to only present in significant amounts in the hippocampus. Therefore to make significant impact on its expression *in vivo* we would have had to inject the plasmids into the ventral hippocampus. This brain region is large and thus injection of the CPEB plasmids into the ventral hippocampus would not be efficient. Due to this limitation the development of plasmids to manipulate CPEB expression was stopped.

3.7 Protein Expression of CPEB in Control, High- and Low-anxiety brain tissue

To understand if CPEB had a role in anxiety, its protein expression was examined. The protein expression of CPEB in high- and low-anxiety compared to control was investigated using western blot analysis of the brain samples obtained from the elevated plus maze results. In initial western blot experiments CPEB protein expression resulted as very faint bands (Figure 3.9). To ensure that the faint bands were not the result of an error in the western blot protocol, the procedure was repeated with a PC12 sample as a control. Low protein loading was also ruled out by stripping the membranes and re-probing them with β-actin antibody. The β-actin

antibody resulted in strong bands of equal intensity in all lanes (Figure 3.9). The PC12 control cells also showed a relatively strong band specific for CPEB. Thus, it appeared that the weak bands were the result of the CPEB protein being in low concentrations in the brain samples examined. This could be the result of 2 factors: (1) while CPEB is enriched in the hippocampus, the brain samples examined also included the amygdala and nucleus accumbens that contain very low concentrations of the CPEB (Huang *et al*., 2002; Wu *et al*., 1998); and (2) while the CPEB is concentrated in the post-synaptic density but not in other components of a neuron (Huang *et al*., 2002), the brain samples studied contained all the components of neurons. To overcome this problem, an immunoprecipitation protocol was used to concentrate the amount of CPEB in each sample.

The immunoprecipitation procedure resulted in much clearer results. The western blot of samples following immunoprecipitation procedure shows that CPEB levels are increased in both high- and low-anxiety samples compared to control samples (Figure 3.10), which was confirmed by analysis of the repeated CPEB immunoprecipitations using Adobe Photoshop CS (Figure 3.11). One-way ANOVA revealed a significant difference between the groups.

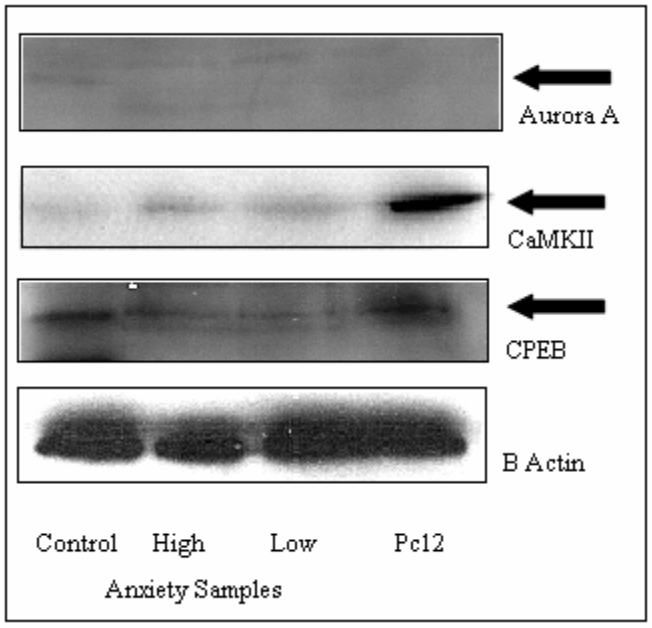

Figure 3.9: Western Blot of Aurora A, CaMKII, CPEB and β actin in Control, High- and Low-Anxiety Brain Samples and PC12 cells. PC12 cells were used as a control. Arrows denote location of faint bands in Aurora A, CaMKII and CPEB western blots.

3.8 Protein expression of CaMKII and Aurora A Kinase in Control, High- and Low- anxiety brain tissue

To catalyze polyadenylation and therefore effect translation, CPEB must be phosphorylated; otherwise it is a repressor of translation. To understand if the increase in CPEB expression results in increased translation or repression, the phosphorylation state of CPEB needs to be established. Unfortunately no antibody to phosphorylated CPEB is available commercially or from another research laboratory. Therefore, in order to gain insight into the activity of CPEB in anxiety, the expression of a known upstream kinase, Aurora A kinase, and a downstream target, calcium/calmodulin-dependent protein kinase II (CaMKII) was examined. Like the CPEB western results, Aurora A kinase and CaMKII also produced very faint bands (Figure 3.9). The CaMKII results were slightly more intense for the brain samples and the PC12 control band was strong. Thus, as in CPEB, an immunoprecipitation was performed, which resulted in much clearer results (Figure 3.10).

