ROLE OF THE TRANSIENT RECEPTOR POTENTIAL CHANNELS IN

MODULATING PREFRONTAL CORTICAL EXCITABILITY

AND BEHAVIORAL RESPONSES TO COCAINE

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DEDICATION

I dedicate this dissertation to my parents who instilled in me the importance of an education and have always provided me with unwavering love and support.

ROLE OF THE TRANSIENT RECEPTOR POTENTIAL CHANNELS IN MODULATING PREFRONTAL CORTICAL EXCITABILITY AND BEHAVIORAL RESPONSES TO COCAINE

By

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DISSERTATION

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ROLE OF THE TRANSIENT RECEPTOR POTENTIAL CHANNELS IN MODULATING PREFRONTAL CORTICAL EXCITABILITY AND BEHAVIORAL RESPONSES TO COCAINE

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Drug addiction is a disease that is influenced by both genetic and environmental factors that result in altered excitability in the key brain regions associated with reward and decision-making. The prefrontal cortex (PFC) processes reward-related information; and pathologies in PFC excitability resulting from prolonged drug use may lead to the loss of control over drug intake associated with drug addiction. We show that layer 5 pyramidal neurons in the PFC exhibit a prolonged depolarizing response to G_q-coupled receptor activation, which

produces a period of heightened excitability of the cell following brief bursts of action potential activity. This burst triggered delayed depolarization enables the cell to convert subthreshold inputs into persistent firing output and may be a way for the cell to hold information in a short term memory buffer. The delayed after-depolarization (dADP) is reduced by dopamine and chronic cocaine, which may serve to bias the cell towards very strong inputs, such as those associated with drug cues, while preventing the cell from responding to smaller, subthreshold inputs. The dADP is induced by activation of G_q-coupled receptors, such as metabotropic glutamate receptors or muscarinic acetylcholine receptors and is mediated by subsequent activation of a non-selective cation channel, which pharmacological data suggested to be a canonical transient receptor potential (TRPC) channel. We used in situ hybridization, immunoblots, and real-time PCR to examine the expression of the TRPC channels and found dense expression of TRPC5 in the pyramidal cell layers of the PFC. Using adeno-associated viral mediated knock-down of TRPC5 in the prefrontal cortex of TRPC5flx mice, we show that TRPC5 channels are necessary for induction of the dADP in the PFC. We show that loss of TRPC5 in the PFC increases the locomotor activating and rewarding effects of cocaine. Knock-out of TRPC1 channels, on the other hand, has no effect on the dADP and does not alter behavioral responses to cocaine, suggesting that TRPC5 homomultimeric complexes rather than TRPC1/5 heteromultimeric complexes underlie the dADP in the PFC. These studies identify the TRPC5 channels as important for modulating neuronal excitability in the PFC and the behavioral responses to cocaine.

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PRIOR PUBLICATIONS

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Fowler MA, Sidiropoulou K, Ozkan ED, Phillips CW, Cooper DC. Corticolimbic expression of the TRPC4 and TRPC5 channels in the rodent brain. PLoS ONE. 2007 Jun 27;2(6):e573.

Cooper DC, Klipec WD, Fowler MA, Ozkan ED. A role for the subiculum in the brain motivation/reward circuitry. Behav. Brain Res. (2006) 174(2): 225-31.

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LIST OF ABBREVIATIONS

AAV: adeno-assciated virus

AMY: amygdala

ASCF: artificial cerebrospinal fluid

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AP5: 2-amino-5-phosphonopentanoic acid

BAPTA: 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid

BSA: bovine serum albumin

CAMKII: Ca^{2+}/calmodulin-dependent protein kinase II

cAMP: cyclic adenosine monophosphate

CIRB: calmodulin/inositol triphosphate receptor binding domain

CPP: conditioned place preference

D1R: dopamine 1-like receptor

D2R: dopamine 2-like receptor

DA: dopamine

dADP: delayed after-depolarization

DAT: dopamine transporter

DG: dentate gyrus

DHPG: dihydroxyphenylglycine

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: dimethyl sulfoxide

DSM-IV: Diagnostic and Statistical Manual IV

DTT: dithiothreitol

ECL: electrochemiluminescence

EDTA: ethylenediamine tetraacetic acid

EPSC: excitatory postsynaptic current

ER: endoplasmic reticulum

EtOH: ethanol

GαM: goat anti-mouse

GαRb: goat anti-rabbit

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GFP: green fluorescent protein

HEK293: human embryonic kidney 293 cells

HIP: hippocampus

i.p.: intra-peritoneal

IP_3: inositol triphosphate

IP_3R: inositol triphosphate receptor

KO: knock-out

LS: lateral septum

mGluR: metabotropic glutamate receptor

NAc: nucleus accumbens

NMDA: N-methyl-D-aspartate

PBS: phosphate-buffered saline

PDZ: postsynaptic density protein, *Drosophila* disc large tumor suppressor, and zonula occludens-1 protein binding domain

PFC: prefrontal cortex

PKA: cAMP-dependent protein kinase, protein kinase A

PKC: protein kinase C

PKG-Neo: phosphoglycerine kinase- neomycin

PLC: phospholipase C

ROC: receptor operated channel

RT: room temperature

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOC: store operated channel

STIM1: stromal interacting molecule 1

SUB: subiculum

TRP: transient receptor potential channel

TRPA: transient receptor potential ankryin-like channel

TRPC: canonical transient receptor potential channel

TRPM: transient receptor potential melastatin channel

TRPML: transiet receptor potential mucolipin channel

TRPN: transient receptor potential nompC channel

TRPP: transient receptor potential polycystin channel

TRPV: transient receptor potential vanilloid channel

vSUB: ventral subiculum

VTA: ventral tegmental area

WT: wild-type

CHAPTER ONE: REVIEW OF DRUG ADDICTION AND POTENTIAL MOLECULAR MECHANISMS UNDE=RLYING ITS PATHOLOGY

Introduction

Drug addiction is a disease which results in over 100,000 deaths annually in the United States and the health and crime related costs associated with drug abuse can reach up to a half a trillion dollars [1]. Drug addicts typically have deficits in attention and decision-making, and often make impulsive decisions that may be to their detriment. The National Institute for Drug Abuse refers to drug addiction as a "chronic, relapsing brain disease in a subject who compulsively seeks drug despite adverse consequences," and the Diagnostic and Statistical Manual IV (DSM IV) states that one or more of the following criteria must be met for the diagnosis of substance abuse: "1) recurrent use resulting in a failure to fulfill major obligations at work, school, or home; 2) recurrent use in situations which are physically hazardous; 3) legal problems resulting from recurrent use; or 4) continued use despite significant social or interpersonal problems caused by the substance use." Addicts will choose to seek drugs even when it causes great detriment to their lives, such as loss of their jobs, friends, family. Drug addicts are often viewed in

a negative light since addiction is commonly and erroneously thought to be due to a lack of willpower or as a personality fault. It is important to realize, however, that there are genetic predispositions that can create a vulnerability for substance abuse and that following drug use, neurological changes occur in the brain that make it extremely difficult for addicts to stop craving and seeking drugs.

Substance abuse is thought be dependent on a number of factors, both environmental and heritable. Though all the genes underlying drug addiction have not been identified, it is clear from family and twin studies that there is a significant genetic component involved which can render an individual more vulnerable for substance abuse [2, 3]. Differences in the genetic makeup of different individuals can lead to altered responses to drugs of abuse through differences in their pharmacokinetics (drug metabolism), pharmacodynamics (altered drug targets), or alterations in the cellular responses to activation at drug targets (changes in intracellular signaling cascades) [2, 4, 5]. Changes seen in pharmacodynamic and cellular responses to drugs appear to be most important for genetic susceptibilities to drug use as these affect the changes in neuronal plasticity seen following repeated drug use [2, 4]. Environmental factors converge with genetic variables to increase or decrease an individual's vulnerability for drug dependence [4]. Recent studies have employed the

use of knock-out mouse model systems to try to identify genes that are important for addiction. These studies examine how loss of drug target genes may affect responses to drugs of abuse by examining both neurophysiological and behavioral outputs [4]. Identifying the genes involved and understanding how they contribute to the neurological changes following repeated drug use is the first step in the search for a treatment for drug addiction.

Due to the adverse effects drug addicts pose on our society, efforts to understand the genes and mechanisms underlying addiction and what might be done to treat it have been subjects of great interest. A great wealth of research has pinpointed a particular circuitry in the brain, the mesolimbic/mesocortical dopamine pathways, commonly referred to as the brain reward circuitry, as a crucial piece to understanding drug addiction. Repeated drug use has been shown to cause long-lasting neuroadaptations in the brain reward circuitry, and understanding the changes that take place is fundamental to understanding the pathology of drug addiction and how it may be treated [6].

The reward circuitry

Understanding the physiological changes that occur following chronic drug use requires an understanding of motivation for natural rewards, both physical, such as food and sex, and psychological, such as friendship. The circuitry involved in eliciting the emotional response and seeking of rewarding behaviors is the brain reward circuitry, and consists of several regions including the ventral tegmental area (VTA), nucleus accumbens (NAc), hippocampus (HIP), prefrontal cortex (PFC) and amygdala (AMY), [7]. Dopamine has long been identified as the key neurotransmitter involved in modulating the activity within this circuitry [7]. Dopaminergic neurons located in the VTA send their projections to the NAc, PFC, HIP, and AMY. The PFC, HIP, and AMY send excitatory glutamatergic projections to the NAc, which has two main GABAergic outputs to the ventral pallidum and the VTA. The ventral pallidum sends GABAergic projections to the medial dorsal thalamus, which closes the circuit with a glutamatergic output to the PFC [7]. The flow of DA is critical for modulating the information within this circuitry, and changes in DA signaling affect behavioral output in many important ways. Psychostimulants are a class of drug that act by increasing DA levels in the brain through various mechanisms; for example, cocaine acts by blocking DA transporters (DAT) to prevent dopamine re-uptake from the

synapse. The increase in DA associated with cocaine use is so high that it exceeds that elicited by natural rewards and causes neglect of naturally rewarding activities in favor of drug use [8].

There are two main classes of DA receptors: D1-like DA receptors (D1R and D5R), and D2-like DA receptors (D2R, D3R, and D4R). The D1 family of receptors are G_s-coupled receptors that activate adenyl cyclase to increase levels of cyclic adenosine monophosphate (cAMP) which leads to activation of the protein kinase A (PKA) intracellular signaling pathway [9]. Activation of the PKA pathway by D1R increases surface expression of the glutamate receptors, NMDA and AMPA, which may enhance their signaling [9]. In contrast, the D2 family of receptors are G_i-coupled receptors that inhibit adenyl cyclase and the PKA pathway while activating the phospholipase C (PLC) pathway [9]. The D2Rs have been shown to decrease AMPA signaling in the striatum by promoting phosphorylation of the receptors and their subsequent trafficking out of the synaptic membrane [9]. Genetic differences in expression and variants of the DA receptors have been linked to the addiction phenotype in humans [10], and animal models have shown DA signaling to be important for mediating the rewarding properties of drugs.

There are two widely used animal models for studying the rewarding properties of psychostimulants: conditioned place preference

and self administration. Conditioned place preference involves the repeated pairing of a drug with a specific environment until a drug-context association is formed. In parallel, a neutral stimulus is paired with a different environment. Following conditioning, the animal is exposed to both environments for a defined period of time. An increase in the amount of time spent in the drug-paired context indicates that the animal found the drug to be rewarding. Two caveats for this method of measuring drug reward are that it is dependent on the ability to learn drug-context associations, and that the drug is given non-contingently so the motivational aspects of drug seeking seen with voluntary administration of drug are not measured. Self administration on the other hand measures the motivational aspects of drug intake by allowing the animal to voluntarily respond for infusions of the drug. In this paradigm, a catheter surgically implanted into the jugular vein allows the animal to intravenously administer small infusions of the drug by pressing a lever or performing a nose-poke in the self-administration box. The frequency of lever pressing for an infusion of the drug indicates the rewarding or reinforcing properties of the drug. In some cases, the animals are trained to lever press for the drug using a progressive ratio schedule where over time, the number of lever presses necessary to receive an infusion of drug increases. The

persistence of an animal to respond for the drug is a measure of both motivation for drug seeking and an indication that the drug is rewarding.

These animal models have been useful in identifying the genes underlying the addiction phenotype. Agonists/antagonists of key drug targets and knock-out animals can be used in these systems to examine the role those genes play in addiction. Uses of these animal models have implicated DA transmission to be critical for the rewarding properties of cocaine. Both D1R and D2R agonists are self administered by rats [7, 11]; and agonists of D2R increase cocaine seeking, while agonists of D1Rs seem to decrease the reinforcing properties of cocaine [12, 13]. D2R antagonists also decrease the reinforcing properties of cocaine [7, 12, 14, 15]. These results indicate that both DA receptors types contribute to the reinforcing properties of psychostimulants; however, their mechanisms of action are not clearly understood.

It is clear that increased DA transmission to the NAc is necessary for maintenance of self-administration of psychostimulants in rats; and that lesion of the NAc or administration of DA receptor antagonists prevent self administration and conditioned place preference to psychostimulants [7, 8, 16, 17]. However, the activity of the NAc is modulated by other brain regions, such as the HIP, AMY, and PFC. The HIP and AMY are memory centers of the brain that store cocaine related memories and the feelings

associated with cocaine in drug addicts [8]. The HIP is primarily involved in recording contextual information associated with drug use; and the AMY is associated with the conditioned emotional responses elicited by drugs [18]. The PFC is involved in higher order thinking and integrates the information provided by the other regions of the limbic system to decide a course of action. Under normal conditions, the PFC can act as a modulator that inhibits pleasurable activities, such as drug intake to avoid negative consequences associated with drug use [8]. However, it is known that after repeated drug use in humans and animals models, the excitability in the PFC becomes modified and its ability to regulate behaviors is reduced [6, 8, 18, 19].

The prefrontal cortex and drug addiction

The PFC is an area involved in decision-making, impulsivity, and reasoning, all of which are impaired in drug addicts. Studies have shown reduced basal activity of the PFC and orbitofrontal cortex in cocaine addicts compared to normal subjects, indicating that normal PFC function is impaired in addicts [20]. However, there is strong activation of the PFC and NAc in cocaine addicts following presentation of cocaine-paired cues that is associated with feelings of craving [21]. In addition, work from animal models indicate that drug seeking behavior in animals trained in

cocaine SA is dependent on the glutamatergic projection from the PFC to the NAc [22]. It's possible that the strong activation of the NAc by the PFC following drug cues in addicts is due to the dysregulation of glutamatergic signaling in the PFC in the addicted state [18, 20, 23, 24]. In addition, the activation seen in the PFC is restricted to that elicited by drugs or drug cues; and the activation of the PFC normally seen with naturally rewarding activities or during higher order cognitive thinking is reduced in drug abusers [18, 25-27]. These studies provide evidence for dysregulation of PFC excitability in the pathological seeking of drugs seen in addicts.

Following chronic drug use, a number of neuroadaptations have been shown to occur in the PFC. The two key areas where these changes can occur are the DA outputs from the VTA to the PFC, and the glutamatergic outputs from the PFC to the NAc. It is likely this plasticity that occurs following chronic drug use in these regions that results in the uncontrollable drive for drug seeking that is responsible for drug reinstatement [21].

The PFC contains both the $G_{s\alpha}$-coupled D1Rs and $G_{i\alpha}$-coupled D2Rs which have been hypothesized to regulate glutamatergic output from the PFC to elicit two different excitability states of the PFC which are reviewed by Kalivas *et al* [21]. In the D2R dominated state, the PFC is less inhibited and can respond to multiple emotional and motivational

stimuli. In the D1R dominated state, the PFC is acting under increased inhibition in which only very strong inputs will elicit glutamatergic output from the PFC to the NAc [21, 28]. Following chronic cocaine use, there is a decrease in $G_{i\alpha}$-coupled signaling in the PFC [21]. This reduction in D2R signaling pushes the PFC neurons into a D1R dominated state of increased inhibition, or hypofrontality, in which only powerful DA enhancing stimuli, like cocaine, will drive output from the PFC. The reduction in D2R is also paired with increased metabotropic glutamate receptor (mGluR) signaling in the PFC which results in dysregulation of the glutamatergic outputs from the PFC to the NAc [29]. These studies provide evidence that changes in PFC plasticity and dopaminergic tone occur following chronic drug use; however few studies have looked closely at the mechanisms of how synaptic excitability in the PFC is altered.

In addition, activation of D1R and D2R alters the intrinsic properties of neurons in the PFC by altering the function of ion channels which can disrupt the normal ability of the PFC to process reward related information and to perform cognitive functions [9, 30-33]. Repeated psychostimulant use disrupts attention and working memory tasks, possibly through these disruptions in the intrinsic excitability of the PFC neurons [32, 34]. During working memory tasks in monkeys, PFC neurons are capable of persistent action potential output during the delay period between the presentation of

the stimulus and the behavioral response [35]. This persistent activity may be a way for the cell to retain salient information for making a behavioral response. Proper DA tone and glutamatergic signaling in the PFC are necessary for performance on working memory tasks; and both types of signaling are disrupted following psychostimulant abuse [36-40]. Our work aims at understanding the mechanism underlying persistent activity in the PFC and how cocaine and DA alter PFC functioning.

In our studies, we examined an intrinsic, cellular mechanism for persistent activity in pyramidal neurons in the PFC in which brief bursts of activation concomitant with activation of G_q-coupled receptors, activates Ca^{2+}-activated non-selective cation channels to induce a delayed, sustained after-depolarization (dADP) sufficient to induce persistent action potential output. This induction in persistent activity could serve as a mechanism for retaining the cellular information during a delay period. The dADP is reduced by chronic cocaine and by activation of D1R through a PKA-dependent pathway. This DA-induced reduction in a persistently activated state may be important in facilitating the rewarding effects of cocaine by biasing the cell towards the very strong inputs associated with drug intake. In studying the molecular mechanism underlying the dADP, we became interested in identifying the non-selective cation channel mediating the phenomenon. Pharmacological studies in the lab led us to

name the non-selective cation channels, TRPC, as candidates for underlying the dADP.

Discovery of the TRP and TRPC channels

The first TRP channel was discovered in *Drosophila melanogaster* in 1989 [41]. In *Drosophila*, light is perceived through activation of rhodopsin and phospholipase C (PLC), which induces a sustained membrane depolarizing current. A *Drosophila* mutant was discovered in which activation of PLC resulted in only a transient rather than a sustained current in response to light [41, 42]. The gene responsible was later cloned and named a transient receptor potential (*trp*) channel [41]. The mammalian homologue of *Drosophila trp*, TRPC, was discovered in 1995 [43] and since then, six other families of mammalian TRP channels have been discovered, and the TRP ion channel superfamily now consists of the seven subfamilies: TRPC, TRPV, TRPM, TRPML, TRPP, TRPA, and TRPN. All of the TRP channels are selective for cations; however their affinities for Ca^{2+} and Na^{+} differ [44]. The channels all contain six transmembrane domains and function as either homomultimeric or heteromultimeric complexes with other members of the same TRP family [44]. We identified the TRPC channels as possible candidates for the non-selective cation channel underlying the dADP because they are

activated by G_q-coupled receptor signaling and their activation is dependent upon release of intracellular Ca^{2+}.

The TRPC channels

The TRPC family consists of seven members, TRPC1-7. They serve to regulate Ca^{2+} homeostasis in the cell and have been implicated in a number of functions including salivary gland secretion, diabetic nephropathy, neuronal development and plasticity, cardiac hypertrophy, and renal functions [45].

Until recently, the expression pattern of the TRPC channels in the mature mammalian brain was widely unknown. A variety of expression studies have now been performed and show high expression of TRPC channels in almost every area of the body, including liver, kidney, heart, endothelial cells, and the brain. At the start of this dissertation work, the expression of the TRPC channels throughout the brain was lacking. Since then, there has been a wealth of information regarding the expression of these channels in the brain, including our paper published in PLoS ONE in 2007 [46]. Interestingly, many of the TRPC channels have been shown to be expressed in the corticolimbic areas of the brain, such as the PFC, orbitofrontal cortex, entorhinal cortex, striatum, lateral septum, HIP,

subiculum, substantia nigra, and cerebellum [47, 48]. The expression pattern of the TRPC channels in the corticolimbic areas of the brain, paired with their ability to regulate Ca^{2+} homeostasis, identifies them as candidates for modulating neuronal excitability in the brain.

The TRPC channels are activated following PLC activation and were initially proposed as store-operated channels (SOC), or channels that sense release of intracellular stores of Ca^{2+} from the endoplasmic reticulum (ER) and are subsequently activated [49-53]; however, later reports have shown otherwise [54-58]. The current prevailing hypothesis is that the TRPC channels function as receptor operated channels (ROC) that interact with a calcium sensor protein, stromal interacting molecular 1 (STIM1), which senses release of intracellular Ca^{2+} from the ER, which activates the TRPC channels [44, 59, 60]. In addition, TRPC3 and 5 channels are activated by growth factors resulting in vesicular translocation of the TRPC channels into the membrane [61, 62]. It is proposed that these channels may be involved in dendritic outgrowth during development. The TRPC channel-mediated changes in neuronal processes may be involved in altered learning related plasticity.