The expression of CaMKII is increased in high-anxiety only. Analysis of the blots shows that CaMKII is expressed in similar levels in control and low-anxiety (Figure 3.12). One-way ANOVA analysis showed significant difference between groups. This suggests increased translation of CaMKII and therefore an increase in CPEB activity in high-anxiety.

The results for the immunoprecipitation of Aurora A Kinase were more difficult to interpret. The western blot of the initial immunoprecipitation showed a slight increase in expression in control and high-anxiety over low-anxiety (shown in Figure 3.10). Each repetition of the immunoprecipitation and western blot resulted in more uniform bands. These experiments were always done in parallel with CaMKII, which showed very little variability while the uniformity of Aurora A blots grew with each repetition. Thus, the overall expression levels of Aurora A among control and high- and low-anxiety groups are not statistically significant (Figure 3.13).

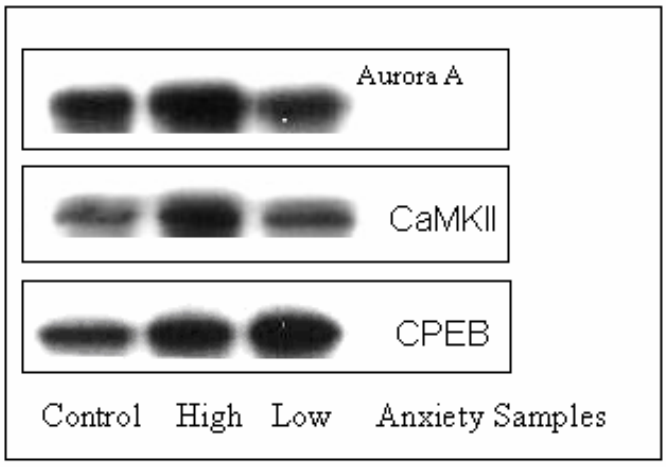

Figure 3.10: Western Blot of Immunoprecipitation of Control, High- and Low-Anxiety Brain Samples against Aurora A, CaMKII, CPEB

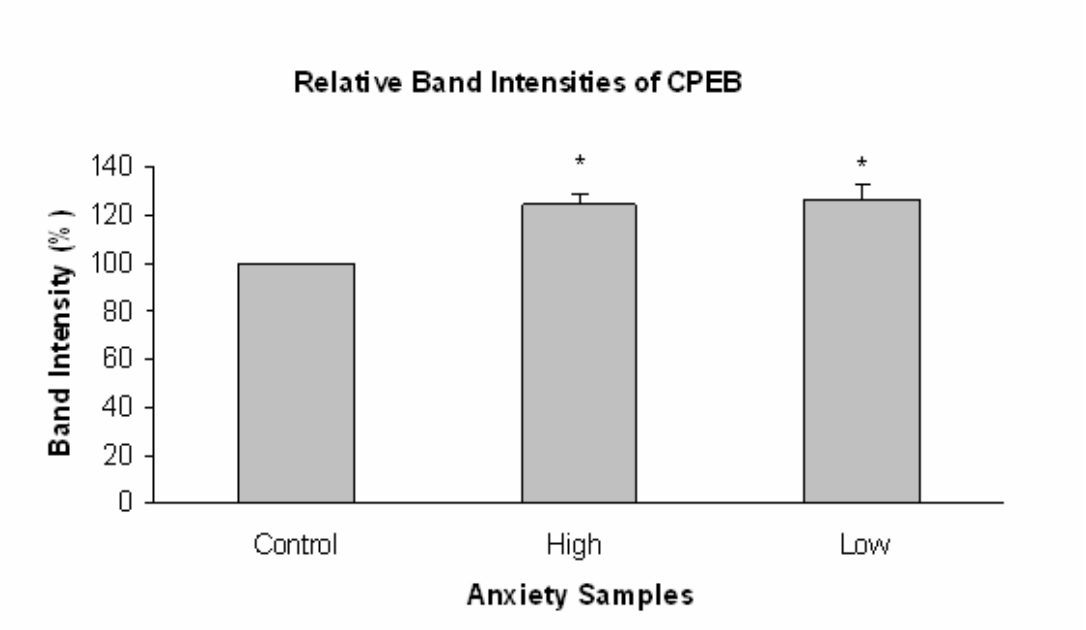

Figure 3.11: Relative Band intensities of Western Blot of CPEB in control, high- and low-anxiety samples. *p<0.05 n=3

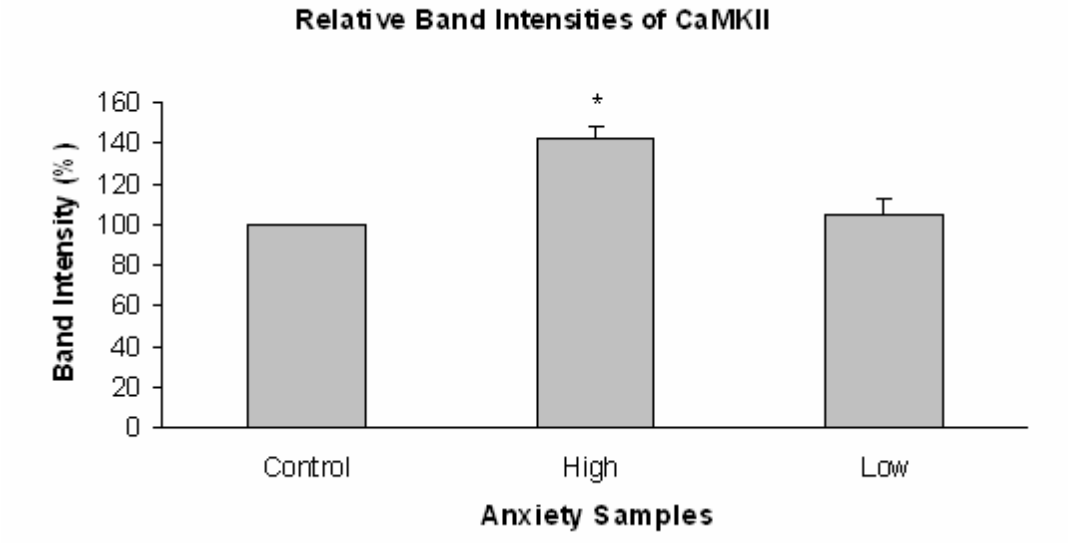

Figure 3.12 Relative Band intensities of Western Blot of CaMKII in Control, High and Low anxiety samples. *p<0.05 n=3

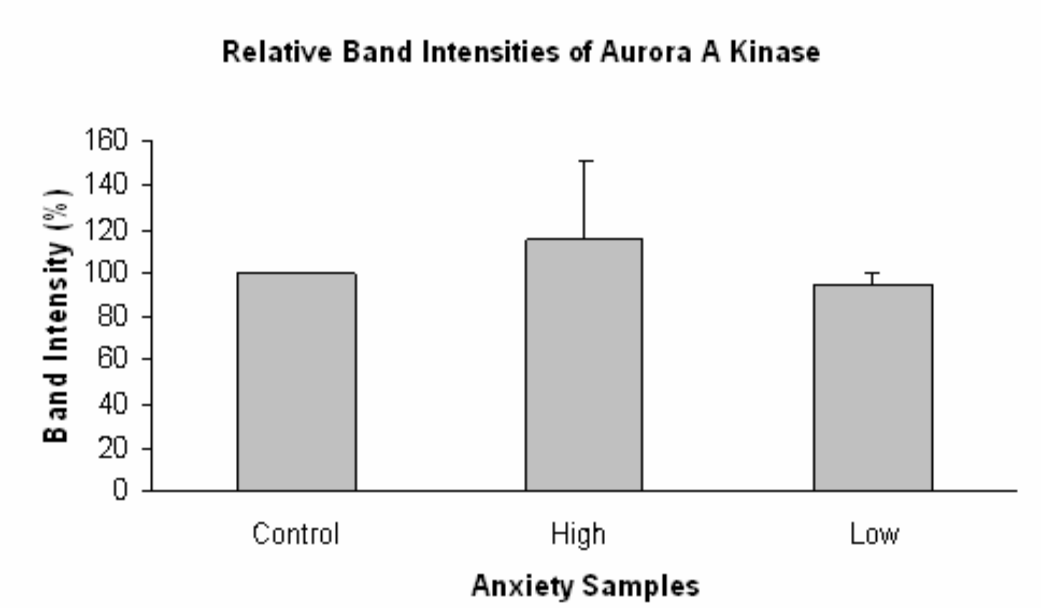

Figure 3.13 Relative Band intensities of Western Blot of Aurora A Kinase in control, high- and low-anxiety samples. *$p<0.05$ n=3

4 Discussion

Anxiety disorders are one of the most common psychiatric disorders. Yet little is known about the molecular mechanisms behind the genesis or maintenance of these disorders. An understanding of these mechanisms is the key to developing better diagnostic techniques and treatments. The increased availability of gene sequence information and new molecular techniques have made it easier than ever to study these complex molecular workings.