The TRPC channels can be classified into four different subgroups of TRPC1, TRPC2, TRPC4/5, and TRPC3/6/7. All of the TRPC channels are expressed in humans with the exception of TRPC2, which is a

pseudogene in humans, but not in other mammals. TRPC1 has been shown to heterotetramize with TRPC3, 4, 5 [54, 63-65], but its ability to form homomultimeric complexes has been controversial. However, it has been suggested that TRPC1 can form a homomultimeric complex which associates with STIM1 in lipid rafts where the complex acts as an SOC. In situations where TRPC1 is not associated with STIM1, it forms heteromultimeric complexes with other TRPC channels in the plasma membrane where it acts as an ROC [66]. This finding fits with previous studies in which TRPC1 homomultimeric complexes were shown to have deficits in trafficking to the plasma membrane when not co-expressed with TRPC4 or 5.

Currently, there is no pharmacological way of distinguishing between the TRPC channels. In vitro, the only method for distinguishing between the two major groups, TRPC4/5 and TRPC3/6/7 is by their response to La^{3+}. TRPC4/5 currents measured in HEK 293 cells are potentiated by 100 uM concentrations of La^{3+} and TRPC3/6/7 currents are completely blocked by the same concentration [67]. The lack of pharmacological distinction makes it difficult to examine the function of the individual TRPC channels *in vivo* or *in vitro*.

TRPC channels in modulating PFC excitability and reward behavior

The dense corticolimbic expression pattern of the TRPC channels and their role in regulating Ca^{2+} homeostasis suggests that they may be involved in modulating neuronal plasticity in the brain. The TRPC channels are activated by G_q-coupled receptor such as mGluR5s, which are important for working memory function [36]. Pharmacological and expression data pointed to TRPC5 as the non-selective cation channel underlying the mGluR5-mediated dADP in the PFC (See Chapter 2, Figure 2.11). We propose that the dADP in the PFC is an intrinsic cellular mechanism for persistent activity seen in the PFC that facilitates working memory. DA and repeated cocaine modulate mGluR function and the behavioral responses to cocaine. We seek to identify the TRPC5 channel as the non-selective cation current underlying the dADP and implicate a role for the TRPC5 channel and the dADP in modulating the behavioral responses to cocaine

CHAPTER TWO: EXPRESSION OF TRPC4 AND TRPC5 IN THE RODENT BRAIN

This chapter was taken from the PLoS ONE publication in 2007 by Fowler *et al* [46].

Summary

The TRPC channels are a family of non-selective cation channels that are activated by increases in intracellular Ca^{2+} and G_q/phospholipase C-coupled receptors. Quantitative real-time PCR, in situ hybridization, and immunoblots were used to examine the expression of the predominant TRPC channels in the rodent brain. Quantitative real-time PCR of the seven TRPC channels in the rodent brain revealed that TRPC4 and TRPC5 channels are the predominant subtypes in the adult rat brain. In situ hybridization and immunoblotting further resolved a dense corticolimbic expression of the TRPC4 and TRPC5 channels. Total protein expression of HIP TRPC4 and 5 proteins increased throughout development and peaked in adulthood (6-9 weeks). In adults, TRPC4 expression was high throughout the frontal cortex, lateral septum, pyramidal cell layer of the hippocampus, dentate gyrus, and ventral subiculum. TRPC5 was highly expressed in the frontal cortex, pyramidal

cell layer of the hippocampus, and the dentate gyrus. Detailed examination of the frontal cortical layer mRNA expression indicated that TRPC4 mRNA is distributed evenly throughout layers 2-6 of the prefrontal, motor, and somatosensory cortices. TRPC5 mRNA is concentrated specifically in the deep layers 5/6 and superficial layers 2/3 of the prefrontal cortex and anterior cingulated. Overall, the dense corticolimbic expression pattern suggests that these G_q/PLC coupled nonselective cation channels may be involved in learning, memory, and goal-directed behaviors.

Introduction

The dynamic homeostatic mechanisms that neurons use to regulate intracellular Ca^{2+} signaling have received much attention recently, due to the important role Ca^{2+} plays in cellular processes including gene expression, axon growth, synaptic plasticity and cell death. TRPC channels have been identified as important channels that may be involved in maintaining intracellular Ca^{2+} concentrations in response to a range of signaling modalities [68]. Despite the recent interest and potential importance of the TRPC channels, there have been no thorough

descriptions of the expression pattern of these channels in the mammalian brain.

The TRPC non-selective cation channels consist of seven members that are organized into four groupings based on sequence homology and functional similarities: TRPC1, TRPC2, TRPC3/6/7, and TRPC4/5 [69, 70]. These channels are mixed cation (K^+, Na^+, and Ca^{2+}) channels that are activated by G_q-coupled receptors, such as group 1 mGLuR and muscarinic acetylcholine receptors [56, 69, 71-73]. To date, there are no selective drugs capable of distinguishing between the TRPC subtypes. However, micromolar concentrations of the trivalent lanthanoids (La^{3+}, Gd^{3+}) block TRPC3/6/7, but potentiate TRPC4/5 channels [67]. In cell culture expression systems, TRPC4 and 5 channels function to modulate cellular excitability, neuronal growth and axon guidance, and regulation of Ca^{2+} homeostasis [56, 61, 69, 74]. The TRPC4 and 5 channels have been proposed to be activated by G_q/PLC signaling, release of intracellular Ca^{2+} stores, or vesicular translocation to the membrane (Figure 2.1; [61, 69, 75]). G_q-signaling mediated activation of PLC increases inositol triphosphate (IP_3) that binds to the IP_3 receptor located on the ER and release intracellular Ca^{2+} [50]. A conformational change opens the TRPC channel bound to IP_3 receptors, which bind to the C-terminal end of the TRPC channels via the calmodulin/IP_3 receptor

binding domain [75, 76]. Another mechanism of activation for TRPC5 channels is through vesicular translocation to the membrane following stimulation by neuronal growth factors [61, 77]. TRPC5 is expressed in neuronal growth cones and is rapidly inserted into the membrane following stimulation by neuronal growth factors where it leads to cessation of growth in cultures cells [61, 77]. These functional in vitro studies suggest that TRPC5 channels may be expressed presynaptically in axon terminals and be involved in axon guidance [78].

Although a few reported studies have examined TRPC channels in select brain regions, an extensive and comprehensive description of the brain-wide expression of TRPC4 and 5 channels is lacking. In this study, we sought to identify the expression pattern and suggest a possible function of the TRPC4 and 5 channels in the rodent brain.

Experimental Procedures

Microdissections of brain regions

For all brain regions, slices were taken from male rats and mice (8-10 weeks old for all experiments unless otherwise indicated). Animals were anesthetized by halothane inhalation and their brains quickly

removed and placed into ice-cold artificial cerebral spinal fluid (ACSF). The brain was mounted and coronal slices (500 μM for rat, 250 μM for mouse) were cut using a vibratome. Each region was dissected while the slices were in ice chilled ACSF and immediately frozen on dry ice upon extraction.

RNA extraction and reverse transcription

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad CA, USA) in accordance with the protocol. The RNA was resuspended in DEPC-treated H_2O and DNase-treated to eliminate any DNA contamination using DNA-free (Ambion, Austin, TX, USA). The concentration of RNA was determined by UV absorbance using a Nanodrop spectrophotometer. The RNA was reverse transcribed with random hexamer primers (Invitrogen, Carlsbad, CA, USA) using the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNA was purified on a spin purification column (Qiagen, Valencia, CA, USA) and the concentration was determined by UV absorbance using a Nanodrop spectrophotometer.

Quantitative real-time PCR

The real-time primers were designed as 23-25 oligonucleotide sequences that amplify a 100-150 base pair gene product. Primers were checked against all known rat gene sequences to ensure specificity and were designed to maximize detection of all known splice variants of the TRPC genes. The real-time PCR reaction was optimized by running a standard curve for each primer set in dilutions of whole brain rat cDNA (1, 0.5, 0.25, 0.125, and 0.0625), with 100 ng of cDNA in the 1X concentration. From this standard curve, the efficiency for each gene was determined using the slope and R^2 value according to the method proposed by Pfafl. The reactions were carried out using SYBR Green PCR Master Mix (Ambion, Austin, TX, USA) in a Stratagene MXP3000 real-time PCR thermal cycler (Stratagene, La Jolla, CA, USA). The amplification plots, melting curves, and standard curve were assessed for primer quality and the product was run on a gel to ensure amplification of a single product of the correct size. The TRPC channel expression was assessed in the different brain regions by running real-time PCR reactions of 100 ng of the cDNA transcribed from total RNA isolated from the dissections. In all reactions GAPDH was used as a positive control to which the results were normalized.

For the relative TRPC channel expression analysis, the cycle threshold values for the reactions were normalized to GAPDH and then expressed as a percentage of 100 relative to each other. For the region-specific TRPC channel expression, the cycle threshold values for the reactions were normalized to GAPDH and expressed as a fold increase relative to expression levels in whole rodent brain. This analysis emphasizes that TRPC4 and 5 have specific expression in those regions compared to levels in whole brain and are not expressed at equal levels throughout the brain.

Molecular cloning of TRPC4 and 5

The TRPC4 and 5 channels were cloned from cDNA reverse-transcribed from total RNA isolated from adult rat HIP tissue using Trizol (Invitrogen, Carlsbad, CA, USA). Primers to each of the channels were designed to the 3' and 5' ends of the mRNA sequences. The 5'primer for TRPC4 contained a XhoI site and the 3'primer contained a BamHI site for cloning into pBluescript (Stratagene, La Jolla, CA, USA). The 5'primer for TRPC5 contained a XhoI site and the 3'primer contained a XbaI site for cloning. The TRPC4 and 5 sequences were amplified using Long Template DNA polymerase (Roche, Indianapolis, IN) and the products

were cloned into pBluescript and confirmed by sequencing. The TRPC5 sequence was cloned into pcDNA-FLAG as a fusion protein with a FLAG tag on the C-terminal end. The clone was confirmed by sequencing.

In situ hybridization of TRPC4 and 5

The in situ hybridization experiments were carried out according to the methods described by Gold and Zachariou [79]. Slide-mounted, fresh-frozen tissue sections were used. Halothane-anesthetized animals were decapitated and their brains removed and rapidly frozen on dry ice. Coronal, fresh-frozen sections were cut at 14 µm in a cryostat, thaw-mounted onto Superfrost Plus (Fisher Scientific, Pittsburgh, PA, USA) glass slides, and stored at −80°C until use. The sections were fixed in 4% paraformaldehyde, and washed in PBS, PBS+glycine, and 0.25% acetic anhydride in 0.1M triethanolamine. The sections were then dehydrated and delipidated in 50%, 75%, 95%, and 100% EtOH and 100% chloroform. Sections were hybridized for 18 hours at 60°C in hybridization buffer containing deionized formamide (40%), dextran sulfate (10%), 1X Denhardt's solution, 4x SSC, denatured and sheared salmon sperm DNA (1 mg/mL), yeast tRNA (1 mg/mL), dithiothreitol (10 mM), and either S^{35}-labelled RNA probe (S^{35}, Perkin Elmer, Waltham, MA, USA) or an

identical, unlabeled RNA probe as a control. The probes used were a 188 bp S^{35}-labeled probe for TRPC4 and a 300 bp S^{35}-labeled probe for TRPC5 which were transcribed using T3 polymerase (Ambion, Austin, TX, USA) from the TRPC4 and 5 pBluescript clones described above and purified on a spin purification column. The counts per million (cpm) of the probes were assessed using a scintillation counter and added to the hybridization buffer at a concentration of $10x10^6$ cpm/ml. After hybridization, the sections were washed in sodium citrate buffer (SSC) + sodium thiosulfate and placed on film for 3-7 days before development.

Quantification of TRPC4 and 5 expression

The expression of TRPC4 and 5 from the in situ hybridization film in the cortex layers was quantified by measuring the signal intensity of a 125 μm^2 region of interest being quantified. The signal intensity of each region was normalized to an identically sized background region, where no tissue was present. The rationale for using the background region as the control is that because we are examining the expression of TRPC4 and 5 in different brain regions within the same slice, instead of the differences in expression in the same region across different slices, using a region of the tissue without a specific signal has an advantage because it does not

assume expression or lack of expression in any brain regions. The controls show a homogenous background level.

For the qualitative assessment of the TRPC4 and 5 signal from the in situ hybridization film, the signal intensity in various brain regions was scored by three independent investigators according to the scale of 0<+<++<+++. The average intensity for each brain region was recorded.

Expression of TRPC5 in HEK293 cells

One day before transfection, 0.5-2 X 10^5 HEK293 cells in 1 ml of Opti-MEM Reduced Serum Medium (Invitrogen, Carlsbad, CA, USA) without antibiotics were plated into 6-well plates so that the cells would be 90-95% confluent at the time of transfection. One µg of TRPC5-Flag plasmid DNA (pTRPC5-F) or control pGFP DNA was diluted into 50 µl of Opti-MEM Reduced Serum Medium (Invitrogen, Carlsbad, CA, USA). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in a ratio of 1:3 (plasmid: reagent) was diluted into 50 ul Opti-MEM I Medium (Invitrogen, Carlsbad, CA, USA) and incubated for 5 min at RT. The Lipofectamine and DNA dilutions were combined and incubated at room temperature for 20 min, then added to the plates containing the HEK293 cells. The cells were incubated at 37°C with 95% O_2 and 5% CO_2. To identify the optimal

transfection conditions, a time course of 18, 24, 36, and 48 hours post-transfection harvesting was performed. A mock transfection control of empty vector and a nontransfected control were included.

Cross-linking Procedure

C57/Bl6 P0 and P48 day old-mice were rapidly decapitated and their brains were extracted and placed into ice-cold ACSF. The whole hippocampus was dissected from 300 µm slices cut on a vibratome (Leica, Nussloch, Germany) and placed into tubes containing ice-cold ACSF on ice. The slices were treated with 2 mM BS^3 (in 5mM NaCitrate buffer, pH 5.0; Pierce, Rockford, IL, USA) for 30 minutes at 4ºC with shaking. The reaction was quenched by the addition of 100 mM glycine for 10 minutes at 4ºC with shaking. Control slices that did not receive BS^3 were also incubated for 30 minutes at 4ºC with shaking and received 100 mM glycine for 10 minutes at 4ºC with shaking. The tubes were spun down, the supernatant was aspirated, and the cells were immediately lysed by sonicating in boiling 1% sodium dodecyl sulphate (SDS) lysis buffer. The samples were boiled for 10 minutes. The protein concentration was determined using the BCA assay (Pierce, Rockford, IL, USA) and 20 µg of protein was loaded onto a 5-16% gradient sodium dodecyl sulfate–

polyacrylamide gel electroporesis (SDS-PAGE) gel. The protein was transferred from the gel to Hybond ECL nitrocellulose membrane (Amersham, Piscataway, NJ, USA) for immunoblotting.

Immunoblot Procedure

Homogenates were obtained by sonicating tissue suspensions in boiling 1% SDS lysis buffer. Protein concentrations were determined by the BCA assay (Pierce, Rockford, IL, USA). Samples are loaded onto 10% SDS–PAGE gels (100 µg protein/lane). Preliminary experiments were performed to demonstrate that these amounts are in the linear range as determined by densitometric analysis. Gels were transferred to Hybond ECL nitrocellulose membrane (Amersham, Piscataway, NJ, USA) for immunoblotting. The membranes were blocked in 5% milk for 1 hour, then primary antibody (TRPC4, 1:1000, gift from Dr. Bonanno; TRPC5, 1:400, Alomone, Jersusalem, Israel or Sigma-Aldrich, St. Louis, MO, USA; β-actin monoclonal, 1:1000, Sigma-Aldrich, St. Louis, MO, USA) for either 1 hour at room temperature or overnight at 4°C with shaking. The membranes were incubated in the appropriate secondary antibody (GαRb, 1:8000 or GαM, 1:8000, Pierce, Rockford, IL, USA) for 1 hour at room temperature with shaking and developed with enzymatic

chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ, USA) reagent.

Results

Quantitative real-time PCR expression of the seven TRPC channels in the rat brain

Results of real-time PCR performed on RNA isolated from adult rat whole brain (excluding the cerebellum) show that TRPC4 and 5 are the two predominantly expressed TRPC channels in the brain, comprising approximately 41% and 24% of the TRPC channel population respectively (Figure 2.2). TRPC3, TRPC1, and TRPC6 are moderately expressed at 18%, 12%, and 5% of the population respectively. TRPC2 and TRPC7 show very little expression in the brain at less than 1%. The real-time PCR primers were designed to target all known splice variants of the TRPC channels and to produce a single product (Table 2.1). The efficiency for each primer set was calculated in order to account for any differences in efficiency and the results were normalized to the housekeeping gene, GAPDH, in order to compare the expression of the different TRPC channels.

Distribution of TRPC4 and 5 in the rodent brain

The qualitative expression of TRPC4 and 5 mRNA throughout the rat and mouse brain is presented in Table 2.2. The results of in situ hybridization of TRPC4 were observed to be similar between the species and are therefore shown together. Coronal and horizontal brain slices show that TRPC4 mRNA is highly expressed in the LS and the cell body layer of the CA1-Ca2 sub-regions of the HIP, ventral subiculum, and dorsal tenia tecta (Figure 2.3). Moderate expression was observed in the frontal cortex including the PFC (infralimbic and prelimbic), anterior cingulated, motor cortex, somatosensory cortex, entorhinal cortex, piriform cortex, orbitofrontal cortex, amygdale, ventral hypothalamus, and Purkinje and granule cell layers of the cerebellum. No specific labeling was detected in slices that were co-incubated with unlabeled control in situ hybridization probes for TRPC4 or 5 (Figure 2.3).

To examine the cortical layer-specific expression of TRPC4 and 5 in the frontal cortex, we performed quantitative analysis of the in situ hybridization signal in the superficial (layers 2/3) and deep (layers 5/6) cortical layers. Results show that TRPC4 mRNA is expressed at similar levels throughout layers 2, 3, 5, and 6 in all regions of the frontal cortex,

including the PFC, anterior cingulated, motor, somatosensory, and orbitofrontal cortices. In layers lacking pyramidal neurons, such as layer 1 of the frontal cortex, there is no TRPC4 mRNA expression (Figure 2.4).

Quantitative comparisons of TRPC4 mRNA expression in the cell body layer of the dorsal HIP of adult rat and mouse were similar. In both species, TRPC4 expression is very high in CA1 while moderate in CA2 and DG, and little expression in CA3 and the hilus is observed. Analysis of ventral HIP showed that TRPC4 is highly expressed in the subiculum with less expression in the entorhinal cortex (Figure 2.5). In subcortical structures, there is moderate expression of TRPC4 mRNA in the amygdala and ventral hypothalamus. In contrast, very low expression of TRPC4 mRNA is seen in either the dorsal striatum or nucleus accumbens, which matches the real-time PCR data indicating low expression of TRPC4 and 5 mRNA in the striatum relative to whole brain TRPC4 and 5 levels (Figure 2.6).

TRPC5 mRNA in situ hybridization indicates high expression concentrated in the deep layers 5/6 and superficial layers 2/3 of the PFC, ventral subiculum, piriform, orbitofrontal, and entorhinal cortices (Figure 2.4). As with TRPC4, no significant TRPC5 expression was detected in layer 1 of the PFC where there are few neuronal cell bodies. TRPC5 had little mRNA in the motor or somatosensory cortices. The cell body layer of

the HIP had robust expression of TRPC5 in CA1, CA2, CA3, and hilus, with moderate expression in the DG and vSUB (Figure 2.5).

In subcortical structures, high TRPC5 expression was detected in the Islands of Caleja, paraventricular hypothalamus, and dorsal and ventral hypothalamus. Little expression of TRPC5 was seen in the motor and somatosensory cortices, and no expression is seen in the dorsal striatum although, interestingly, moderate expression of TRPC5 was detected in the nucleus accumbens (Figure 2.3, Table 2.2).

Relative TRPC channels expression in the prefrontal cortex and lateral septum

In situ hybridization results indicate that TRPC4 or 5 mRNA is highly expressed in the LS and PFC. To validate this using real-time PCR, we examined the relative expression of all TRPC channels in the PFC and LS of age matched adult rats. PCR of the seven TRPC channels in microdissections of the PFC indicate that TRPC4 and 5 mRNAs are the most highly expressed TRPC channels in the PFC, representing 38% (TRPC4) and 34% (TRPC5) of the TRPC channel population. TRPC6, TRPC3, and TRPC1 had low mRNA expression in the PFC with 12%, 9%,

and 6% respectively. TRPC7 and TRPC2 comprise <1% of the TRPC channel population in the PFC (Figure 2.6).

Real-time PCR of the seven TRPC channels in microdissections of the LS of adult rat confirms that TRPC4 is the predominantly expressed channel in the LS, comprising 66% of the TRPC channels population. TRPC3 is moderately expressed in the LS with 22%, and TRPC5, TRPC6, and TRPC1 have lower expression with 4%, 4%, and 3% respectively. TRPC2 and TRPC7 comprise <1% of the TRPC channel population in the LS (Figure 2.6).