4.1 Anxiety Model

Studying a complex psychiatric disorder in a rodent is inherently difficult, yet it is often the only method available to gain insight into the molecular mechanisms underlying these disorders. The results of this project are not only influenced but possibly limited by two decisions made at the beginning. First is the choice of behavior test. There are many different behavior tests for anxiety in rodents and each one has draw backs. The conditioned tests, while more amenable to experimental manipulation (Flint, 2003), measure a conditioned or learned response (Hitzemann, 2000). As the anxious response measured by the test is learned it does not seem to be the appropriate choice to study different levels of inherent anxiety. The unconditioned tests on the other hand do not involve training and so are believed to measure un-learned or inherent fear. While there are

numerous unconditioned tests for anxiety that may have been equally appropriate the EPM was chosen for this project as it is extensively used and highly validated in anxiety research (Gordon and Hen, 2004; Hogg, 1996; Pellow *et al.*, 1985; Rodgers and Dalvi, 1997).

Like all other behavior tests, the EPM is extremely sensitive to outside factors. Housing conditions, lighting, test duration and prior handling have all been shown to affect the test results (Rodgers and Dalvi, 1997). To reduce the impact of these factors great care was taken to ensure that all the rats were treated identically and strict testing standards were maintained. Even with the dedicated attention to detail our testing procedure produced an interesting effect; the open arm times were higher than expected for the EPM. This has been attributed to the presence of the observers in the room which may have decreased the anxiogenic properties of the open arms. From these results it is highly recommended that observation of the maze be carried out by a video capture system.

Validation of the rats selected for their high- or low-anxiety by other behavior tests may have been beneficial, although Ho *et al.* (2002) has shown that the behavior of Wistar rats on the EPM has been shown to reliably predict their behavior in other anxiety tests. Also, different anxiety tests may measure different aspects of anxiety (Hitzemann, 2000) so additional testing using a different test may have confounded the results instead of strengthening them.

The second design decision that has a large impact on this study is the brain areas selected for investigation. Anxiety is controlled by many brain areas,

including various brain stem, limbic and cortical regions. While each brain area is an important contributor to anxiety it was not possible to study them all. As a result we chose to investigate three brain areas associated with the limbic system, the amygdala, the hippocampus and the nucleus accumbens. The limbic system is believed to play a central role in anxiety (Sandford *et al.,* 2000). The amygdala was chosen due to its role in processing fearful stimuli and its extensive interconnections controlling locomotor, neuroendocrine, autonomic, and respiratory responses (Gordon and Hen, 2004; Lang *et al.*, 2000; Sandford *et al.*, 2000). The hippocampus was of interest due to its role in learning and memory, especially the evidence that a hypersensitive hippocampus may be biased towards negative associations (Gray and McNaughton, 2000). Lastly, the nucleus accumbens was chosen due to its central position between many of the brain regions involved in the development of anxiety disorders and its involvement in aversive reinforcement (Sturm *et al*., 2003).

After isolation of the three selected brain regions they were pooled together before the isolation of the mRNA. This was to ensure there was enough mRNA for making the cDNA samples and for the subsequent suppression subtractive hybridization experiment. While this ensured a high mRNA concentration it may have influenced the final result. It is possible that certain genes were regionally expressed in one brain structure and not in the other two. Not only could we not detect regional changes but this may also have masked genes only upregulated in

one brain region. Future investigations using isolated brain regions should be investigated and could possible identify such genes.

4.2 Application of Molecular Techniques in the Study of Anxiety

4.2.1 cDNA Microarray

cDNA microarray is one molecular technique that has recently become quite popular. A single hybridization step occurs between the test sample and a group of labeled, known cDNAs placed on a substrate. This method allows a wide array of gene expression levels to be examined in a relatively short period of time. A recent study employed cDNA microarray to examine genes over-expressed in the rat model of anxiety (Wang *et al*., 2003). Unfortunately there are drawbacks to cDNA microarray that limit its usefulness in this investigation. cDNA microarray cannot be used as a tool to examine unknown sequences as known sequences are applied to the array substrate for hybridization (Strakhova and Skolnick, 2001). The cDNA can only be probed by one test population. Using this approach, we would require two arrays; one for high-anxiety and one for low-anxiety. With the requirement of specialized scanners and software for their analysis this makes the cost quite prohibitive (Strakhova and Skolnick, 2001). Also, even with the proper equipment, analysis of gene expression using cDNA microarray is plagued by external inconsistencies such as pixel intensity and spot shape (Wang *et al.,* 2003).

4.2.2 Suppression Subtractive Hybridization

An attractive alternative to the popular cDNA microarray is suppression subtractive hybridization. This technique uses two complete populations of cDNA for its hybridization steps, as opposed to cDNA microarray's one population. Also while cDNA identifies upregulated genes in one population, suppression subtractive hybridization allows the identification of up- and down-regulated genes by using two populations as both tester and driver in parallel (Strakhova and Skolnick, 2001). Suppression subtractive hybridization is also more sensitive to rare transcripts than cDNA microarray due to its PCR based suppression step (Strakhova and Skolnick, 2001). Thus, while more labor intensive, suppression subtractive hybridization appears to be the more appropriate choice in this quest to gain new insight into the molecular mechanisms of anxiety.

A recent study examined anxiety-related genes in the rat amygdala using suppression subtractive hybridization (Koks *et al*., 2004). It has been shown that anxiety disorders involve multiple brain areas (such as the amygdala, hippocampus and nucleus accumbens) working together to process anxiogenic stimuli and generate the appropriate response (Bannerman *et al*., 2004; Graeff *et al*., 1993, Lesch, 2001; Pezze and Feldom, 2004; Swift and Swift, 2005). Therefore, our present study on anxiety-related genes in the amygdala, hippocampus and nucleus accumbens would provide a better understanding of mechanism underlying anxiety

development. As previous studies have induced an anxious state, either using a cat or its odor, before the application of cDNA microarray (Wang *et al*., 2003) or suppression subtractive hybridization (Koks *et al*., 2004), genes identified in these studies may include those related to stress itself but not anxiety. To exclude this possibility and to identify the genes that confer a possible predisposition to anxiety, we chose to identify rats with inherently high or low levels of anxiety prior to suppression subtractive hybridization.

4.3 Anxiety-related Genes

By using suppression subtractive hybridization in this project numerous genes were found upregulated in high- and low-anxiety. Some genes upregulated in high-anxiety have also been identified in another study of gene expression using suppression subtractive hybridization. These include tyrosine 3-monooxygenase/tryptophan 5-monooxygenase, CaMKII, calmodulin and gelsolin (Koks *et al*., 2004). Many other genes identified in this study have never been reported to be associated with anxiety before.

This extensive list of candidate genes provides a starting point for future studies of the molecular mechanisms of anxiety and may prove to be exciting targets for future pharmacotherapies. For example, of the 102 low-anxiety genes, the wolframin gene (accession number AJ011971.1|MMU011971**)** appears to be of great interest (Table 3.2). The wolframin gene is highly expressed in the amygdala and the CA1 region of the hippocampus. Carriers of a single wolframin mutation

have been shown to have a predisposition to psychiatric illness (Swift and Swift, 2005). The wolframin gene was found to be upregulated in anxiety in the Koks study using suppression subtractive hybridization (Koks *et al*., 2004). Perhaps the difference in expression levels may be accounted for by our inclusion of the nucleus accumbens and the hippocampus in our samples.