Region-specific protein expression of TRPC4 and TRPC5

To examine whether the mRNA expression of TRPC4 and TRPC5 protein corresponds with the protein expression of these channels, I performed immunoblots for TRPC4 and TRPC5 from microdissections of striatum, PFC, and LS. I did not examine the relative protein expression of the TRPC4 and TRPC5 in the brain due to potential differences in antibody affinities between the two channels. Due to these inherent limitations in immunoblotting, I restricted the protein analysis to a region-specific analysis[80]. The antibody used for TRPC4 has previously been validated and shown to be specific [81]. To verify the specificity of the

TRPC5 commercial antibody, we cloned the TRPC5 into the mammalian vector, pCDNA3.1-FLAG that inserts a FLAG tag on the C-terminal region of the protein. Immunoblots of protein harvested from HEK293 cells 48 hours after transfection with TRPC5-FLAG were blotted with both α-FLAG and α-TRPC5 antibodies. The detected bands were identical in size (inset, Figure 2.7), confirming that the FLAG-tagged protein expressed in the HEK293 cells is TRPC5 protein. No bands were detected on immunoblots of protein harvested from cells transfected with empty vector or from non-transfected cells. In addition, immunoblots of protein harvested from TRPC5-FLAG transfected HEK293 cells and rat whole brain show identical bands, thus confirming the specificity of the TRPC5 antibody in rat brain lysates (inset, Figure 2.7).

Results of TRPC5 protein expression from immunoblots from rat striatum, PFC, and LS show that TRPC4 protein is most highly expressed in the PFC and moderately expressed in the LS with lower expression in the striatum (Figure 2.7). These results show that, in general, the protein expression of these channels matches the mRNA expression levels in those regions.

Developmental expression of TRPC4 and TRPC5 proteins

To determine if TRPC4 or 5 protein expression is developmentally regulated, we examined expression at different stages of development in the HIP. Immunoblots performed for TRPC4 and 5 in microdissections of HIP from post-natal day 0 (P0) and post-natal day 48 (P48) mice show that TRPC4 and 5 protein levels are higher at P48 compared to P0 (Figure 2.8C). HIP TRPC5 protein is present at low levels from embryonic day 18 to post-natal day 20 and increase robustly to P48 (Figure 2.8B). Similar results were observed in rat PFC, vSUB, and entorhinal cortex comparing post-natal day 21 and post-natal day 63 rats (Figure 2.9).

To examine the surface expression of TRPC5 protein in P0 and P48 mice, we used the irreversible, membrane impermeable crosslinker, BS^3, to cross-link surface-expressed TRPC5 proteins. Cross-linked samples were blotted and the ~100kD band representing the remaining intracellular pool of TRPC5 was quantified. These methods are similar to those described by Grosshans et al [82]. Cross-linking resulted in immunoblots that showed a TRPC5 labeled high molecular weight smear (>206 kD) in cross-linked samples that was not present in non cross-linked samples, indicating successful surface cross-linking (Figure 2.8B). To ensure that cross-linking produces no non-specific changes in protein, we examined levels of the intracellular protein, beta-actin, and found that the

total amount of protein was no significantly different across cross-linked and non cross-linked samples for P0 or P48 (Figure S1). This confirms previous work demonstrating the lack of membrane permeability of BS^3 [83].

Expression of TRPC5 channels is associated with a burst-induced delayed after-depolarization

Given the robust expression of these channels in adults and their presence on the surface of the membrane, we sought to identify a function for these channels in the corticolimbic system using whole-cell patch clamp recording in the deep layer pyramidal neurons. Using the Group I mGluR agonist, DHPG, others in the lab observed an action potential burst-induced delayed after-depolarization (dADP) lasting several seconds after the burst [32]. This dADP correlated with the presence of TRPC4 and 5 expression (Figure 2.10). It was robust in areas such as layer 5 pyramidal neurons in the medial PFC, LS, and SUB where mRNA expression in high, yet it was absent in the nucleus accumbens and striatum where mRNA expression is low (Figure 2.11A-B, Figure 2.10A-C). Na^+ replacement by 80% choline-Cl substantially reduced the dADP, but did not eliminate it, indicating a substantial Na^+ ion component to the

dADP (Figure 2.11B). Bath application of the voltage gated Na^+ channel blocker, tetrodotoxin (1 mM), had no effect on the dADP amplitude, indicating that the current underlying the dADP is not a voltage gated TTX-sensitive Na^+ current (Figure 2.11C-D). Intracellular application of the Ca^{2+} chelator, BAPTA (10 mM), significantly reduced the dADP, demonstrating the role of intracellular Ca^{2+} in activation of the dADP (Figure 2.11C-D). Application of the Na^+/Ca^{2+} exchanger blocker, benzamil (100 mM), failed to reduce the dADP (Figure 2.11D), ruling out the involvement of a Na^+/Ca^{2+} exchanger. The dADP was reduced by the protein kinase C activator, PdBU (1 mM),which has also been shown to deactivate the TRPC channels [84-86] (Figure 2.11D). Application of the non-selective cation channel blocker, flufenamic acid (100 mM), and IP_3 receptor blocker, heparin (2 mg/ml), significantly reduced the dADP (Figure 2.11D). A small dADP was induced in the absence of DHPG by the trivalent cation La^{3+} (100 mM), which has been shown to potentiate TRPC4 and 5 and block TRPC3, 6, and 7 current in transfected HEK293 cells [67] (Figure 2.11D inset). This induction of the dADP by La^{3+} was blocked by the non-specific TRP channel blocker, SKF96365 (100 mM) (Figure 2.11 D inset).

Discussion

Expression of TRPC4 and TRPC5 channels in the rodent brain

The TRPC family of nonselective cation channels has been implicated in a number of neuronal processes such as calcium homeostasis, cellular excitability, and axon guidance, yet despite their potential importance, a thorough characterization of these channels in the brain is currently lacking and their exact function and method of activation remains unclear. Determining the regional distribution of these channels in the brain is a fundamental step in understanding their function. In this study, we provide an extensive description of the expression of the TRPC4 and 5 channels, which were found to be the most predominantly expressed TRPC channel mRNAs in the brain. The region-specific expression of TRPC4 and 5 mRNA throughout the brain was concentrated in the areas of the corticolimbic system, such as the PFC, orbitofrontal cortex, LS, HIP, and amygdala. For TRPC4, the highest expression was located in the LS, consistent with previous work by Otsuka et al [87]. We performed detailed quantification of TRPC4 and 5 in the cortical cell layers and found a uniform distribution of TRPC4 throughout layers 2-6 with no labeling in layer 1, while TRPC5 showed dense labeling in layers 2/3 and

layers 5/6. These results suggest that TRPC5 mRNA is localized to pyramidal neurons found in high density in these two regions of the PFC although further work is needed to demonstrate this directly. The uniform distribution of TRPC4 in the cortical layers suggests it is distributed throughout many cell types. Expression of both TRPC4 and 5 in the cell body layers of the HIP provides evidence that these channels are present in pyramidal neurons in the HIP. These results are consistent with previous immunohistochemical work by Chung et al showing high expression of TRPC5 in the cell body layer of the HIP [88]. The striking absence of either mRNA or protein in the striatum radiatum and striatum lacunosum moleculare suggests a lack of TRPC4 or 5 expression in the interneurons in these regions, although this would best be examined using immunohistochemistry.

TRPC5 channels transport along neuronal projections

The TRPC channels have been implicated in both presynaptic and postsynaptic neuronal processes, however, the synapse specific expression and function of these channels is currently unknown. In our studies, we observe a disparity in the mRNA and protein levels of TRPC5 in some brain regions, suggesting that the protein may be transported

along neuronal projections into the presynaptic terminals, thus providing evidence for a presynaptic function of the channel. The protein expression of TRPC4 and TRPC5 correspond with the mRNA expression for each channel in regions such as the PFC, LS, and HIP; however, in the nucleus accumbens TRPC5 mRNA is expressed at low to moderate levels yet the protein is expressed at moderate to high levels. Also, previous studies by De March et al show that TRPC5 protein expression in the substantia nigra using immunohistochemistry, however, our studies show a lack of TRPC5 mRNA expression in this region [89]. It is not uncommon that mRNA and protein levels do not match [90]. These differences can be attributed to regulation at the transcriptional or translational level, differences in half-lives of the mRNA and protein due to differences in rate of synthesis or degradation, or mRNA and protein transport and trafficking [80]. However, a more likely explanation for the results in the nucleus accumbens is a differential pre vs. postsynaptic expression of the TRPC5 mRNA and protein. For example, the deep layer pyramidal neurons in the PFC send a substantial projection to the nucleus accumbens, therefore it is possible that TRPC5 mRNA is transcribed and translated in the cell bodies located in the PFC and the protein is subsequently transported into the presynaptic terminals located in the accumbens [91-93]. Future studies examining the sub cellular localization of TRPC5 protein may help

elucidate whether the channel is expressed in the presynaptic or postsynaptic terminals or in the dendrites.

TRPC channels regulation of neuronal growth and plasticity

The transport of the TRPC5 channels into the presynaptic terminals and the up-regulation of TRPC4 and 5 protein levels in the HIP of P48 compared to P0 mice suggests that these channels may be involved in neuronal development. TRPC5 is thought to be involved in regulating neuronal growth in HIP primary culture cells [61, 77]. Stimulation of cultured E18 HIP neurons by growth factors invokes a negative feedback mechanism involving the vesicular translocation and activation of TRPC5 channels in the membrane of neuronal growth cones leading to the cessation of growth [61]. This work in cell culture systems is consistent with our finding of increased developmental expression of TRPC5 in the HIP, PFC, SUB, and entorhinal cortex. It's intriguing, though speculative, that TRPC5 may serve as a switch to terminate neuronal growth as the animal reaches maturity and neural circuitry becomes fully developed.

Since growth factors modulate synaptic activity in the adult mouse HIP, TRPC5 activation by growth factors may be important for synaptic plasticity [94-97]. It's interesting that there is a fraction of TRPC5 that

remains intracellular at both P0 and P48. It's possible that TRPC channel trafficking to the surface may be subject to other signals, such as G_q-coupled receptor/PLC cascades that have been shown to activate TRPC channels and modulate neuronal plasticity in the HIP and cortex [98-100].

TRPC channel modulation of neuronal excitability in the corticolimbic system

The dense pyramidal neuron-specific corticolimbic expression of the TRPC4 and 5 channels in the brain suggests that they may be involved in modulating synaptic plasticity associated with learning and memory. TRPC5 channels are activated in vitro by mGluR receptor stimulation, which are receptors that have been implicated in neuronal plasticity in the HIP [84, 99, 101, 102]. In brain regions expressing high levels of TRPC5 mRNA and protein, others in the lab stimulated G_q-coupled receptors using the group I mGluR agonist, DHPG to induce a delayed after-depolarization (dADP) that is triggered by Ca^{2+} entry coming from a brief burst of action potentials. Action potential bursting is a neuronal output mode thought to be relevant for routing information and signaling novel and salient events [85, 86]. Work by our laboratory indicated that the DHPG-induced burst-triggered dADP in the PFC is

mediated specifically by mGluR5 receptors. Furthermore, we report that the dADP is mediated by a non-selective cation channel current that is dependent on intracellular Ca^{2+} and IP_3 receptor function, which suggests that the dADP is activated through a G_q/PLC coupled signaling pathway. The blockade of the dADP by the TRPC channel blocker, SKF96365, and the ability of the TRPC4 and 5 potentiator, La^{3+}, to induce the dADP in the absence of any group I mGluR stimulation provides evidence for TRPC4 or 5 involvement in mediating the dADP. Furthermore, we observed a substantial reduction in the dADP amplitude in response to PKC activation, which is consistent with the report that in HEK cells, TRPC5 channels are desensitized by PKC phosphorylation of T972 in the C-terminal region [103-105].

Further support for the hypothesis that the dADP is mediated by TRPC4 or 5 channels comes from patch-clamp recordings in brain regions where TRPC4 or 5 mRNA was not detected. For example, the dADP is completely absent in striatal medium spiny neurons where TRPC4 or 5 mRNA is not present, yet this is not due to lack of mGluR expression in the medium spiny neurons [106, 107]. In fact, mGluR1 and 5 receptors are expressed and their activation induces long term depression in this area [107, 108].

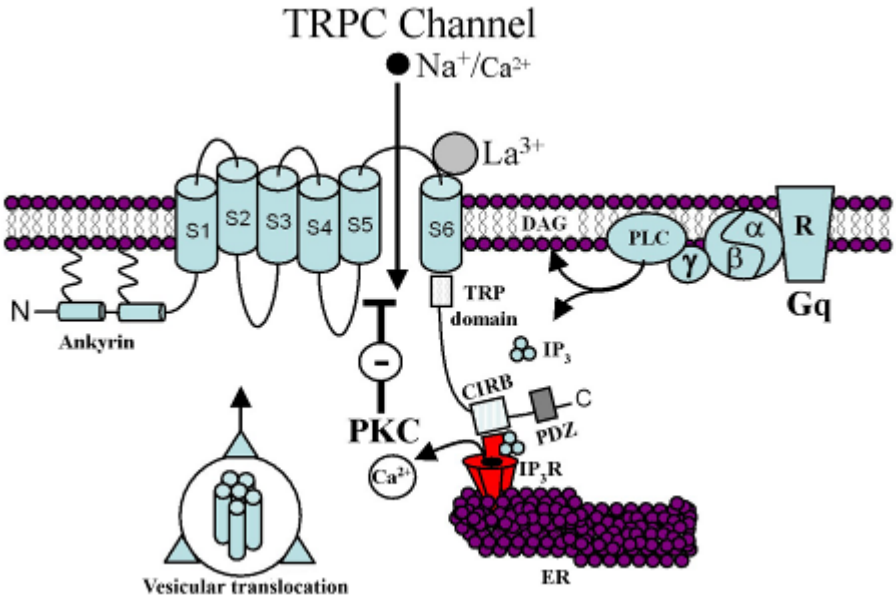

Figure 2.1 TRPC channel structure and mechanisms of activation.

Schematic showing the six transmembrane structure of the nonselective cation channels, TRPC4 and 5, and the conserved N-terminal ankryin and C-terminal TRP, CIRB, and PDZ domains. The channels are proposed to be activated by released of intracellular Ca2+ stores from the ER, conformational changes following binding of IP3 to IP3R, and vesicular translocation. The channels are inhibited by PKC and potentiated by La3+.

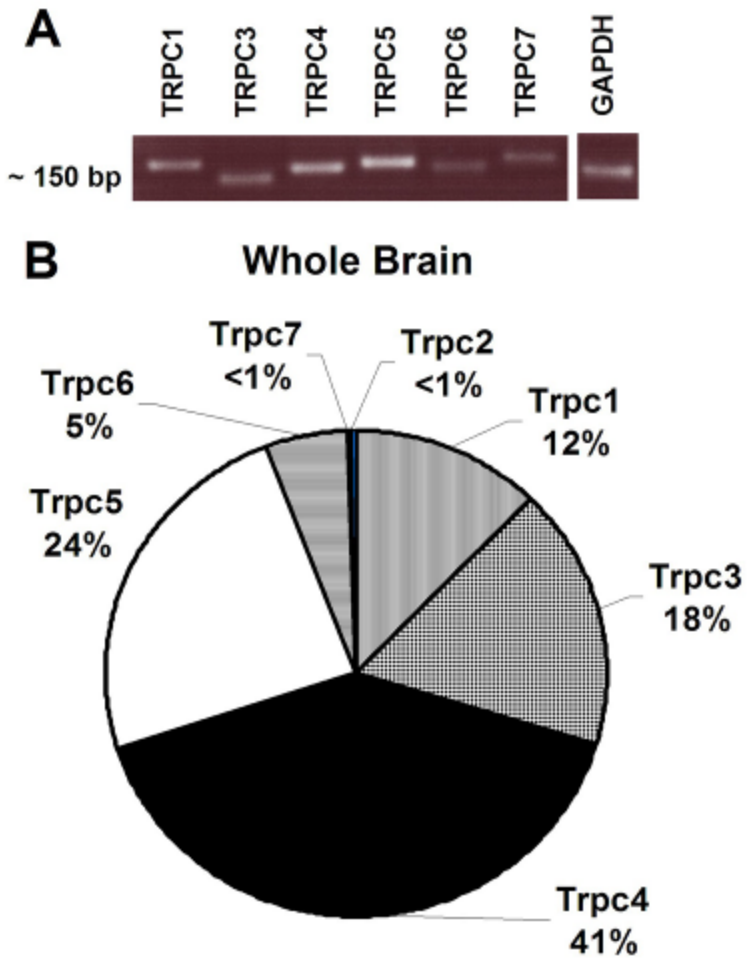

Figure 2.2 Expression of the TRPC channels in the rat brain

(A) Agarose gel showing a single product from real-time PCR of the TRPC channels. **(B)** Pie chart showing the relative expression of the seven TRPC channels in the rat whole brain determined using real-time PCR. Results were normalized to GAPDH mRNA levels (n=4)

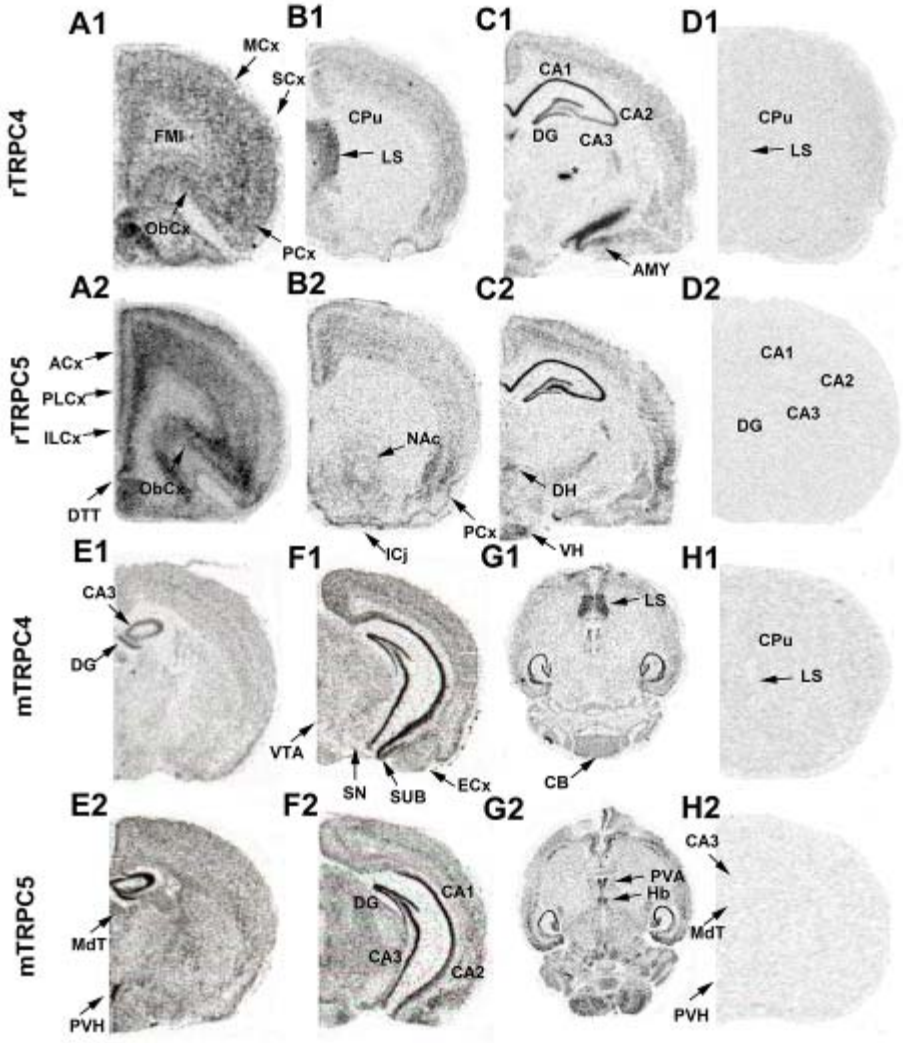

Figure 2.3 Expression of TRPC4 and 5 in the rat and mouse brain

(A1, B1, C1) In situ hybridization of TRPC4 in rat coronal brain slices (Bregma, mm): A1, 3.70; B1, 1.00; C1, 24.16. **(A2, B2, C1)** In situ hybridization of TRPC5 in rat coronal brain slices. Coordinates same as A1, B1, C1. **(D1, H1)** Unlabeled probe control in situ hybridization of TRPC4 and TRPC5 in rat coronal brain slices. **(E1, F1)** In situ hybridization of TRPC4 in mouse coronal brain slices. **(G1)** In situ hybridization of TRPC4 in horizontal mouse brain slices. **(E2, F2)** In situ hybridization of TRPC5 in mouse coronal brain slices. **(G2)** In situ hybridization of TRPC5 in mouse horizontal brain slices. **(D2,H2)** Unlabeled probe control of in situ hybridization of TRPC4 and 5 in mouse coronal brain slices.