Of 62 genes upregulated in the high-anxiety group (Table 3.2), several genes may play a role in anxiety. Activating transcription factor 4 (ATF4, accession number BC061546.1) is homologous to the CREB-2 protein. It has been shown that ATF4 hinders the transfer of short-term synaptic potential and memory storage to their long-term forms (Chen *et al*., 2003). Neuronal protein 25 (NP25, accession number M84725.1|RATNP25GN) is highly expressed in the hippocampus, frontal cortex, cerebellum and midbrain (Ren *et al*., 1994). Immobilization stress has been shown to increase the activity of NP25 in the frontal cortex and hippocampus, suggesting a role in stress-mediated mental disorders (Morinobu *et al*., 2003). The tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (accession number NM_013053.1) is part of the 14-3-3 protein family that is found to be highly expressed in the large projection neurons of the hippocampus, cerebellum and spinal cord (Malaspina *et al*., 2000) and was also found to be upregulated in the Koks study (Koks *et al*., 2004). The 14-3-3 proteins play essential roles in key signaling pathways of apoptosis and cell proliferation (Malaspina *et al*., 2000). Synaptotagmin I (accession number NM_001033680) is a regulator of calcium-dependent exocytosis and endocytosis (Nicholson-Tomishima

and Ryan, 2004). Synaptotagmin IV, a protein of the same family, has been shown to be involved in anxiety (Ferguson, *et al*., 2004). The gene encoding the prion protein (accession number NM_012631.2) was found to be upregulated in high-anxiety rats. Further investigation into the literature revealed the prion protein may play a role in excitatory neurotransmission (Carleton *et al.,* 2001) and a role modulating anxiety (Nico *et al.,* 2005).

Any of the genes identified in the suppression subtractive hybridization study would have made for fascinating research subjects but we decided to follow a lead brought to our attention by the prion protein literature. CPEB was first brought to our attention as a prion-like protein. Further investigation into the literature showed CPEB to be involved in experience-dependent protein translation and as a result could be a key component of learning and memory (Huang *et al*,. 2002; Wu *et al.,* 1998). It was also revealed that CPEB-dependent polyadenylation (and subsequent translation) of specific mRNAs was dependent on activation of the NMDA receptor. As excessive neuronal hyper-excitability is anxiogenic (Lydiard, 2003) and CPEB activity is regulated by the NMDA receptor we decided to investigate the possibility that CPEB is overactive in anxiety. While CPEB was not directly identified by the suppression subtractive hybridization it would not have been brought to our attention had it not been for the identification of the prion protein in the list for high-anxiety.

The results of this project have shown CPEB to be present in the adult rat hippocampus in both mRNA and protein form by PCR and western blot analysis,

respectively. To understand the role of CPEB in anxiety, its normal role in the brain should be discussed. Results from the work done by other research groups have highlighted a key role for CPEB in the long-term maintenance of synaptic plasticity. CPEB is located in the center of a pathway critical for long-term potentiation (LTP). In normal function it perhaps plays a significant part in the mechanism of long-term memory formation.

4.4 Synaptic Plasticity

The neural mechanism of memory has been an intriguing subject for many years. Synaptic plasticity is thought to be a key component of the formation of memories (Si *et al*., 2003; Wells *et al*., 2001). It can be defined as a modulation of synaptic strength and is based on the history of that synapse (Huang *et al*., 2002; Si *et al*., 2003; Wells *et al*., 2001). The long-term form of synaptic plasticity, which is believed to underlie the long-term maintenance of memory, has been shown to require new protein synthesis and the formation of new connections.

A large collection of evidence has shown that the new protein synthesis required for synaptic plasticity occurs within the activated synapse itself. The dendritic compartments of neurons contain all the necessary cellular machinery to translate mRNAs locally (Huang *et al.,* 2002; Richter, 1999). Electron microscopy has shown polyribosomes associating with postsynaptic structures. Hippocampal dendrites have been shown to contain RNA molecules and protein components

required for protein synthesis (Huang, 1999). More conclusive studies have shown protein synthesis to occur in isolated dendritic or synaptic compartments (Huang *et al.,* 2002). Long-lasting phase of LTP, which requires new protein synthesis, can be established in dendritic layers alone (Huang *et al.,* 2002).

Only a select group of mRNAs are translated at the synapse. More specifically, dendrites contain specific mRNAs that may be involved in synaptic plasticity. High levels of mRNAs, including those for microtubule-associated protein MAP2, α subunit of the calcium/calmodulin-dependent protein kinase II (CaMKII) and an immediate early gene, Arc, have been found to be co-localized with the protein synthesis machinery in dendrites (Huang, 1999).

The translation of these specific mRNAs does not occur in all the dendrites of a given neuron. Synaptic activity has been shown to trigger the translation of these mRNAs only at specific synapses within the same neuron. Arc mRNA has been shown to be concentrated only in the active dendrites of a single neuron. Strong stimulation of hippocampal and cortical neurons resulted in the increase of the Arc protein within their dendrites (Huang, 1999). CaMKII has the same pattern of expression. Strong stimulation of hippocampal slices resulted in increased protein expression of CaMKII specifically in the dendrites (Huang, 1999).