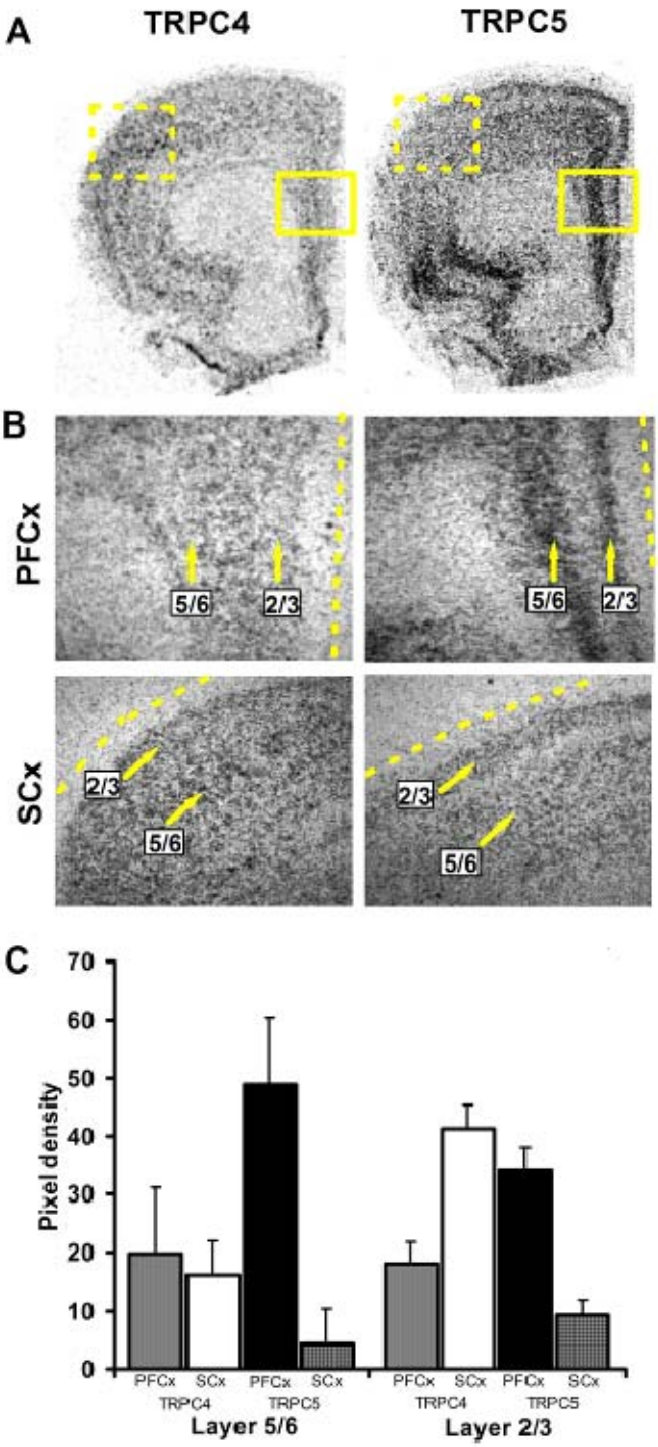

Figure 2.4 Quantification of the cRNA labelling densities of TRPC4 and 5 in the cortical layers of rat

(A) (Left) In situ hybridization of TRPC4 in a rat coronal brain slice. (Right) In situ hybridization of TRPC5 in a rat coronal brain slice. Scale bars in mm. **(B)** (Upper) Magnification of TRPC4 5 cRNA labeling in the PFC layers. (Lower) Magnification of TRPC4 5 cRNA labeling in the SCx layers. **(C)** Quantification of the cRNA labeling densities of TRPC4 and 5 in the PFC and SCx layers expressed as a % change from background (n = 3). Scale bars in µm.

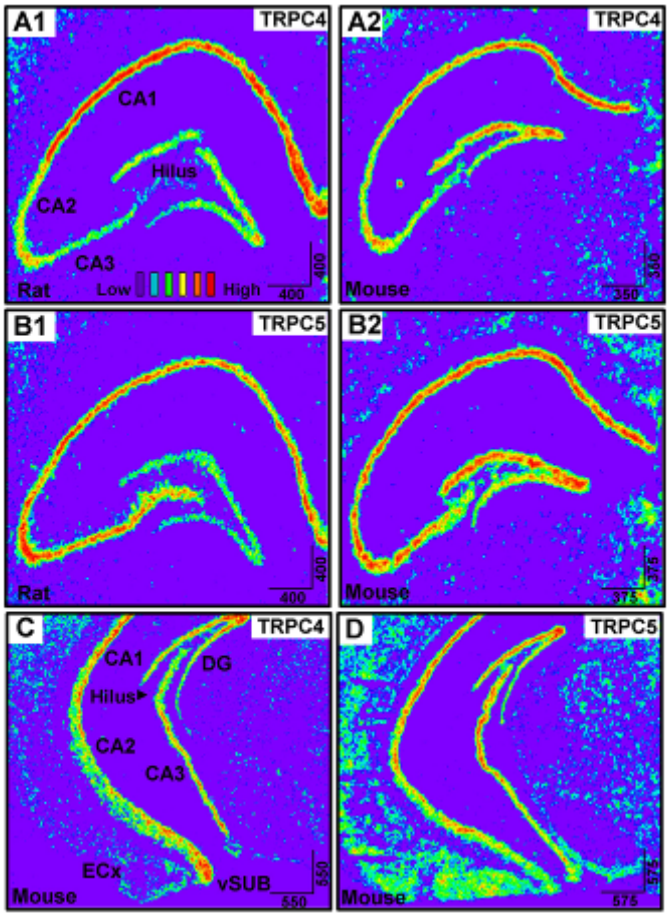

Figure 2.5 Expression of TRPC4 and 5 in the hippocampal formation

(A1, A2) cRNA labeling of TRPC4 mRNA in rat and mouse CA1-3, hilus, and dentate gyrus. **(B2, B2)** cRNA labeling of TRPC5 mRNA in rat and mouse CA1-3, hilus, and dentate gyrus. **(C, D)** cRNA labeling of TRPC4 and 5 in the ventral mouse hippocampal formation including the entorhinal cortex and ventral subiculum

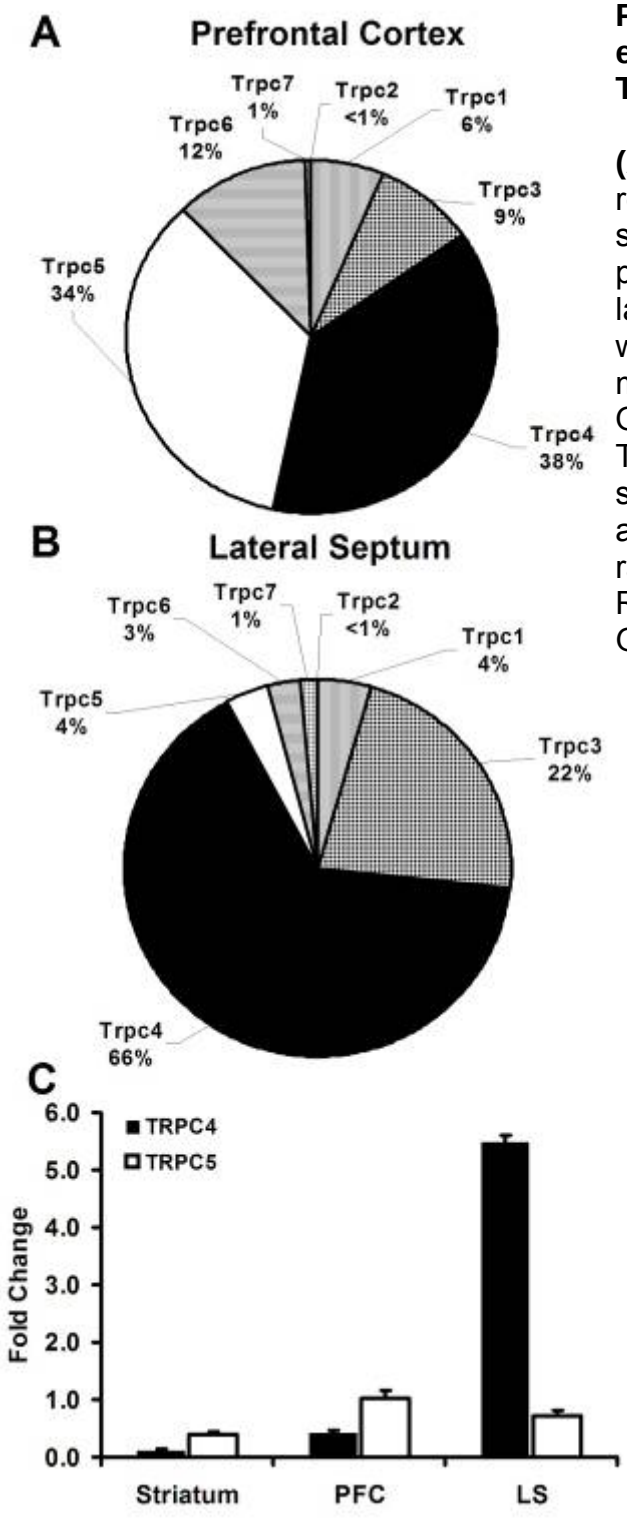

Figure 2.6 Region-specific expression of TRPC4 and TRPC5 mRNA

(A, B) Pie charts showing the relative expression of the seven TRPC channels in rat prefrontal cortex **(A)** and lateral septum **(B).** Results were normalized to GAPDH mRNA levels (n=4). **(C)** Quantifica tion of TRPC4 and TRPC5 mRNA levels in the striatum, prefrontal cortex, and lateral septum of adult rat using real-time PCR. Results was normalized to GAPDH mRNA levels.(n=4)

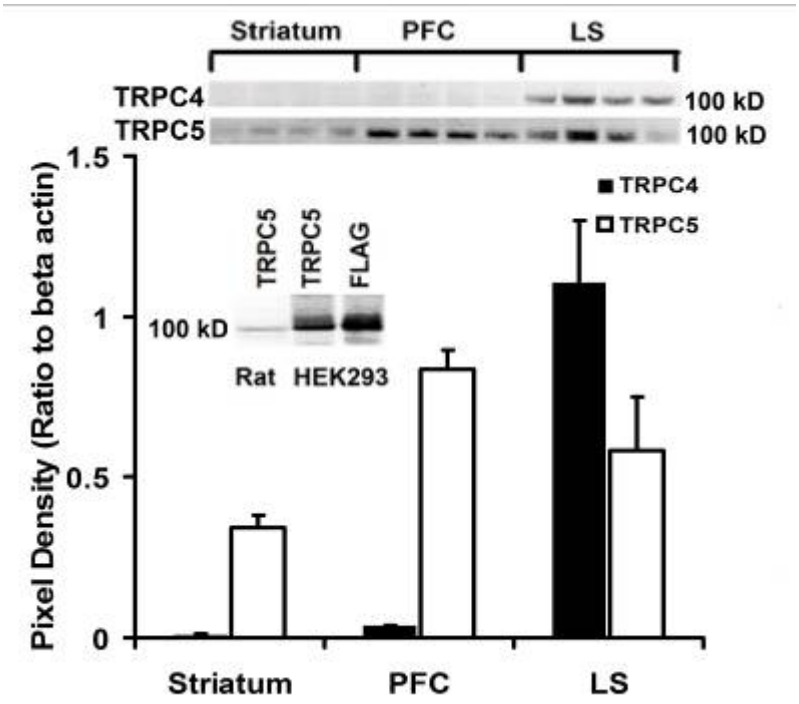

Figure 2.7 Region-specific expression of TRPC4 and TRPC5 protein . Graph shows the quantification of TRPC4 and TRPC5 protein levels in the striatum, prefrontal cortex, and lateral septum in the adult rat. Results was normalized to b-actin protein levels (Top) Representative bands of the immunoblots (n = 4). (Left inset) Immunoblot of rat brain tissue lysates and TRPC5 expressing HEK293 cells blotted with a-TRPC5 and of TRPC5 expressing HEK293 cells blotted with a-Flag

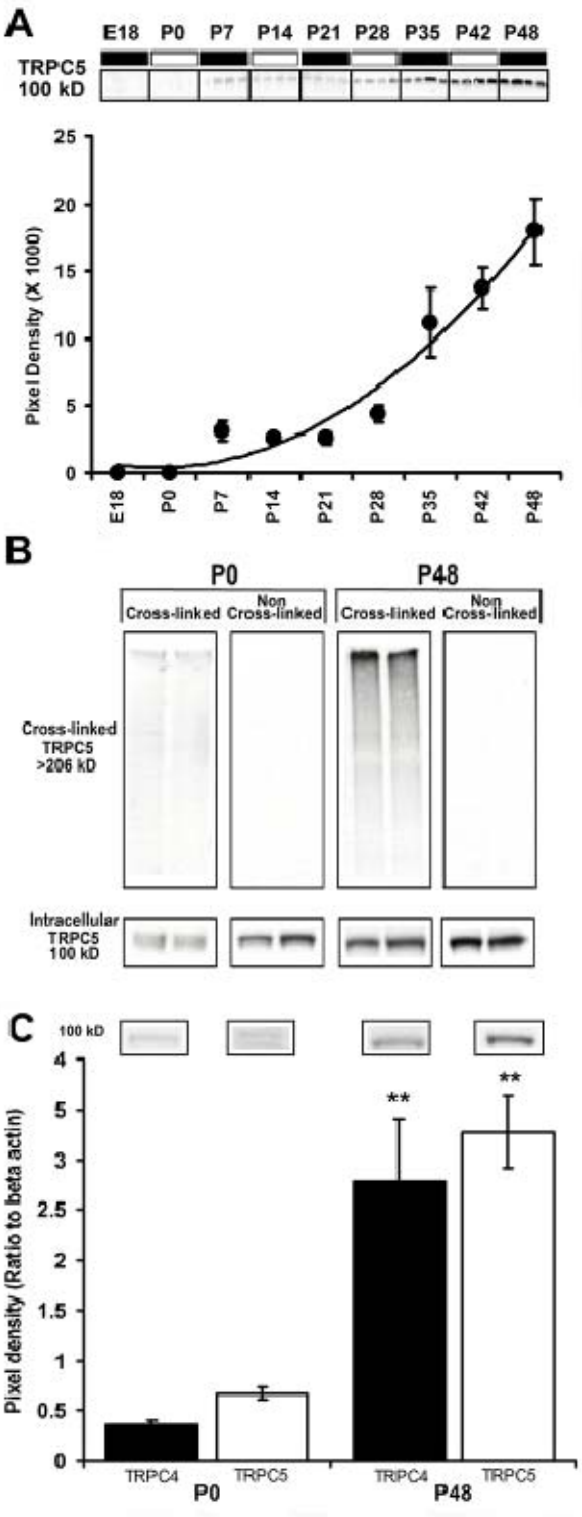

Figure 2.8 Developmental and surface expression of TRPC5 protein in the mouse hippocampus.

(A) Quantification of TRPC5 protein levels in the HIP of E18 through P48 day old mice. (n = 4) (Top inset) Representative bands from the immunoblot showing TRPC5 expression in the HIP of E18-P48 mice. **(B)** Representative bands from immunoblots showing surface (cross-linked) and intracellular TRPC5 protein in P0 and P48 mice (n = 6). **(C)** Quantification of TRPC4 and 5 protein levels in the HIP of P0 and P48 mice (n= 6; for P0, p = 0.002; for P48, p = 3.16e-5) (top inset) Representative bands from an immunoblots showing TRPC4 and TRPC5 protein in the HIP of P0 and P48 mice (n= 6)

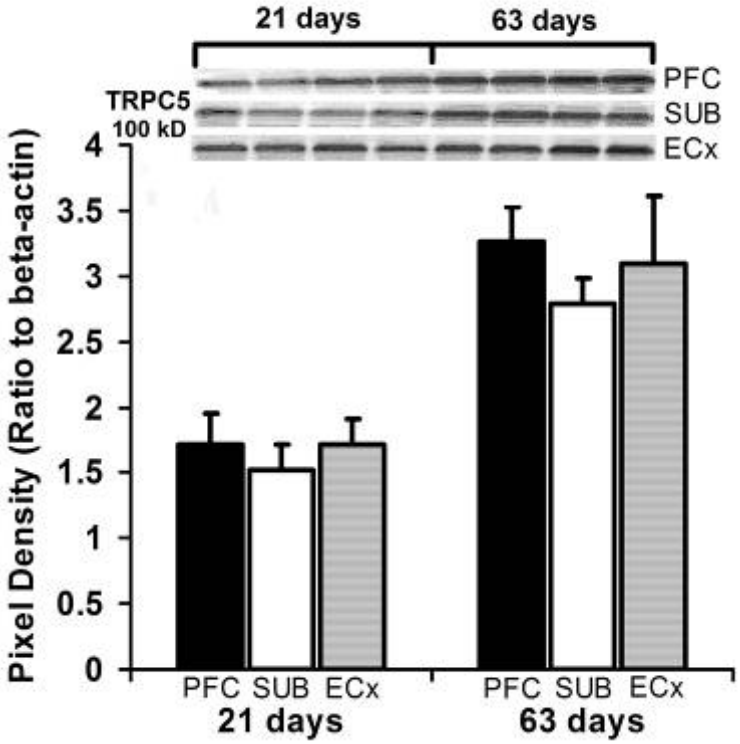

Figure 2.9 Developmental expression of TRPC5 protein in the rat

Quantification of TRPC5 protein levels in the prefrontal cortex (PFC), subiculum (SUB), and entorhinal cortex (ECx) in 21 and 63 day old rats. Results were normalized to b-actin protein levels. (Top left Inset) Representative bands of the immunoblot (n = 4)

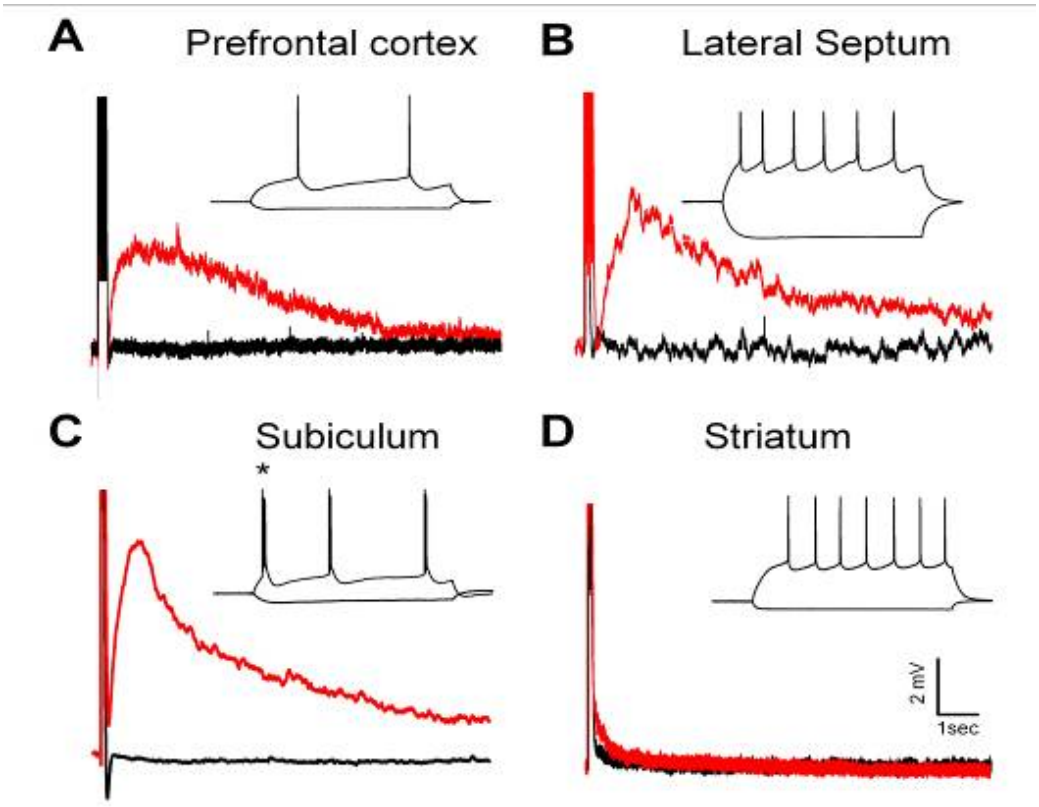

Figure 2.10 Induction of a nonselective cation current-mediated dADP in brain regions expressing high levels of TRPC mRNA and protein.

(A) The group 1 mGluR agonist DHPG (50 uM; Red trace) induces a burst-triggered dADP in the prefrontal cortex (n = 6; p,0.05), **(B)** Lateral septum (n = 4; p,0.05) and **(C)** Subiculum (n = 6; p,0.05). Black traces show the baseline before bath application of DHPG (A–D). The inset shows the response to a suprathreshold 600ms square wave input at 200pA or hyperpolarizing input at 2100 pA in each cell region (A–D). **(D)** The lack of a DHPG-induced burst-triggered dADP in medium spiny neurons from the dorsal striatum (n = 6) or nucleus accumbens (n = 4), together labeled Striatum. All recordings were held between 265 and 270mV during the baseline and DHPG application. There were no qualitative differences in the dADP that varied according to the holding potential during the DHPG application in any brain region. *These experiments were performed by Kyriaki Sidiropoulou (RFUMS) and Emin Ozkan (UTSW).*

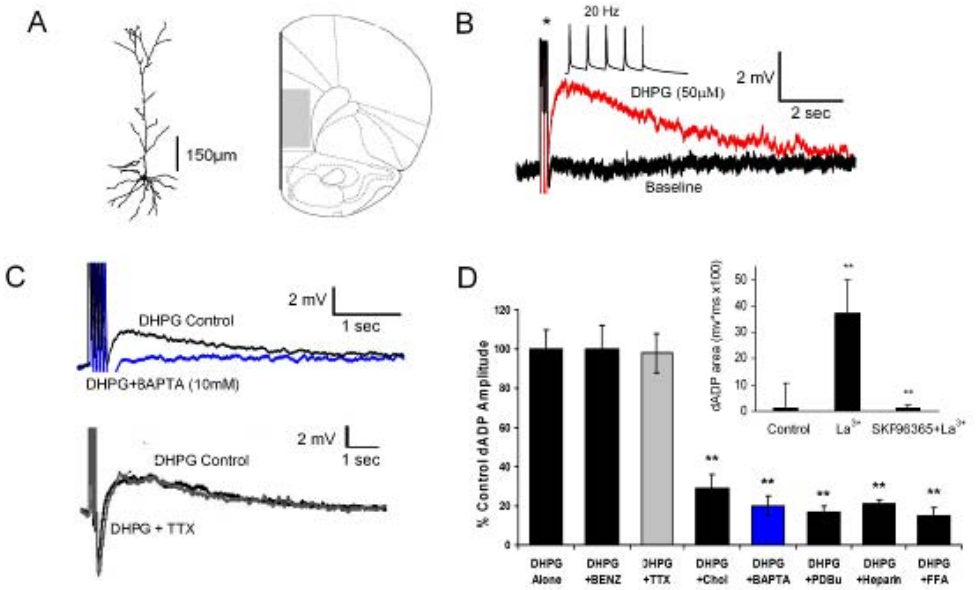

Figure 2.11 Deep layer 5 pyramidal PFC neurons show a burst-induced nonselective cation current-mediated slow afterdepolarization following activation of group 1 mGluR receptors.

(A) Camera lucida reconstruction of a deep layer 5 pyramidal neuron from the PFC (left). (Right) schematic showing the PFC (infralimbic and prelimbic), shaded area where recordings were taken. **(B)** The group 1 mGluR agonist DHPG (50uM) induced a 20Hz burst-triggered (Inset) dADP (Red) compared to baseline before DHPG application to the bath. **(C)** (top) Intracellular Ca2+ chelation with BAPTA (10 mM; Blue) for 10 min after establishing whole-cell configuration substantially reduced the dADP induced by a 20 Hz burst and DHPG (50 uM)+BAPTA (10mM) immediately after establishing whole-cell configuration. (bottom) Bath application of the voltage-gated Na+ channel blocker, TTX (1 mM, Gray) for 10 min and elimination of action potentials at intensities 10 times the rheobase had no effect on the dADP induced by a 20 Hz burst and DHPG (50 uM)+TTX (1 mM). **(D)** The effects of the Na+/Ca2+ exchanger blocker benzamil (100 mM); Na+ ion 80% replacement with choline chloride; voltage-gated Na+ channel blocker, TTX (1 mM); intracellular Ca2+ chelation with BAPTA (10 mM); PKC activation with PdBU (1 mM),; IP3 receptor blockage with heparin (2 mg/ml); and nonselective cation channel blockade with flufenamic acid (100 mM) on the DHPG-(50 mM) and burstinduced dADP. (n.5, **p,0.01) (Inset) The TRPC4/5 potentiator, La3+ (100 mM) induced a small burst triggered (5 action potentials @ 20 Hz) dADP in the absence of mGluR activation and was blocked by the broad spectrum nonselective cation channel blocker, SKF96365 (100 mM). (n.5, **p,0.01). *These experiments were performed by Kyriaki Sidiropoulou (RFUMS) and Emin Ozkan (UTSW).*

Gene	Acc. #	Forward primer	Reverse primer
TRPC1	NM 053558	5' AGGTGAAGGAGGAGAACACCTTG 3'	5' CCATAAGTTTCTGACAACCGTAGTCC 3'
TRPC2	NM 022638	5' AGAAGCTGGGCAATTTCAACG 3'	5' CGATGAGCATGTTGAGTAGCACA 3'
TRPC3	NM 021771	5' CCACATGCAGTGAGACTTTGACTC 3'	5' AGGCCAACCTTGGGATCATTT 3'
TRPC4	NM 053434	5' AATTACTCGTCAACAGGCGGC 3'	5' CACCACCACCTTCTCCGACTT 3'
TRPC5	NM 080898	5' AAGTTTCGAATTTGAGGAGCAGATG 3'	5' AATCTCTGATGGCATCGCACA 3'
TRPC6	NM 053559	5' GCCTCATGATTATTTCTGCAAGTGTAC 3'	5' TGAACTCTTTCTCAATGTTGGCAA 3'
TRPC7	XM 225159	5' ATGACGAGTTCTATGCCTACGACG 3'	5' TTGTAGGCATTCATACGGGAGC 3'

Table 2.1 Real-time PCR primer sequences for the seven TRPC channels in rat.

Table showing the left and right real-time PCR primer sequences for the seven TRPC channels in rat and GAPDH and the NCBI accession numbers for each gene.

	TRPC4	TRPC5		TRPC4	TRPC5
Amygdala	++	+	Motor cortex		
Caudate putamen	0	0	Layer 1	0	0
Cerebellum			Layer 2/3	++	++
Molecular layer	0	+	Layer 5/6	++	++
Purkinje cell layer	++	++	Nucleus accumbens		
Granule cell layer	++	++	Core	0	+
Corpus collosum	0	0	Shell	0	++
Entorhinal cortex	++	+++	Orbitofrontal cortex	++	+++
Habenula	++	+++	Piriform cortex	++	+++
Hippocampus			Prefrontal cortex		
CA1	+++	+++	Layer 1	0	0
CA2	+++	+++	Layer 2/3	++	+++
CA3	++	+++	Layer 5/6	++	+++
Hilus	+	+++	Somatosensory cortex		
Dentate gyrus	++	++	Layer 1	0	0
Hypothalamus			Layer 2/3	++	++
Dorsal	0	+	Layer 5/6	++	++
Ventral	++	++	Subiculum	+++	++
Paraventricular	+	+++	Substantia nigra	+	0
Islands of Caleja	0	+++	Tenia tecta	+++	++
Lateral septum	+++	0	Thalamus	0	++
Medial Septum	+	0	Rank 0 < + < ++ < +++		

Table 2.2 Qualitative analysis of TRPC4 and TRPC5 mRNA expression in the rodent brain.

Qualitative analysis of the in situ hybridizations of TRPC4 and TRPC5 mRNA in rat and mouse coronal brain sections (from Figure 3). (0) no expression, (+) little expression (++) moderate expression, (+++) high expression. (n = 3)

CHAPTER THREE: TRPC5 CHANNEL REGULATION OF PREFRONTAL CORTICAL EXCITABILITY AND BEHAVIORAL OUTPUT

Summary

Drug addiction is a disease that is influenced by both genetic and environmental factors that result in altered excitability of neurons within the reward circuitry that may be associated with drug dependence. The prefrontal cortex is one brain region that is involved in modulating behavioral responses to the rewarding effects of drugs; and alterations in prefrontal cortical excitability mediate the uncontrollable urges for drug seeking associated with drug addiction. Using AAV mediated knock-down of TRPC5 in the prefrontal cortex of TRPC5flx mice, we show that TRPC5 channels are necessary for a G_q-coupled, burst-activated delayed after depolarization (dADP) that induces a depolarizing wave that is capable of producing persistent action potential output. We show that loss of TRPC5 in the PFC results in an increase in the locomotor activating and rewarding effects of cocaine. These studies identify the TRPC5 channels as

important for modulating neuronal excitability in the PFC and the behavioral responses to cocaine.

Introduction

The PFC is an area of the brain involved in higher order cognitive functions, such as decision-making, reasoning, and working memory. People with lesions in the PFC have impaired decision-making and impulsivity [109]. PFC functioning is impaired in cocaine addicts, which is manifested in their inability to make decisions [6, 20]. Genetic traits and environmental factors can influence PFC excitability [2, 4, 5], and repeated cocaine alters the excitability of the PFC by biasing neurons towards strong inputs, such as those associated with drug cues, which may diminish its cognitive function [21]. Understanding the mechanism underlying how PFC excitability influences the behavioral responses to psychostimulants is fundamental to learning how to reverse these neuroadaptations in order to treat addiction. In our studies, we identified an intrinsic mechanism for neuronal excitability and persistent activity in the PFC which is modulated by chronic cocaine and dopamine stimulation that may be involved in mediating drug seeking in cocaine addicts.

We have previously shown that layer 5 pyramidal neurons in the PFC exhibit a dADP, which is a G_q-coupled receptor mediated period of heightened excitability following brief bursts of action potentials [32, 46]. The dADP is induced by activation of G_q-coupled receptors, such as mGluRs or muscarinic acetylcholine receptors and is mediated by subsequent activation of a non-selective cation channel, which is likely to be a TRPC5 channel [32, 46]. Induction of the dADP in the PFC results in an increase in the firing frequency for several seconds following a single burst of 2-5 action potentials delivered at 5X20Hz [32]. The ability of deep layer pyramidal neurons within the PFC neurons to turn sub threshold inputs into persistent firing output may be a way for neurons to temporarily hold salient reward related information. The dADP is modulated by DA in which activation of the D1R/PKA signaling cascade reduces the dADP amplitude. D2R activation on the other hand has no effect [32]. In animals treated repeatedly with cocaine the modulatory role of D1R activation is eliminated.

The reduction in the dADP and loss of D1R modulation following repeated cocaine treatment may serve to increase the input signal to noise ratio which magnifies a bias towards very strong inputs like those associated with drug cues, while simultaneously preventing smaller, sub threshold inputs from influencing neuronal output. The effect of this

lasting intrinsic plasticity of the dADP and D1R modulation may be to promote drug-seeking behavior alter short-term working memory processing.

Pharmacological and expression data pointed towards the TRPC5 channel as a candidate for mediating the dADP in the PFC [46]. In this study, we sought to identify the TRPC5 channel as the non-selective cation channel underlying the dADP and to implicate a role for TRPC5 in modulating drug seeking behaviors.

Experimental procedures

Generation of conditional TRPC5 knock-out mouse lines

Floxed TRPC5 mice were obtained from Lutz Birnbaumer (NIEHS) where loxP sites flank exon 5 of the TRPC5 gene. Recombination at the loxP sites following cre recombinase expression results in loss of exon 5 and a frame shift, resulting in a premature stop codon in the first position of exon 6. The floxed TRPC5 mice were crossed with a CamKII-cre mouse line where cre expressed in controlled by the CamKII promoter as shown by Luikart *et al* [110]. The expression of Cre turns on at ~P21 and peaks at ~P60.

Region-specific knock-down of TRPC5

Adult (8-12 weeks), male mice were anesthetized with ketamine/xylazine and fitted into a stereotaxic apparatus. The scalp was shaved and cut with a sterile scalpel. For the prefrontal cortex, small holes were drilled with a Dremel tool at the following coordinates from bregma: anterior/posterior= +1.9, medial/lateral= +/- 0.3, dorsal/ventral= -2.2, -2.0. The dorsal/ventral coordinate was calculated from the skull surface. One uL of AAV-cre-GFP or AAV-creΔ-GFP was injected with a 5 µL Hamilton syringe (Hamilton Company, Reno, NV) at a rate of 0.1 µL/ 45 seconds at the dorsal/ventral coordinate of -2.2. After one µL was dispensed, the needle was raised to the dorsal/ventral coordinate of -2.0 and an additional 1 µL was dispensed at a rate of 0.1µl/45 seconds. The needle was allowed to rest for 5 minutes post injection. The needle was removed and the scalp was sutured closed and treated with antibiotic. The animals were allowed to recover at least 4 weeks before the start of any behavioral, physiological, or molecular procedure. The accuracy of the injection site was confirmed post-hoc by in situ hybridization for Cre mRNA.

In situ hybridization of TRPC5

The in situ hybridization experiments were carried out according to previous methods [46, 79]. Slide-mounted, fresh-frozen tissue sections were used. Halothane-anesthetized animals were decapitated and their brains removed and rapidly frozen on dry ice. Coronal, fresh-frozen sections were cut at 14 µm in a cryostat, thaw-mounted onto Superfrost Plus (Fisher Scientific, Pittsburgh, PA, USA) glass slides, and stored at − 80°C until use. The sections were fixed in 4% paraformaldehyde, and washed in PBS, PBS+glycine, and 0.25% acetic anhydride in 0.1M triethanolamine. The sections were then dehydrated and delipidated in 50%, 75%, 95%, and 100% EtOH and 100% chloroform. Sections were hybridized for 18 hours at 60°C in hybridization buffer containing deionized formamide (40%), dextran sulfate (10%), 1X Denhardt's solution, 4x SSC, denatured and sheared salmon sperm DNA (1 mg/mL), yeast tRNA (1 mg/mL), dithiothreitol (10 mM), and either S^{35}- labeled RNA probe (S^{35}, Perkin Elmer, Waltham, MA, USA) or an identical, unlabeled RNA probe as a control. The probes used was a 300 bp S^{35}-labeled probe for TRPC5 which were transcribed using T3 polymerase (Ambion, Austin, TX, USA) from the TRPC4 and 5 pBluescript clones described above and purified on a spin purification column. The counts per million (cpm) of the probes were assessed using a scintillation counter and added to the hybridization

buffer at a concentration of $10x10^6$ cpm/ml. After hybridization, the sections were washed in sodium citrate buffer (SSC) + sodium thiosulfate and placed on film for 3-7 days before development.

Cocaine conditioned place preference

Adult male CamKIIcre- or TRPC5flx mice injected with AAV-cre-GFP or AAV-creΔ-GFP were weighed and marked one day prior to the start of the behavioral procedure. The conditioned place preference chamber consisted of two large compartments that differed in both visual and tactile cues, separated by a smaller center compartment (Med Associates, St. Albans, Vermont). The two large compartments were equipped with six photobeams for monitoring movement, and the center compartment with two photobeams. Prior to conditioning, animals were placed into the center chamber and allowed to freely explore the three compartments for 20 min. The animals were paired with a compartment for conditioning so that there was no bias for either side. On training days one through four, partitions were used to block off the two large compartments and each animal was intraperitoneally injected with either cocaine or saline (3.5 or 7.5 mg/kg alternate over 4 days) and placed into its respective compartment for 30 min. On the fifth day, the partitions

were lifted and the animals were placed into the center compartment. The animals were allowed to move freely between the three compartments for 20 minutes and the time spent in each compartment was recorded. The preference for the cocaine-paired side was calculated as the time spent in the cocaine-paired side minus the time spent in the saline-paired side.

Locomotor response to cocaine

Adult male TRPC5flx-CamKII-Cre or TRPC5flx mice injected with AAV-cre-GFP or AAV-creΔ-GFP were weighed and marked one day prior to the start of the behavioral testing. Mice were habituated to the testing room for one hour prior to the start of testing on each day of testing. The locomotor chambers consisted of a cage identical to the home cage placed into a box equipped with five photobeams for recording movement. Ambulatory movement was defined as two consecutive beam breaks. On each day of testing, the mice were placed into the locomotor chambers and movement was recorded for one hour during habituation to the chambers. On days one and two, after one hour of habituation to the chamber, the animals were injected with saline and placed back into the chambers where movement was recorded for an additional hour. Animals were removed from the chambers and placed back into their home cages.

On days three, five, and seven, after one hour of habituation to the chamber, the animals were given ascending doses of cocaine of 5, 10, and 20 mg/kg. A saline injection was interleaved between the cocaine injections to prevent sensitization.

Contextual fear conditioning

Adult male TRPC5flx mice injected with AAV-cre-GFP or AAV-creΔ-GFP were weighed and marked one day prior to the start of behavioral testing. Mice were habituated to the testing room for one hour prior to the start of testing on each day of testing. The fear conditioning chamber is a clear polycarbonate chamber with a removable grid floor (Med Associates, St. Albans, Vermont). For contextual conditioning, each mouse was placed into the chamber and allowed to habituate to the chamber for two minutes before being delivered three 0.5 mA shocks separated by one minute. The freezing behavior was hand recorded every ~3 seconds during the five minute period. Freezing behavior was defined as complete lack of movement besides respiration. After conditioning, the mouse was removed from the chamber and placed back into its home cage. On day two, acquisition of the fear response was measured by placing each mouse back into the fear conditioning chamber

and recording the freezing behavior every ~3 seconds for five minutes. Extinction learning was measured on days three through eight by recording the freezing behavior for five minutes on each day.

Adeno-associated virus production

The plasmids pAAV-cre-GFP and pAAV-creΔ-GFP were obtained from Dr. Ilya Bezprozvanny (UT Southwestern Medical Center, Dallas, TX). The pAAV-creΔ-GFP plasmid contained a cre recombinase gene in which the first 400 bp of the gene have been deleted, rendering the protein nonfunctional. The baculovirus containing the gene of interest was generated by transfecting the pAAV-Cre-GFP and pAAV-CreΔ-GFP plasmids into SF9 culture cells and harvesting 4-5 days post transfection. The baculoviruses were then amplified in SF9 culture cells and stored at 4ºC, protected from light, until use. The AAV virus was then produced by infecting SF9 cells with the amplified AAV baculoviruses, along with the baculoviruses: BacAAVCAP, and BacAAVRep, which are the genes necessary for replication and packaging of the virus. After 72 hours of incubation at 27ºC, the cells were harvested by centrifugation and the pellets resuspended in hypotonic buffer. A French press high pressure homogenizer was used to fractionate the cells and release the AAV virus.

Disruption was carried out at 600 psi of pressure for 2-3 minutes. The cell lysates were then centrifuged and the resulting supernatant was treated with benzonase (Sigma Aldrich, St Louis, MO) at 50 U/ml for 30 min at 37ºC. Following treatment, the cell lysate was clarified by centrifugation and the supernatant was diluted to a final volume of 30 ml with hypotonic buffer. The AAVs were purified by an iodixanol gradient (Sigma Aldrich, St. Louis, MO). Tubes were layered with 15-60% iodixanol, starting with the lightest solution and adding each successive layer under the previous layer at the bottom of the tube. The viral lysate was layered on top of the iodixanol layers. The tubes were centrifuged at 350,000g for 75 min at 18ºC. The 40% layer at the 40-60% interface was collected. The virus was further purified through a Mustang-Q membrane ion exchange (Acrodisc unit, Pall Corporation, East Hills, NY). The virus from the iodixanol gradient was diluted 1:5 with 20mM Tris buffer, pH8.0. The diluted virus was run through the acrodisc at a rate of 1-4 ml/min. The acrodisc was washed with 20 ml of 20 mM Tris buffer, pH8.0. The virus was then eluted with 500 uL elution buffer by passing 2-3 drops of elution buffer through the membrane, then incubating the membrane in elution buffer for 5 min at RT. The remaining elution buffer was run through the membrane twice, each time incubating for 5 min at RT before passing. The buffer was then expelled from the membrane and a second aliquot of

500 uL of elution buffer was passed through the membrane. Both elution fractions are retained, with the first fraction being more concentrated than the second. The concentration of the viruses was determined through infection with dilutions of the virus into HEK293 cells. The infectious particles per ml were calculated by the formula: TU/ml = (# green cells)*(dilution factor)*100.

Micro-dissections of brain regions

For all brain regions, slices were taken from male rats and mice (8-10 weeks old for all experiments with the exception of the developmental and surface expression experiments where the ages are specified). Animals were anesthetized by isoflurane inhalation, decapitated, and their brains quickly removed and placed into ice-cold artificial cerebral spinal fluid (ACSF). The brain was mounted and coronal slices (500 μm for rat, 250 μm for mice) were cut using a vibratome (Leica, Nussloch, Germany). Each region was dissected while the slices were in ice chilled ACSF and immediately frozen on dry ice upon extraction.

RNA extraction and reverse transcription

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the protocol. The RNA was resuspended in DEPC-treated H_2O and DNase-treated to eliminate any DNA contamination using DNA-*free* (Ambion, Austin, TX, USA). The concentration of the RNA was determined by UV absorbance using a Nanodrop spectrophotometer. The RNA was reverse transcribed with random hexamer primers (Invitrogen, Carlsbad, CA, USA) using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNA was purified on a spin purification column (Qiagen, Valencia, CA, USA) and the concentration was determined by UV absorbance using a Nanodrop spectrophotometer.

Quantitative real-time PCR

The real-time primers were designed as 23-25 oligonucleotide sequences that amplify a 100-150 base pair gene product. Primers were checked against all known rat gene sequences to ensure specificity and were designed to maximize detection of all known splice variants of the TRPC genes. The real-time PCR reaction was optimized by running a standard curve for each primer set in dilutions of whole brain rat cDNA (1,

0.5, 0.25, 0.125, and 0.0625), with 100 ng of cDNA in the 1X concentration. From this standard curve, the efficiency for each gene was determined using the slope and R^2 value according to the method proposed by Pfafl. The reactions were carried out using SYBR Green PCR Master Mix (Ambion, Austin, TX, USA) in a Stratagene MXP3000 real-time PCR thermal cycler (Stratagene, La Jolla, CA, USA). The amplification plots, melting curves, and standard curve were assessed for primer quality and the product was run on a gel to ensure amplification of a single product of the correct size. The TRPC channel expression was assessed in the different brain regions by running real-time PCR reactions of 100 ng of the cDNA transcribed from total RNA isolated from the dissections. In all reactions cyclophilin was used as a positive control to which the results were normalized.

Results

Generation and examination of a TRPC5 knock-out mouse line

We obtained a full TRPC5 knock-out line from Dr. Lutz Birnbaumer at NIEHS. First, the TRPC5flx mouse line was created in which loxP sites

flank exon 5 of the TRPC5 gene. Expression of Cre recombinase in the TRPC5flx mouse results in excision of exon 5 and frame shift that creates a premature stop codon in the first position of exon 6 (Figure 3.1). To create a full knock-out, the TRPC5flx line was crossed with a Sox2-Cre mouse line which expresses Cre in the germ line. Knock-out of TRPC5 was confirmed using real-time PCR for TRPC5 mRNA (Figure 3.2). Upon confirmation of knock-out, patch clamp recordings were performed by Ming-Hu Han to examine the dADP in pyramidal neurons of the PFC in TRPC5+/+ and -/- animals. We found no reduction in the dADP amplitude in PFC neurons of TRPC5-/- compared to TRPC5+/+ PFC mice (data not shown).