CPEB may be the regulator of the protein synthesis required for synaptic plasticity. Research into the translational control of *Xenopus* oocytes has shown CPEB can keep mRNAs translationally dormant until it becomes phosphorylated. The phosphorylation event triggers polyadenylation and translation of the bound

mRNA. Within dendrites the same mechanism exists. CPEB has been shown to hold specific mRNAs dormant in the dendrite until the dendrite becomes activated. After stimulation CPEB becomes phosphorylated and triggers the translation of the dormant mRNAs, including CaMKII (Huang *et al.,* 2002; Wu *et al*., 1998).

The signal that triggers CPEB phosphorylation is the *N*-methyl-*D*-aspartate (NMDA) receptor. Huang *et al.,* (2002) found that NMDA receptor signaling triggered Aurora A kinase to phosphorylate CPEB and therefore catalyze the polyadenylation of specific mRNAs at the activated synapse. NMDA receptor is also known to mediate mechanisms required for memory consolidation (Izquierdo and Medina, 1997).

CPEB may be a key regulator of synaptic plasticity, a key component of learning and memory. CPEB may play a role in the development of changes in neural plasticity that may create a "long-term behavioral sensitization" which can lead to anxiety disorders (Rainnie *et al.,* 2004). How these long-term changes in neural plasticity lead to anxiety is unknown. Glutamatergic neurotransmission and the NMDA receptor have already been shown to be key components. CPEB regulation of protein synthesis within specific synapses could be a major part of this process.

4.5 NMDA Receptor in Anxiety Disorders

The involvement of the NMDA receptor in anxiety is well documented. Excessive neuronal hyper-excitability is believed to underlie the development of pathological anxiety (Lydiard, 2003) possibly in reaction to stress. Stress can induce alterations in neural plasticity in a glutamatergic dependent manner (Du *et al.,* 2004). Stress exposure can also increase glutamate levels and NMDA receptor expression in the hippocampus (Boyce-Rustay and Holmes, 2006; Du *et al.,* 2004). The NMDA receptor is believed to mediate mood and emotion-related behaviors as it is highly expressed in cortical and limbic regions (Bergink *et al.,* 2004; Boyce-Rustay and Holmes, 2006).

Manipulation of the NMDA receptor has shown it to be a major player in anxiety. The application of both competitive and non-competitive NMDA receptor antagonists have been shown to be anxiolytic in many rodent behavioral tests, including the EPM (Boyce-Rustay and Holmes, 2006). NMDA receptor knockout mice show lowered anxiety-related behaviors than their wild type counterparts (Boyce-Rustay and Holmes, 2006). The NMDA receptor has been shown to be critical in anxiety-related learning paradigms (Bergink *et al.,* 2004). In fear conditioning, for example, has been shown to be NMDA receptor dependent. Blocking the NMDA receptor with an antagonist blocks the acquisition of fear (Bergink *et al.,* 2004). The same mechanism has been shown to be involved in passive avoidance learning (Izquierdo and Medina, 1997). NMDA dependent

neural plasticity is also a critical component of the acquisition of fear-potentiated startle (Adamec, 1997).

4.6 Role of CPEB in Anxiety Disorders

In this study CPEB has been shown to be upregulated in both high- and low-anxiety rats over control. These results are not in conflict with our data shown in Table 3.2, in which suppression subtractive hybridization revealed no significant difference in the expression levels of CPEB between high- and low-anxiety rats. Upregulation of CPEB in both high- and low-anxiety rats does not reveal any insight into its contribution to anxiety at first glance. However, once these changes are viewed within the context of a pathway an interesting hypothesis emerges. First, let us look at CPEB in high-anxiety. As previously mentioned, the NMDA receptor plays a critical role in anxiety disorders because glutamatergic hyper-excitability is thought to underlie these disorders. CPEB is known to lie downstream of the NMDA receptor and its phosphorylation is dependent upon NMDA activity.

In a control situation NMDA receptor activation catalyzes the polyadenylation of specific mRNAs through CPEB. This leads to the long-term maintenance of synaptic plasticity. In a high-anxiety state, CPEB is over-expressed. As a result, activation of the NMDA receptor may produce a much larger increase in protein synthesis through the increased presence of CPEB. The stronger response could possibly create a much more sensitive synapse.