We suspected that the continued presence of the dADP in the PFC of TRPC5-/- animals may be due to compensation by other TRPC channels, therefore we compared the expression levels of the TRPC channels expressed in the PFC (TRPC1, 3, 4, 5, and 6) of the TRPC5+/+ and -/- mice. Results show a complete an increase in the levels of TRPC3 mRNA in the TRPC5-/- animals compared to the TRPC5+/+ animals (Figure 3.2). Levels of TRPC1, 4, and 6 mRNA were not changed in the TRPC5-/- compared to the TRPC5+/+ animals (Figure 3.2).

Generation of a PFC-specific TRPC5 knock-out model

We turned to a conditional TRPC5 knock-out strategy in order to circumvent difficulties with compensation by TRPC3 seen in the TRPC5 knock-out mouse line. We sought to selectively and temporally knock down TRPC5 channels in the PFC to determine whether TRPC5 is responsible for the dADP. In order to achieve this region specific knock-down, an adeno-associated virus expressing Cre recombinase fused with green fluorescent protein (AAV-Cre-GFP) was delivered into the PFC of TRPC5flx mice. Expression of Cre recombinase mRNA and subsequent loss of TRPC5 mRNA in the PFC was confirmed via in situ hybridization four weeks after injection (Figure 3.3). Results show a knock-down of TRPC5 in areas where AAV-Cre-GFP was delivered (Figure 3.3).

Previous studies have shown that, like many AAV serotypes including 1, 7, and 8, the AAV serotype 5 used in this study undergoes retrograde transport in the CNS [111, 112]. However, no expression of Cre recombinase was seen in the VTA or SUB of AAV-CreΔ-GFP or AAV-Cre-GFP injected animals, suggesting that the AAV does not undergo retrograde transport (Figure 3.4).

Loss of burst-triggered delayed after-depolarization following knock-down of TRPC5 in the prefrontal cortex

Adult TRPC5flx mice were bilaterally injected with AAV-Cre-GFP or a virus containing a mutant, non-functional Cre (AAV-CreΔ-GFP) into the PFC. Four weeks after injection, patch clamp recordings in PFC neurons expressing GFP were performed by Ming-Hu Han to test for loss of the dADP in response to brief bursts of five action potentials at 20Hz in the presence of the muscarinic acetylcholine receptor agonist, carbachol (10 μM). Cells expressing AAV-Cre-GFP showed robust knock-down of the dADP amplitude in contrast to cells expressing AAV-CreΔ-GFP, which showed no difference in the dADP amplitude compared to cells not expressing AAV-CreΔ-GFP in the same slice (Figure 3.5). The lack of difference in the AAV-CreΔ-GFP infected slices shows that neither infection by the virus or GFP is responsible for the reduction in the dADP amplitude. Non-infected cells in slices injected with AAV-Cre-GFP showed no difference in the dADP amplitude compared to AAV-CreΔ-GFP controls, showing that the loss of the dADP is intrinsic within the cell and does not translate across all neurons in the slice. (Figure 3.5)

Generation of a conditional TRPC5 knock-out line

In order to obtain a more generalized knock-out of TRPC5 in a behavioral mouse model, we crossed the TRPC5flx line with the CamKII-Cre mouse line which shows a frontal expression pattern of cre recombinase, turning on at ~P23 and peaking at ~P60 [110]. Knock-down of TRPC5 in the TRPC5flx-CamKII Cre line was confirmed using in situ hybridizations for Cre and TRPC5 cRNA in Cre+ and Cre- mice older than P60. Cre was expressed in areas such as the frontal cortex, striatum, and HIP (Figure 3.3). Corresponding in situ hybridizations for TRPC5 showed knock-down of TRPC5 cRNA in the frontal cortex and the dentate gyrus of the HIP (Figure 3.3). Knock-down of TRPC5 was confirmed using real-time PCR for TRPC5 mRNA in microdissections of the PFC, dorsal striatum, nucleus accumbens, and HIP. The real-time PCR primers were designed to amplify TRPC5 mRNA containing exon5, thereby excluding any TRPC5 mRNA which had undergone exon5 splicing by Cre. Results show ~50% knock-down of TRPC5 mRNA in the PFC; however, there was no reduction of TRPC5 mRNA in the dorsal striatum, NAc, or HIP (Figure 3.3).

To examine possible compensation by other TRPC channels in response to the loss of TRPC5, the expression of the TRPC channels that are expressed in the PFC and HIP (TRPC1, 3, 4, and 6) was examined

using real-time PCR. Results show no significant compensation of TRPC1, 3, 4, or 6 channels in the PFC or HIP, though there was a non-significant trend for an increase in the levels of TRPC4 in Cre+ compared to Cre- mice (Figure 3.6).

Acute responses to cocaine

Acute doses of cocaine induce hyper locomotive activity in animals [113]. We examined the acute responses to cocaine in mice lacking TRPC5 in the frontal cortex by performing a locomotor dose response to 5, 10, and 20 mg/kg i.p. injections of cocaine in TRPC5flx-CamKII Cre- and Cre+ mice. Results show no difference in the ambulatory locomotor response between Cre- and Cre+ mice (Figure 3.7).

However, when we examined the ambulatory movement to the same doses of cocaine in animals that were bilaterally injected with AAV-Cre-GFP or AAV-CreΔ-GFP into the PFC, we found that mice injected with AAV-Cre-GFP, resulting in knock-down of TRPC5 in the PFC, showed significantly more locomotor activity at high doses of cocaine (20 mg/kg) than those injected with AAV-CreΔ-GFP (Figure 3.7)..

Cocaine conditioned place preference

In the conditioned place preference paradigm, a drug is repeatedly paired with a specific context, thus forming an association of the drug with that context. The paradigm mimics the drug-paired contextual associations commonly formed in human drug addicts. The strength of the association is determined by measuring the amount of time spent in the drug-paired context. Before conditioning, there was no preference for the context in which the drug was to be paired in either TRPC5flx-CamKII Cre- or Cre+ mice. After conditioning to a low dose of cocaine of 3.5 mg/kg, the Cre+ mice showed a significantly higher preference for the cocaine-paired context compared to Cre- mice ($p<0.05$, Figure 3.6). At a higher dose of cocaine, 7.5 mg/kg, both Cre+ and Cre- mice showed preference for the cocaine paired side equally (Figure 3.8).

To determine which region is responsible for the effect, TRPC5 floxed mice were bilaterally injected with AAV-Cre-GFP or AAV-CreΔ-GFP into the PFC to specifically knock out TRPC5 in the PFC. Before conditioning, there was no preference for the drug-paired context in Cre or CreΔ mice. However, after conditioning to 3.5 mg/kg, mice injected with AAV-Cre-GFP showed more preference for the cocaine-paired side than AAV-CreΔ-GFP mice (Figure 3.8). At 7.5 mg/kg, both groups showed significant preference for the cocaine-paired side, and no difference

between the two groups was detected. These results suggest that loss of TRPC5 in the PFC is responsible for the increased reward.

Previous work looking at the expression of TRPC5 in the rodent brain showed TRPC5 to have high expression in the pyramidal neurons of both the PFC and HIP [46]. Since the CamKII-Cre line expresses Cre in the cell body layer of the HIP, we wanted to confirm that the conditioned place preference effect is due to loss of TRPC5 in the PFC, and not the HIP. TRPC5 floxed mice were bilaterally injected with AAV-Cre-GFP or AAV-CreΔ-GFP into the CA1 region of the HIP to specifically knock out TRPC5. Before conditioning, there was no preference for the drug-paired side in AAV-Cre-GFP or AAV-CreΔ-GFP injected animals. After conditioning to either 3.5 mg/kg or 7.5 mg/kg of cocaine, there was no significant difference in the preference for the cocaine-paired side in either AAV-Cre-GFP or AAV-CreΔ-GFP mice (Figure 3.9). These results lend further evidence that the increase in preference for the cocaine paired side is due to loss of TRPC5 in the PFC.

Fear conditioning

Cocaine conditioned place preference depends on two factors: the rewarding aspects of the drug and learning of the context in which the

drug is paired. To determine whether the increase in preference seen in mice lacking TRPC5 in the PFC is due to changes in the rewarding aspects of the drug or contextual learning, fear conditioning was performed. No differences in acquisition or extinction of contextual fear memory between AAV-Cre-GFP and AAV-CreΔ-GFP animals were observed, suggesting that the increase in conditioned preference in AAV-Cre-GFP mice is due to an increase in the rewarding aspects of the drug rather than improved contextual learning (Figure 3.10).

Discussion

Compensation in the TRPC5 knock-out line

Before conditional knock-outs became available, the traditional way of studying a gene by knock-out strategy was by creating a congenic knock-out line, such as the TRPC5 knock-out described here. These lines have advantages in that they allow you to fully knock-out a gene without the caveats of incomplete knock-down. However, the disadvantage of these types of model systems is that they allow time for compensation by genes similar to the gene of interest. This is the case with the TRPC5 KO mouse where we see increased expression of TRPC3 channel mRNA to

compensate for the loss of TRPC5 in the brain. Evidence to support a potential role of TRPC3 in mediating a similar phenomenon comes from recent work by Hartmann *et al* (2008*)* in which they show that TRPC3 is necessary for a slow excitatory postsynaptic current in Purkinje cells of the cerebellum [114]. It's likely that the dADP is still present in PFC neurons of TRPC5 KO mice due to the compensatory actions of TRPC3.

Conditional loss of TRPC5 in the adult mouse brain

To circumvent the difficulties with compensation in the TRPC5 -/- mouse line, we implemented a conditional knock-out method in which the knock-down occurs only in a short time window following complete development of the mouse. We employed two different types of conditional knock-out models in these studies. We used AAV-Cre-GFP injection into a TRPC5flx mouse line to selectively knock out TRPC5 in a regionally specific manner and a CamKII-Cre mouse line to temporally knock out TRPC5 in the frontal regions of the brain beginning at P23 and peaking at P60. With both methods, the expression of Cre and subsequent knock-down of TRPC5 occurs within four weeks, which does not produce compensation by other channels as evidenced by the lack of compensation in the TRPC5flx-CamKII Cre mouse line. This gives us the

ability to study the effects of TRPC5 knock-down without the complication of mRNA compensation by other TRPC channels.

TRPC5 has been predicted to be involved in neuronal outgrowth and development as well as neuronal plasticity [61] so it is possible that we see compensation by different TRPC channels depending on the developmental time point at which knock-down occurs. Cre expression turns on around P23 in the CamKII Cre line and peaks in adulthood at P60. This is in contrast to the TRPC5 KO line where KO occurs from the embryonic stage. It would be interesting to look at mRNA compensation by TRPC channels in the TRPC5flx-CamKII Cre mouse line at different developmental time points.

One difficulty we encountered with the TRPC5flx-CamKII Cre mouse line is the ambiguity with where the knock-out of TRPC5 occurs. The expression of Cre in this mouse line is primarily frontal and in the corticolimbic areas of the brain, including the PFC, striatum, and HIP [110]. However, when we examined the loss of TRPC5 mRNA in different regions of the TRPC5flx-CamKII-Cre mouse line, we saw ~50% loss of TRPC5 mRNA in the PFC, but no loss of TRPC5 in the dorsal striatum, NAc, or HIP. We have previously shown that TRPC5 mRNA expression is very low in the striatum, so it is possible that we failed to detect a loss in that area because TRPC5 expression was already so low. However, we

would have expected to see loss of TRPC5 mRNA in the HIP due to the high expression of both TRPC5 and Cre in that area. One possible explanation for the discrepancy is that the real-time PCR experiment is done with whole hippocampus, including all regions of both dorsal and ventral hippocampus. The Cre expression in the CamkII-Cre line is primarily in the dorsal HIP and is also variable across the different sub regions of the HIP [110]. Perhaps a more specific dissection and analysis of the dorsal sub regions of the HIP would elucidate a loss of TRPC5 in one of the sub regions of the HIP. Another possibility is that Cre and TRPC5 are not expressed in the same population of neurons in the HIP. Dual-labeled flouresecent in situ hybridization studies of Cre and TRPC5 in the HIP would be useful to determine whether Cre and TRPC5 co-localize in the HIP.

Loss of the burst-triggered delayed after-depolarization in the prefrontal cortex following knock-down of TRPC5

We have identified the TRPC5 channels as necessary for the induction of the dADP in layer 5 pyramidal neurons in the PFC using AAV-mediated knock-down of TRPC5 in the PFC of TRPC5flx mice. The loss of the dADP in AAV-Cre-GFP infected neurons is not due to the viral

infection itself, or by infection with GFP as shown by the normal dADP in AAV-CreΔ-GFP infected neurons. In addition, the loss of the dADP are limited to the neurons that are infected and does not translate to uninfected neurons in the same slice, suggesting that the effect of TRPC5 loss on the dADP is cell autonomous. These results have important implications for the role of TRPC5 in intrinsic neuronal excitability in the PFC. As shown by previous studies, the dADP is a mechanism for persistent activity in the PFC, which is known to be involved in working memory and attention, both of which are disrupted following drug use [34, 115]. The dADP could serve as a memory buffer to retain information during a delay period before a response can be made and the system resets. A reduction in prefrontal cortical functioning following repeated cocaine usage is consistent with reports that chronic cocaine abusers have deficits in working memory, attentional disorders, and hypofrontality in the PFC [20, 34].

Enhanced behavioral responses to cocaine following loss of TRPC5 in the prefrontal cortex

The reward circuitry consists of several regions which are connected by a number of inhibitory and excitatory inputs which provide

both positive and negative feedback. The PFC is an integral part of the circuitry which is fundamental in deciding the behavioral output resulting from drug stimuli. Loss of TRPC5 and a subsequent propensity of the PFC to respond to strong synaptic inputs associated with drug stimuli causes mice to be more sensitive to the effects of cocaine, which is exhibited by the increased locomotor response to cocaine in mice lacking TRPC5 in the PFC. The failure to see the same phenotype in the TRPC5flx-CamKII Cre mouse line may be due to incomplete knock-down of TRPC5 in the PFC. Though robust expression of Cre mRNA is seen in the PFC, the cellular localization of Cre is unknown. It's possible that Cre is only expressed in a subset of neurons in the PFC that may not all also express TRPC5. In contrast, the AAV injections into the PFC may infect a larger proportion of neurons, thus resulting in a stronger behavioral effect. Furthermore, the CamKII Cre line also expressed Cre in other brain areas, such as the motor cortex, striatum, and HIP. It is possible that the lack of enhanced locomotor response to cocaine is due to reduced activity in other regions of the reward circuitry [116]. We were unable to definitively show a reduction in TRPC5 mRNA levels in the striatum due to its low level of expression. In addition, our previous studies indicate that while TRPC5 mRNA expression in the NAc is low, TRPC5 protein is expressed in moderate levels, potentially through trafficking of the TRPC5 from the

PFC. It is possible that there is a reduction in TRPC5 protein levels that is causing a reduction in striatal activity that prevents the increased locomotor response to high doses of cocaine.

The AAV serotype (5) used in these studies has been shown to undergo retrograde transport in the CNS [111, 112, 117]. For retrograde transport to occur, the virus must infect a cell terminal and be transported to the nucleus of the cell body [112]. Retrograde labeling for AAV serotype 5 has been observed in the nigrostriatal pathway, the HIP, and in the cerebellum [112, 117]. Therefore, it is also possible that we see a locomotor phenotype in the AAV-Cre-GFP injected animals and not the TRPC5flx CamKII Cre animals due to retrograde labeling of the AAV. However, we detect no evidence of retrograde labeling in the AAV-Cre-GFP injected animals, suggesting that the phenotype is due to specific knock-down of TRPC5 in the PFC and not due to nonspecific effects form knock-down in regions that send inputs to the PFC.

We find that the loss of TRPC5 in the PFC results in an increase in the rewarding properties of cocaine in both conditional knock-out lines. We show that this increase in drug seeking is specifically due to loss of TRPC5 in the PFC and knock-down of TRPC5 in the HIP has no effect on CPP behavior. The increase in preference for the drug-paired side is not due to improved contextual learning as evidenced by fear conditioning

studies looking at the animals' ability to make context-paired associations. However, the increase in CPP is seen only at low doses of cocaine (3.5 mg/kg); no difference is seen between the two groups at higher doses of cocaine. It's possible that high doses of cocaine provide strong enough inputs that are able to elicit a behavioral response regardless of the activity state of the PFC. It seems that loss of the dADP allows low doses that are normally filtered out by the PFC to be rewarding, thus lowering the threshold for cocaine reward.

These findings are an important step in understanding how the PFC can determine drug induced behaviors and provides a potential mechanism for the how alterations in PFC excitability can contribute to the uncontrollable drug seeking seen in drug addicts. We show that the PFC can retain information for salient events through induction of a G_q-coupled burst-actived dADP which provides a depolarizing wave sufficient to produce single-cell persistent firing output. We show that cocaine and dopamine can reduce activity of the PFC by decreasing the dADP and that this loss biases the neurons towards very strong inputs, such as those possibly associated with drug cues. We identify the non-selective cation channel, TRPC5, as the channel responsible for mediating the dADP in PFC pyramidal neurons using a novel conditional knock-out mouse system. We show that loss of TRPC5 in the PFC results in an increased

sensitivity to cocaine and increased drug seeking, consistent with our hypothesis.

The search for genes associated with drug addiction is ongoing and these studies implicate the TRPC5 channels as important for mediating the rewarding effects of cocaine. Reduced TRPC5 expression in the PFC or mutations in these channels that inhibit their function may contribute to susceptibility for drug dependence; and it would be interesting to examine the expression levels of TRPC5 channels in normal and addicted subjects to establish a genetic link. Conversely, treatments which increase TRPC5 expression or function may help alleviate the uncontrollable urges for drug seeking seen in addicts that lead to reinstatement of drug use. Future studies may focus on over expression of TRPC5 in the PFC or the development of TRPC5 agonists as treatments or preventative measures for addiction.

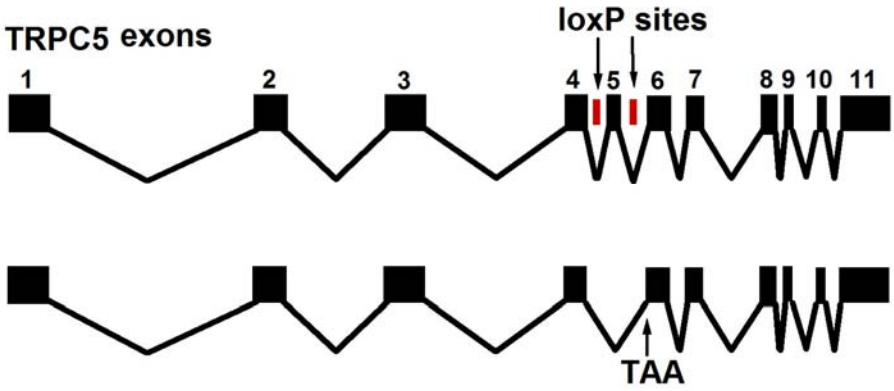

Figure 3.1 Creation of a conditional TRPC5 knock-out mouse line.

Knock-down strategy of TRPC5 in TRPC5flx mice. The schematic represents the *Mus musculus* TRPC5 gene before and after expression of Cre recombinase. The flanking loxP sites are placed into the introns surrounding exon5 of the TRPC5 gene (top, TRPC5flx). After homologous recombination by Cre, exon 5 is eliminated, resulting in a frame shit and a premature stop codon in the first position of exon 6 (bottom)

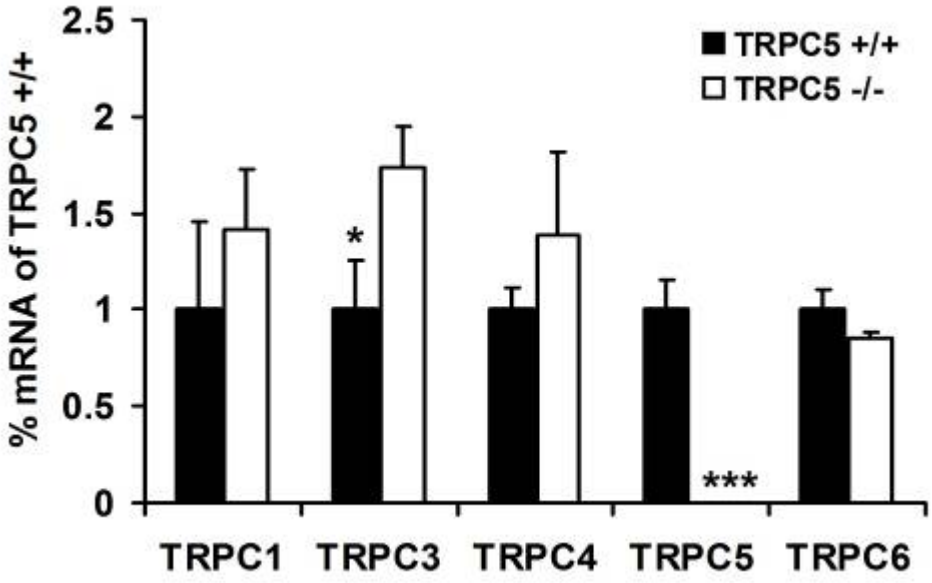

Figure 3.2 Knock-out and compensation of TRPC channel mRNA expression in the TRPC5 knock-out mouse line.

Quantitative real-time PCR of the TRPC channels expressed in the PFC of TRPC5+/+ and -/- mice (n=6 each; TRPC3, $p<0.05$; TRPC5, $p<0.001$)

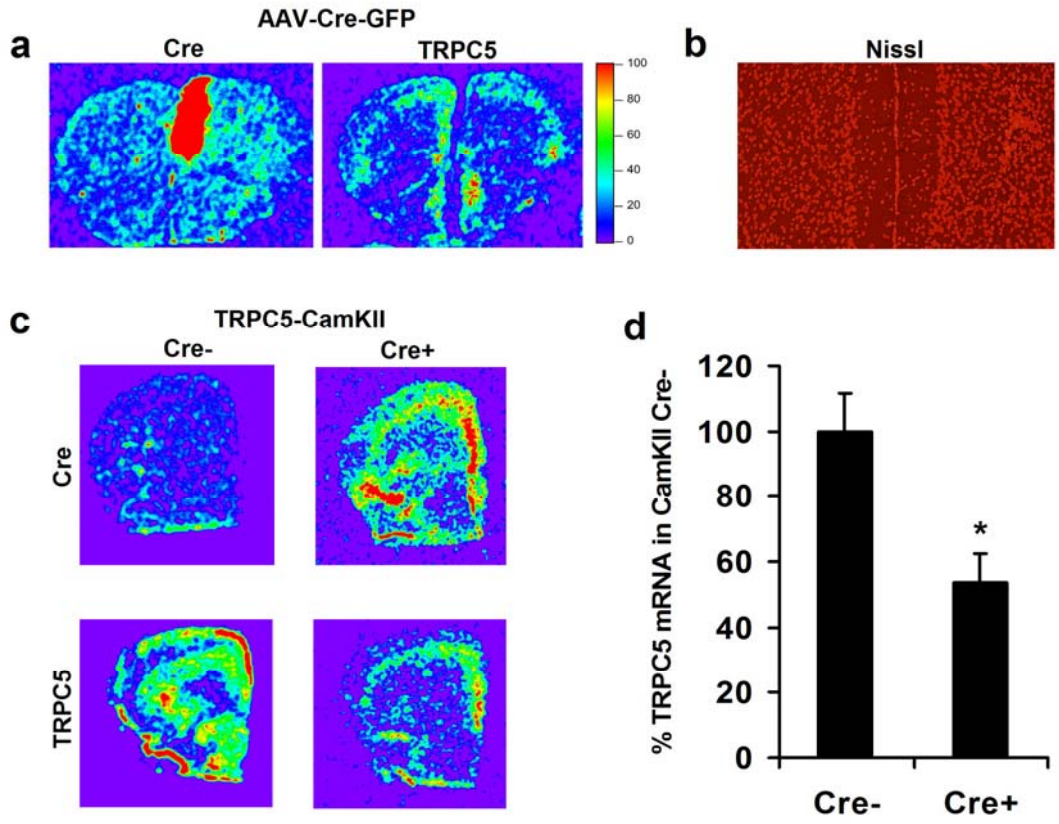

Figure 3.3 Knock-down of TRPC5 in PFC pyramidal neurons.

(a) In situ hybridizations showing Cre (left) and TRPC5 (right) cRNA signals in mice unilaterally injected with AAV-Cre-GFP into the PFC **(b)** Nissl staining in adjacent slices shown in b. **(c)** In situ hybridizations showing the Cre (top) and TRPC5 (bottom) cRNA signals in TRPC5-CamKII Cre- (left) and Cre+ (right) mice **(d)** Quantitative real-time PCR shows a 46% reduction in TRPC5 mRNA levels in the PFC of TRPC5-CamKII Cre+ (n=12) compared to Cre- mice (n=12; p=0.006).

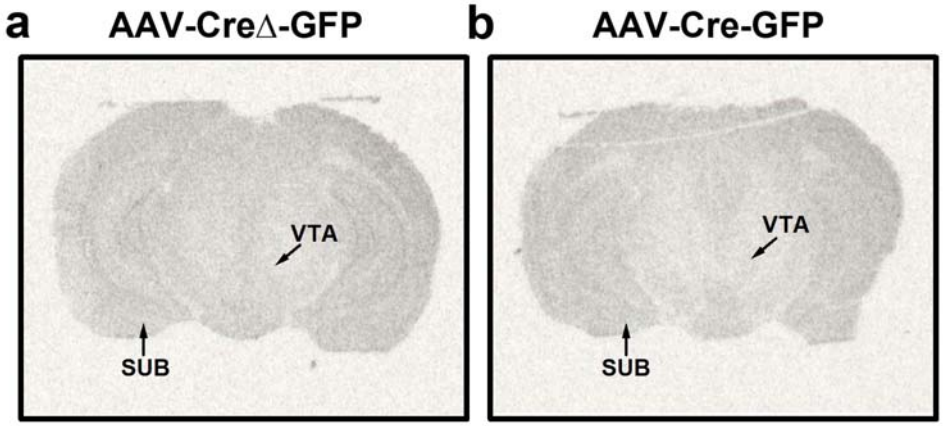

Figure 3.4 Retrograde transport of AAV.

In situ hybridization for Cre recombinase in the SUB and VTA of mice injected bilaterally in the PFC with AAV-CreΔ-GFP **(a)** and AAV-Cre-GFP **(b)** injected animals.

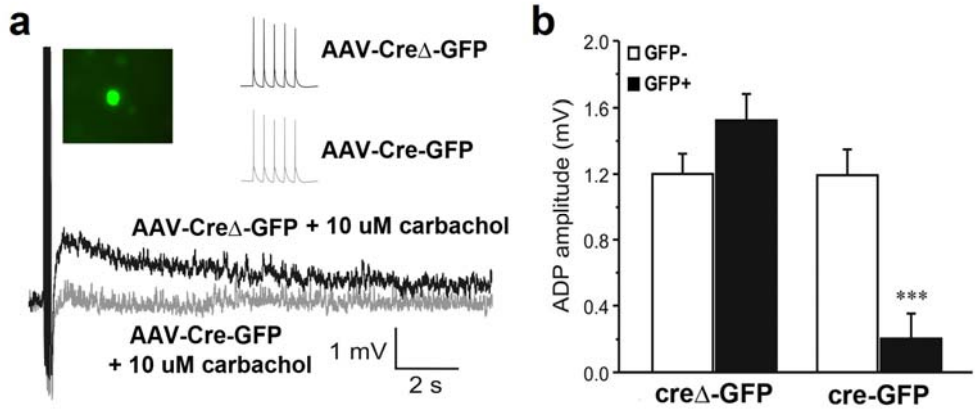

Figure 3.5 Loss of a muscarinic acetylcholine mediated dADP in PFC pyramidal neurons lacking TRPC5 channels.

(a and b) Carbachol-induced (10 μM), burst-triggered dADP is eliminated in PFC neurons infected with AAV-Cre-GFP (n=6) compared to PFC neurons infected with control AAV-CreΔ-GFP (n=3) and uninfected neurons in AAV-Cre-GFP slices (n=7) and AAV-CreΔ-GFP slices (n=7, $p<0.001$) **(inset)** Example of a GFP-expressing PFC neuron infected with AAV-Cre-GFP**.** *The patch-clamp recordings were performed by Ming-Hu Han (UTSW).*

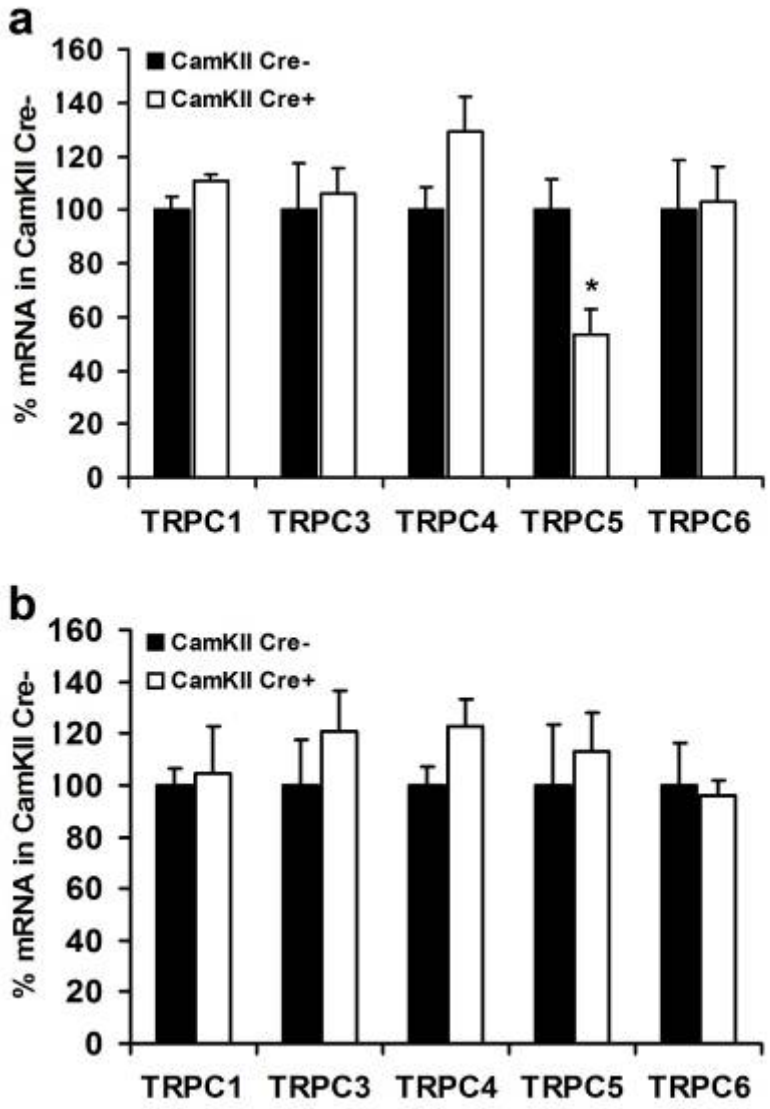

Figure 3.6 Compensation of TRPC channel mRNA expression in TRPC5 CamKII Cre- and Cre+ mice.

(a-b) Quantitative real-time PCR of the TRPC channels expressed in the PFC (a) and HIP (b) in TRPC5 CamKII Cre- (n=12) and Cre+ (n=12; *$p<0.05$).

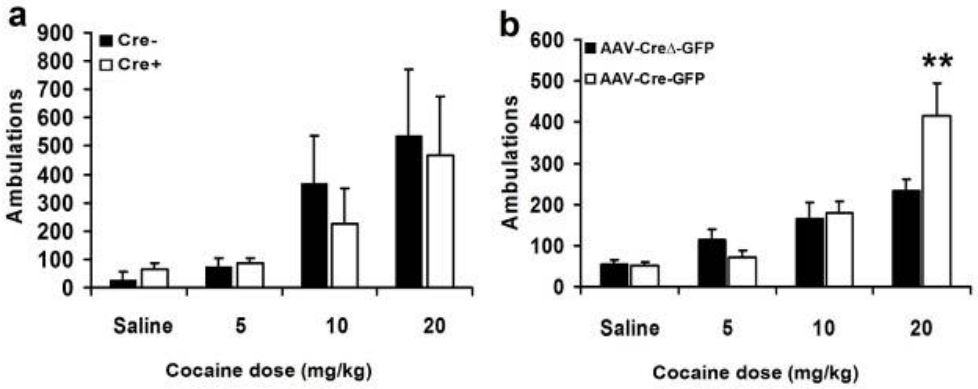

Figure 3.7 Mice lacking TRPC5 in PFC neurons show a heightened locomotor response to cocaine.

(a) The ambulatory locomotor response to i.p. injections of 5, 10, and 20 mg/kg cocaine in TRP5flx-CamKII Cre+ (n=6) and Cre- mice (n=6). **(b)** The ambulatory locomotor response to i.p. injections of 5, 10, and 20 mg/kg cocaine in mice that were bilaterally injected with AAV-CreΔ-GFP (n=11) or AAV-Cre-GFP (n=10) into the PFC ($p<0.01$).

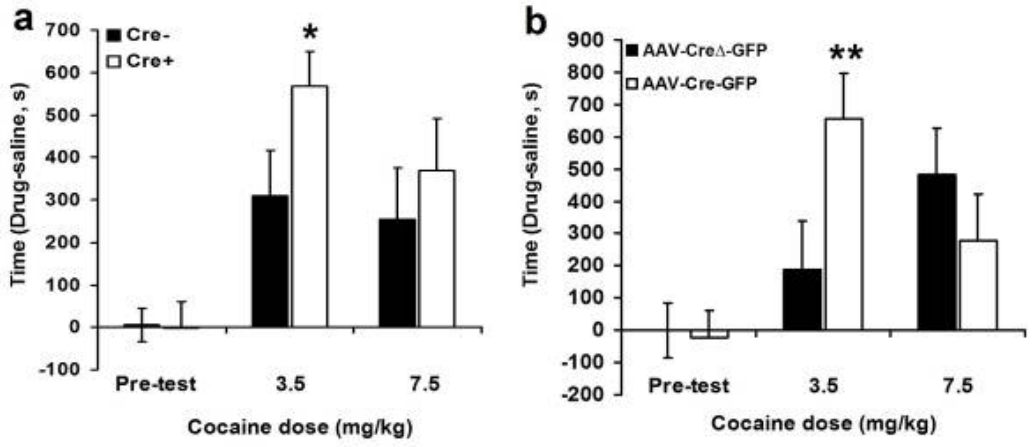

Figure 3.8 Mice lacking TRPC5 in PFC neurons show a heightened response to the rewarding properties of cocaine.

(a) Cocaine conditioned place preference to 3.5 and 7.5 mg/kg cocaine in TRPC5 CamKII Cre- (n=18) and Cre+ mice (n=16; $p<0.05$). **(b)** Cocaine conditioned place preference to 3.5 and 7.5 mg/kg cocaine in mice that were bilaterally injected with AAV-CreΔ-GFP (n=11) or AAV-Cre-GFP (n=10) into the PFC ($p<0.01$).

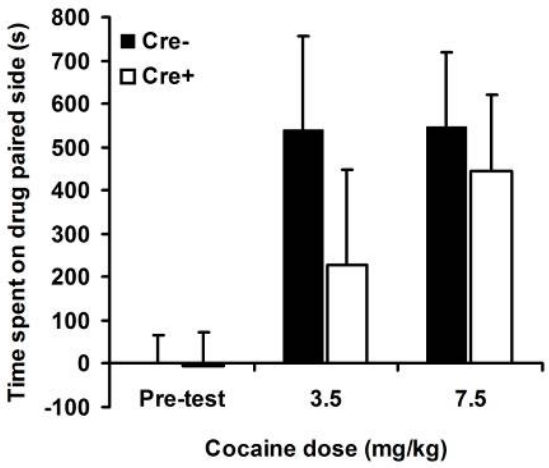

Figure 3.9 Mice lacking TRPC5 in HIP neurons show no difference in their response to the rewarding properties of cocaine.

Cocaine conditioned place preference to 3.5 and 7.5 mg/kg cocaine in in mice that were bilaterally injected with AAV-CreΔ-GFP (n=7) or AAV-Cre-GFP (n=7) into the CA1 region of the HIP.

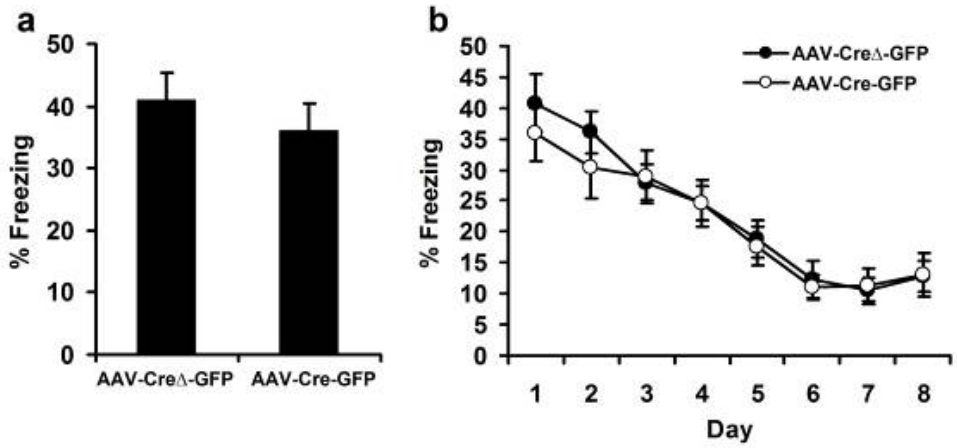

Figure 3.10 Loss of TRPC5 in the PFC does not alter contextual fear conditioning acquisition or extinction

(a) Acquisition of the fear response in mice bilaterally injected with AAV-CreΔ-GFP (n=11) and AAV-Cre-GFP (n=10) into the PFC. **(b)** Extinction of contextual fear over 8 days in animals in (a).

CHAPTER FOUR: METABOTROPIC GLUTAMATE RECEPTOR 5 INVOLVEMENT IN ADDICTION-RELATED BEHAVIORS

Summary

Metabotropic glutamate receptor 5 is important for mediating the behavioral responses to psychostimulants. A previous study examining the mGluR5 knock-out mouse line showed that mGluR5-/- mice fail to show rewarding psychomotor activation in response to cocaine. Our work shows that mGluR5-/- mice show no difference in the locomotor response to low to moderate doses of cocaine (5, 10, or 20 mg/kg) but instead show a blunted response to a high dose of cocaine (40 mg/kg) compared to wild type mGluR5+/+ mice. Our conditioned place preference studies indicated no difference in conditioning to cocaine between mGluR5-/- and +/+ mice, suggesting normal rewarding properties of cocaine. Differences in strain backgrounds or paradigms may account for the conflicting results, but our results indicate that mGluR5 receptors exert a modulatory rather than necessary role in the locomotor stimulating or rewarding properties of cocaine.

Introduction

The group 1 metabotropic glutamate receptors, mGluR1 and mGluR5, are G_q-coupled receptors that play an important role in mediating the neurological responses to psychostimulants [118-120]. The mGluR5s function as homodimers and activation of the receptors induces an intracellular cascade involving activation of PLC and subsequent release of intracellular Ca^{2+} from the endoplasmic reticulum which then activates various other intracellular pathways [121]. Expression of mGluR5 in the brain is widespread, with some of the highest expression in the striatum, an area important for mediating the behavioral responses to psychostimulants [118, 122]. In addition, glutamatergic signaling from the PFC to the NAc is important for regulation drug seeking behavior [21]. Furthermore, studies have shown that administration of antagonists of mGluR5 reduce cocaine self-administration and reinstatement [119, 123, 124]. A study by Chiamulera *et al.* (2001) shows a complete loss of the locomotor activating effects of cocaine in mGluR5 knock-out mice. They tested cocaine self-administration (SA) and found that mGluR5 knock-out mice do not self-administer cocaine, suggesting a deficit in the rewarding effects of cocaine [119]. However, they did not thoroughly test for deficits in instrumental learning leaving the possibility that mGluR5 receptors are

involved in instrumental learning in general rather than cocaine specific reward. We sought to further explore the role of mGluR5 in cocaine locomotor responses and measure cocaine reward using a conditioned place preference (CPP) paradigm that does not involve instrumental learning.

Experimental procedures

Locomotor response to cocaine

Adult male mGluR5+/+ and -/- mice were weighed and marked one day prior to the start of the behavioral testing. Mice were habituated to the testing room for one hour prior to the start of testing on each day of testing. The locomotor chambers consisted of a cage identical to the home cage placed into a box equipped with five photobeams for recording movement. Ambulatory movement was defined as two consecutive beam breaks. On each day of testing, the mice were placed into the locomotor chambers and movement was recorded for one hour during habituation to the chambers. On days one and two, after one hour of habituation to the chamber, the animals were injected with saline and placed back into the chambers where movement was recorded for an additional hour. Animals

were removed from the chambers and placed back into their home cages. On days three, five, and seven, after one hour of habituation to the chamber, the animals were given ascending doses of cocaine of 10, 20, and 40 mg/kg. A saline injection was interleaved between the cocaine injections to prevent conditioned locomotion.

Cocaine conditioned place preference

Adult male mGluR5+/+ and -/- mice were weighed and marked one day prior to the start of the behavioral procedure. The CPP chamber consisted of two large compartments that differed in both visual and tactile cues, separated by a smaller center compartment (Med Associates, Vermont, Georgia). The two large compartments were equipped with six photobeams for monitoring movement, and the center compartment with two photobeams. Prior to conditioning, animals were placed into the center chamber and allowed to freely explore the three compartments for 20 min. The animals were paired with a compartment for conditioning so that there was no bias for either side. On training days one through four, partitions were used to block off the two large compartments and each animal was given an i.p. injection of either cocaine or saline (10 mg/kg alternate over 4 days) and placed into its respective compartment for 30

min. On the fifth day, the partitions were lifted and the animals were placed into the center compartment. The animals were allowed to move freely between the three compartments for 20 minutes and the time spent in each compartment was recorded. The preference for the cocaine-paired side was calculated as the time spent in the cocaine-paired side minus the time spent in the saline-paired side.

Results

Locomotor response to cocaine

Previous studies have shown that mGluR5-/- mice have a reduced ambulatory locomotor response to cocaine at doses of 10, 20, and 40 mg/kg compared to mGluR5+/+ mice [119]. However, our results showed no decrease in ambulatory movement in the mGluR5-/- compared to the mGluR5+/+ mice at 5, 10, or 20 mg/kg of cocaine. However, there was a significant decrease in the ambulatory movement of mGluR5-/- mice at 40 mg/kg cocaine compared to mGluR5+/+ (Figure 5.1).