In low-anxiety state, there is also an increase in CPEB. In this case, the result could be quite opposite. Research has shown that blocking the NMDA receptor prevents lasting increases in anxiety behavior (Adamec, 1998). It can be hypothesized that the NMDA receptor is less active in low-anxiety, either by its own downregulation or lower glutamatergic neurotransmission. In either case less NMDA signaling would mean less CPEB would be phosphorylated. Unphosphorylated CPEB has been shown to act as a repressor. When bound to specific mRNAs, unphosphorylated CPEB keeps mRNAs dormant by preventing the 40s subunit of the ribosome from interacting with and translating the mRNA. In this case an excess of CPEB in a synapse would mean a smaller proportion of mRNAs would be translated after NMDA activation. This could be protective against anxiety by creating stricter limitations on synaptic plasticity.

Our working hypotheses described above are, at least partially, confirmed by our further findings in this study about CaMKII. We have shown that high-anxiety rats, but not low-anxiety rats, showed significantly increased expression levels of CaMKII, the downstream effector of CPEB. CaMKII was found to be upregulated in both the suppression subtractive hybridization (Table 3.2, number 47 in high-anxiety gene list) and immunoprecipitation experiments (Figures 3.10, 3.12), suggesting the reliability of these data. Another recent study by Koks *et al.,* using suppression subtractive hybridization, also showed similar results in the rat amygdala after cat odor-induced anxiety (Koks *et al*., 2004). All these results strongly suggest an important involvement of CaMKII in inducing anxiety.

Like its upstream effectors NMDA receptor and CPEB, CaMKII is a known mediator of synaptic plasticity. CaMKII is believed to play a role as a "memory switch" as it can orchestrate the long-term strengthening of a synapse through many signaling cascades (Du *et al.,* 2004). CaMKII has been shown to be critical for the long-term maintenance of the LTP (Du *et al.,* 2004). Like the NMDA receptors (Boyce-Rustay and Holmes, 2006), CaMKII has also been shown to be increased after stress exposure (Suenaga *et al.*, 2004). Thus, it appears as though increased activity of the NMDA-CPEB-CaMKII pathway may prove to be important in the development and/or maintenance of anxiety disorders

4.7 CPEB in Man

CPEB has been found to be present in many organisms from invertebrates, like *Aplysia californica*, to frogs, zebrafish, mice and rats. In 2001, the human CPEB was cloned. It was found to be in high concentrations in adult ovary and brain (Welk *et al.,* 2001). The human CPEB bears potential to function like the CPEB found in adult rodents. It is able to bind RNA in a sequence-specific manner just like *Xenopus* and *Mus* CPEBs (Welk *et al.,* 2001). Thus it is likely that the findings of CPEB activity in rodents may hold for humans also.

4.8 Future Directions

This project has provided two sets of results that hold potential to uncover important and novel molecular mechanisms involved in anxiety. Firstly, the gene lists derived from the suppression subtractive hybridization hold great promise. While there are many genes that have no known link to anxiety disorders some are beginning to accumulate promising evidence such as CaMKII and the cellular prion protein. Many of the identified genes may be investigated in detail from molecular work back to behavior using *in vivo* studies. Any one may result in new insight into the molecular mechanisms of anxiety.

Secondly, the CPEB results presented here are preliminary. Due to the lack of an antibody specific to phosphorylated CPEB we were only able to infer that CPEB is overactive in high-anxiety. Development of a phosphorylated antibody will allow confirmation of the phosphorylation state of the over-expressed CPEB in high-anxiety. Identification of other CPE containing targets of CPEB would be an asset. Finally the role of CPEB in synaptic plasticity, learning and memory and its relation to anxiety should be investigated possibly through the use of a conditioned-fear paradigm.

Further investigation into either the gene list presented in this project or CPEB may lead to a better understanding of the molecular mechanisms underlying both “normal” and “pathological” anxiety. Ideally this work will lead to more effective treatments for such a prevalent and disabling disorder.

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Jiang W*, **Van Cleemput J***, Sheerin AH, Ji S, Zhang Y, Saucier DM, Corcoran, ME, Zhang X. (2005) Involvement of Extracellular Regulated Kinase and p38 Kinase in Hippocampal Seizure Tolerance. J. Neurosci. Res. 81, 581-588. (* these authors contribute equally to this work)

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