Cocaine conditioned place preference

Cocaine self-administration studies by Chiamerula *et al* have shown that mGluR5-/- mice will not self-administer cocaine at any dose while mGluR5+/+ self-administer cocaine normally [119]. Self-administration is a behavioral paradigm used to directly measure the rewarding properties of cocaine. Alternative methods, such as CPP have been developed to measure the conditioned reinforcing properties of cocaine. The CPP procedure involved repeated injections of cocaine to mice paired with a specific context and explicit unpairing in a different context. Then, in the absence of the drug the mice are allowed to move freely between the two contexts with the amount of time spent on the cocaine paired context as an indirect measure of the rewarding property of cocaine. We sought to use CPP as an alternate method to assess cocaine reward. Unlike cocaine self-administration, CPP assesses cocaine reward memory in the absence of the locomotor activating properties of cocaine. Before conditioning, neither group showed preference to the cocaine paired side. After conditioning to a 10 mg/kg dose of cocaine, both mGluR5-/- and +/+ mice showed significant preference for the cocaine paired side and there were no group differences (Figure 5.2).

Discussion

Our results indicate that the mGluR5-/- mice have a normal locomotor activation response to low to moderate doses of cocaine. This is inconsistent with previous studies in which the mGluR5-/- mice did not activate to 10, 20, or 40 mg/kg doses of cocaine [119]. We see a reduction in the ambulatory response to high doses (40 mg/kg) cocaine in mGluR5-/- mice compared to +/+ mice, which is consistent with previous reports [119]. However, it is important to note that the previously mentioned study did not see locomotor activation of mGluR5-/- mice to 40 mg/kg cocaine dose compared to a saline control, while we do see significant activation compared to saline in our studies. Furthermore, we find that the mGluR5-/- mice show normal CPP to cocaine while the authors of the previous study found that mGluR5 -/- mice do not self-administer cocaine.

Differences in the paradigms used to measure cocaine reward could be responsible for the differences between the studies. Using CPP we found that the mGluR5-/- mice show normal cocaine reward memory as evidenced by their increased time spent on the cocaine-paired side after conditioning with cocaine. Chiamulera *et al.* (2001) tested cocaine self-administration and found that the mGluR5-/- mice fail to self-

administer cocaine at any dose. CPP is a method of measuring cocaine reward that is partly based on the animal's ability to form drug-context associations. Once the association is formed through repeated pairings of the drug with a particular context, the animal is allowed to choose between the drug-paired context and an unpaired context. If the drug is considered rewarding, the animals will spend the majority of the time in the drug-paired context. There are caveats to this method of measuring drug reward. First, it relies on the ability of the animal to form these associations, which rely on intact contextual learning ability and drug-associated learning and memory. Also, in CPP, the drug is administered through non-contingent intraperitoneal injections. In CPP the animal does not have ability to voluntarily administer the drug during training, so it does not rely on instrumental learning to establish cocaine intake. In cocaine SA, on the other hand the animal responds for intravenous infusions of the drug into the jugular vein and this requires instrumental learning. Cocaine SA has different kinetics for elevating brain dopamine levels compared to CPP and measures both the motivational and rewarding properties of cocaine by enabling the animal to volitionally titrate their drug intake. Both CPP and SA are accepted forms of measuring the rewarding effects of cocaine; however it has been shown that there are processes involved

that are unique to each paradigm. For example, D1R knock-out mice fail to show CPP yet will self-administer cocaine [5].

Previous studies have shown that mGluR5 antagonists are capable of reducing cocaine CPP. Pretreatment with an i.p. injection of the selective mGluR5 antagonist, MPEP reduced CPP in mice at both low (5 mg/kg) and high (20 mg/kg) conditioning doses of cocaine [123]. Pretreatment with MPEP itself was not responsible for the reduced CPP to cocaine because conditioning to MPEP alone did not produce preference or an aversion for the MPEP-paired context [123]. This study suggests that mGluR5 receptors are important for acquisition of CPP. However, these studies with MPEP measure an acute reduction of mGluR5s, while our studies are following chronic loss of mGluR5 in a mouse KO model. It's clear that acute loss of mGluR5s in normal adult mice reduces cocaine mediated reward, but chronic loss of mGluR5 may not produce the same effect. It is also possible that we fail to see an effect of the loss of mGluR5 on cocaine reward due to developmental compensation in the mGluR5 KO mouse. It would be important to examine whether compensation is occurring in the mGluR5 KO mouse. Furthermore, nonspecific pharmacological effects of MPEP may have contributed to the reduction in cocaine CPP. In light of this possibility it would be interesting to test MPEP in the mGluR5 -/- mice to answer this question.

Although cocaine CPP appears to be normal in the mGluR5-/- mice at the dose we tested, we found a reduction in the locomotor activating effects of cocaine at high doses. This is seemingly inconsistent with our previous findings that loss of TRPC5 and the mGluR5-mediated dADP in the PFC results in an increased sensitivity for the rewarding properties of cocaine. However, the method of knock-down in these two mouse lines is vastly different, and could account for the differences in their responses to cocaine. The TRPC5 knock-down was acute and PFC-specific, whereas the mGluR5 line is a chronic global knock-out which may have compensatory increases in gene expression due to the prolonged knock-out. Furthermore, mGluR5 expression is especially high in the NAc and levels of mGluR5 mRNA have been shown to increase in the NAc following repeated cocaine, suggesting that mGluR5 may be important for mediating the behavioral responses to cocaine through its actions in the NAc [125-127]. Therefore, loss of mGluR5 in the NAc would be expected to reduce the locomotor response and the rewarding properties of cocaine. It's possible that mGluR5 receptors exert oppositional roles in the NAc and PFC, and that loss of mGluR5 in NAc reduces cocaine reward whereas loss in the PFC enhances cocaine reward. Our results showing increased CPP in the TRPC5 KO studies could reflect how loss of mGluR5 selectively in the PFC enhances cocaine reward. In those

studies, mGluR5 function in the NAc is left intact due to the lack of TRPC5 expression there. The knock-out of mGluR5 in both regions may result in normal cocaine CPP because the effects of the loss of mGluR5 in the NAc and PFC would cancel each other out. An interesting and informative next step would be to selectively knock out mGluR5 in the PFC and the NAc to examine how loss of mGluR5 signaling might affect behaviors in each of those regions.

Another possible reason to consider for the discrepancies in our results compared to previous reports is that we may be testing two different strains of mGluR5 mice. A growing body of research has found that there is a large amount of behavioral variability between strains, and that brain regions can be differentially activated in different strains [128-133]. A study performed by Wahlsten *et al* examined the behavior of eight different rodent strains on six different behaviors in three different laboratories. Their results found that for many behaviors, such as ethanol preference and water escape learning, there was little difference between the strains. However, for other behaviors, such as locomotor activity, cocaine-induced locomotor, and elevated plus maze there was a large strain dependence [134]. In addition, it has become clear that prenatal care, early life development, housing, gender, age, and social activity can alter the behavior of inbred strains [130-132, 135]. We obtained our

C57Bl/6J back-crossed mGluR5 mouse line from a different source than the authors of the previous studies who did not disclose the identity of the mGluR5 strain used. Therefore, potential strain differences could be partially responsible for our conflicting reports.

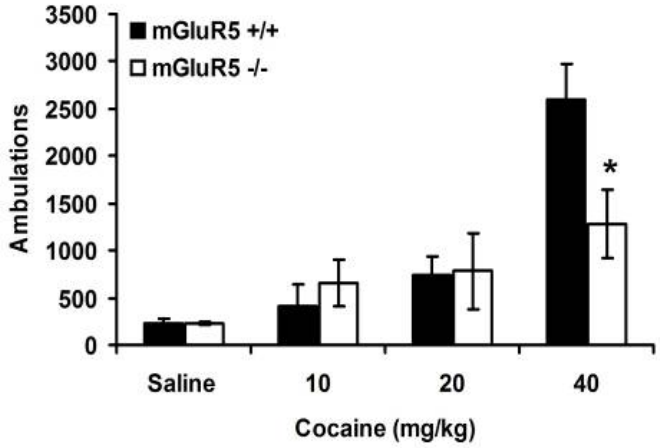

Figure 4.1 mGluR5-/- mice show a decreased locomotor response to cocaine at high doses.

The ambulatory locomotor response to i.p. injections of 5, 10, and 20 mg/kg cocaine in mGLuR5+/+ (n=8) and -/- (n=5) mice ($p<0.05$ at 40 mg/kg).

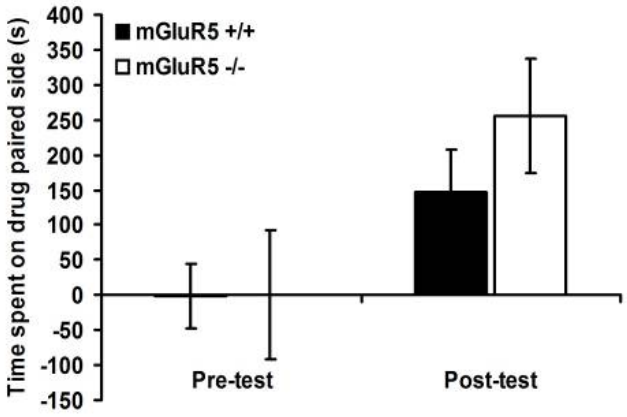

Figure 4.2 Loss of mGluR5 does not affect cocaine conditioned place preference.

Cocaine conditioned place preference at 10 mg/kg of cocaine in mGLuR5+/+ (n=13) and -/- (n=14) mice.

CHAPTER FIVE: BEHAVIORAL CHARACTERIZATION OF THE TRPC1 KNOCK-OUT MOUSE

Summary

TRPC channels have been implicated in a number of neuronal functions in the brain such as neuronal development, axon guidance, and slow excitatory synaptic transmission in the cerebellum. The slow excitatory postsynaptic potentials (EPSC) reported to be mediated by TRPC1 channels are similar to the dADP we have found to be mediated by mGluR5/TRPC5 signaling in the PFC. Like the TRPC5 channels, TRPC1 channels are widely expressed in the mammalian brain. TRPC1 subunits form heteromultimeric channels with TRPC3, 4, and 5 subunits that are activated following G_q-coupled signaling cascades. Robust expression of TRPC1 was found in the corticolimbic areas of the brain including the PFC, striatum, HIP, and LS. Knock-out of TRPC1 in the brain of TRPC1-/- mice was confirmed via immunoblots. Real-time PCR experiments show no compensation in the PFC by other TRPC channels following loss of TRPC1. The TRPC1 channels were proposed to modulate a slow EPSC in the cerebellum, however we saw no differences

in motor skills of the TRPC1-/- compared to +/+ mice. The dense expression of the TRPC1 channels in the PFC, LS and HIP led us to examine behaviors associated with those regions. Surprisingly the results showed no differences in anxiety, depression, cocaine responses, contextual fear learning, or spatial learning in TRPC1-/- compared to +/+ mice. We found an increase in the acoustic startle response at high decibel levels that may indicate deficits in sensorimotor gating in the TRPC1-/- mouse. Though we were unable to elucidate a definitive role for TRPC1 in modulating behavior, the robust expression of the TRPC1 channels in the corticolimbic areas of the brain suggests that they perform some function in the brain that has yet to be determined. Future studies using dual knock-outs of TRPC1 with TRPC3, 4, or 5 may be useful in determining the function of TRPC1 in the brain.

Introduction

TRPC1 channels are expressed widely throughout the mammalian system and have been shown to have a diverse array of functions. TRPC1 is expressed in the salivary glands, liver, endothelial cells, vascular smooth muscle, heart, skeletal muscle, testis, and brain [45]

where it serves to regulate Ca^{2+} homeostasis. TRPC1 forms heteromultimeric complexes with TRPC3, 4, 5 where it functions as a receptor operated channel (ROC) [54, 63-65]; and there is controversy regarding whether the TRPC1 channels can be properly trafficked to the plasma membrane without co-assembly with another TRPC channel. However, it has recently been suggested that TRPC1 can form a homomultimeric complex that associates with STIM1 in lipid rafts where the complex acts as a store operated channel (SOC). The formation of homomultimeric complexes by TRPC1, however, is not yet widely accepted, and research currently focuses on heteromultimeric TRPC1 complexes and their physiological roles.

TRPC1 channels are expressed in the mammalian brain, but few studies have elucidated their role. One of the first examinations of TRPC1 in the brain looked at its role in the cerebellum where it was hypothesized that TRPC1 was involved in mediating a mGluR1-evoked slow EPSC in Purkinje cells [73]. The involvement of TRPC1 in modulating excitability of Purkinje cells lends evidence that TRPC1-/- mice may show deficits in motor skills due to loss of cerebellar functioning. .

We provide evidence in previous studies for the role of TRPC5 in modulation a G_q-coupled evoked dADP in pyramidal neurons in the PFC; and show that loss of TRPC5 channels in the PFC results in an increase

in the locomotor activating and rewarding properties of cocaine. The ability of TRPC1 to form heteromultimeric complexes with TRPC5 lends evidence that TRPC1 channels may have a similar function. The loss of the TRPC5-mediated dADP in the PFC results in an enhancement of the locomotor activating and rewarding properties of cocaine; and it's possible that TRPC1-/- mice also show an altered response to cocaine.

Previous studies have suggested that TRPC1 is involved in neuronal development in the hippocampus [45, 136]. In addition, robust expression of TRPC1 is seen in the HIP, suggesting that the TRPC1 channels may be important for hippocampal-dependent learning. Furthermore, the dense expression of TRPC1 in the LS, an area implicated in stress and anxiety mechanisms, prompts the study of TRPC1 involvement in anxiety or depressive behaviors. The development of the TRPC1 knock-out mouse line has provided a valuable tool in examining the role of TRPC1 in the mammalian brain. In this study, we seek to examine how TRPC1 channels may be modulating neuronal behavior.

Experimental procedures

Immunoblot procedure

Homogenates were obtained by sonicating tissue suspensions in boiling 1% SDS lysis buffer. Protein concentrations were determined by the BCA assay (Pierce, Rockford, IL, USA). Samples were loaded onto 10% SDS-PAGE gels (50-100 µg protein/lane). Gels were transferred to Hybond ECL nitrocellulose membrane (Amersham, Piscataway, NJ, USA) for immunoblotting. The membranes were blocked in 5% milk for 1 hour, then primary antibody (TRPC1, 1:500, gift from Dr. Tsiokas) overnight at 4ºC with shaking. The membranes were incubated in secondary antibody (GαM 1:8000, Pierce, Rockford, IL, USA) for 1 hour at room temperature with shaking and developed with ECL (Amersham Biosciences, Piscataway, NJ, USA) reagent.

Rotarod

Adult TRPC1-/- and TRPC1+/+ littermates were habituated to the rotarod testing room for one hour prior to testing. Each mouse was placed onto an accelerating rotarod from 0-40 rotations per minute over five minutes. The latency to fall from the rotarod was recorded. The mice underwent four trials per day for two days. Each mouse had at least 30 minutes between each trial to prevent fatigue.

Acoustic startle

Adult TRPC1-/- and +/+ mice were habituated to the startle testing for one hour prior to testing. Startle responses in were measured at 120, 110, 100, 90, 80 and 0 decibels. The mice were each placed into a small acrylic cylinder in a sound-proof chamber that measures the startle response of the animal (Med Associates, St. Albans, VT). The startle testing was precluded with a 5 minute white noise habituation period, followed by a 10 minute startle threshold paradigm in which the decibel level is randomized.

Cocaine conditioned place preference

Adult male TRPC1+/+ and -/- mice were weighed and marked one day prior to the start of the behavioral procedure. The conditioned place preference chamber consisted of two large compartments that differed in both visual and tactile cues, separated by a smaller center compartment (Med Associates, Vermont, Georgia). The two large compartments were equipped with six photobeams for monitoring movement, and the center compartment with two photobeams. Prior to conditioning, animals were placed into the center chamber and allowed to freely explore the three

compartments for 20 min. The animals were paired with a compartment for conditioning so that there was no bias for either side. On training days one through four, partitions were used to block off the two large compartments and each animal was intraperitoneally injected with either cocaine or saline (5 mg/kg alternate over 4 days) and placed into its respective compartment for 30 min. On the fifth day, the partitions were lifted and the animals were placed into the center compartment. The animals were allowed to move freely between the three compartments for 20 minutes and the time spent in each compartment was recorded. The preference for the cocaine-paired side was calculated as the time spent in the cocaine-paired side minus the time spent in the saline-paired side.

Locomotor response to cocaine

Adult male TRPC1+/+ and -/- mice were weighed and marked one day prior to the start of the behavioral testing. Mice were habituated to the testing room for one hour prior to the start of testing on each day of testing. The locomotor chambers consisted of a cage identical to the home cage placed into a box equipped with five photobeams for recording movement. Ambulatory movement was defined as two consecutive beam breaks. On each day of testing, the mice were placed into the locomotor

chambers and movement was recorded for one hour during habituation to the chambers. On days one and two, after one hour of habituation to the chamber, the animals were injected with saline and placed back into the chambers where movement was recorded for an additional hour. Animals were removed from the chambers and placed back into their home cages. On days three, five, and seven, after one hour of habituation to the chamber, the animals were given ascending doses of cocaine of 5, 10, and 20 mg/kg. A saline injection was interleaved between the cocaine injections to prevent sensitization.

Contextual fear conditioning

Adult male TRPC1+/+ and -/- mice were weighed and marked one day prior to the start of behavioral testing. Mice were habituated to the testing room for one hour prior to the start of testing on each day of testing. The fear conditioning chamber is a clear polycarbonate chamber with a removable grid floor (Med Associates, St. Albans, VT). For contextual conditioning, each mouse was placed into the chamber and allowed to habituate to the chamber for two minutes before being delivered three 0.5 mA shocks separated by one minute. Conditioning took place over two consecutive days and acquisition of the freezing

behavior was recorded on the third day. The freezing behavior was hand recorded every ~3 seconds during the five minute period. Freezing behavior was defined as complete lack of movement besides respiration. After conditioning, the mouse was removed from the chamber and placed back into its home cage. On day two, acquisition of the fear response was measured by placing each mouse back into the fear conditioning chamber and recording the freezing behavior every ~3 seconds for five minutes. Extinction learning was measured on days three through eight by recording the freezing behavior for five minutes on each day.

Novel object location task

Adult TRPC1+/+ and -/- mice were habituated to the testing room one hour prior to testing. The testing apparatus consisted of a large square, white box with different visual cues placed on each wall of the box. The objects were two 50mL conical tubes filled with water that were placed on their ends in the top-right and top-left corners of the box. The trial and testing periods were videotaped and hand scored for the time spent interacting with each object. The investigator was blind to the genotype of the mice.

The test day consisted of five five-minute trials in which the two objects were placed into the top-right and top-left corners of the box. For the sixth five-minute trial, one object was moved to the bottom-right corner of the box. The time spent interacting with each object (seconds) was scored for the fifth and sixth trials.

Open field

Adult TRPC1+/+ and -/- mice were habituated to the open field testing room for one hour prior to testing. The open field arena consisted of a large, square white box with defined arenas for the center, middle, and perimeter of the box. The movement of each mouse was videotaped and tracked using Ethovision 3.1 (Noldus Information Technology, Leesburg, VA). The time spent in each arena was recorded over the five minute testing period.

Elevated plus maze

Adult TRPC1+/+ and -/- mice were habituated to the elevated plus maze testing room for one hour prior to testing. The elevated plus maze consists of two open arms and two closed arms of the maze, connected in the center. The movement of each mouse in the arms was videotaped

and tracked using Ethovision 3.1 (Noldus Information Technology, Leesburg, VA). The time spent in each arm was recorded over the five minute testing period.

Social defeat

Adult TRPC1+/+ and -/- mice were habituated to the social defeat room for one week prior to starting the defeats. These intruder mice were single housed in one side of a large rat cage with a clear, Plexiglas partition in the middle of the cage. On the opposite side of the cage, a male CD1 aggressor mouse was housed. The CD1 mice were screened for aggressive behavior with the criterion that they attack a test mouse within 60 seconds of introduction on three consecutive days. For the defeats, an intruder mouse was introduced into the aggressor's side of the cage where it was physically defeated for 10 minutes before being removed to the opposite side of the cage. The defeats continued for 10 days, with each intruder mouse seeing a new aggressor each day. Control mice were housed in similar cages, but experienced no defeats. One day after the last defeat, the mice were tested for social interaction.

Social interaction

Mice were habituated to the social interaction room for 1 hour prior to testing. The social interaction arena consisted of a large square box containing a small wire mesh cage large enough to fit a male CD1 mouse in the top center of one side of the arena. Interaction zones were defined for the area directly next to the mesh cage, the corners farthest from the cage, and the areas between the mesh cage and the corners. The amount of time spent in each of the interaction zones over five minutes was recorded for the first (no CD1 mouse present) and second (CD1 mouse present) trials.

Sucrose preference

Mice were single housed in standard mouse cages without food. Two bottles containing either water or a 30% sucrose solution were placed in the cage. The amount of liquid consumed from each bottle was recorded each day for two days.

Results

Generation of the TRPC1 knock-out mouse line

The TRPC1 knock-out mouse line was developed by Dr. Lutz Birnbaumer at NIEHS by inserting a PKG-neo cassette containing a stop codon in exon 8 of the TRPC1 gene into a transgenic mouse line [137]. Loss of TRPC1 was confirmed using immunoblots for the TRPC1 protein from TRPC1+/+ and -/- mice in microdissections of the PFC, motor cortex, dorsal striatum, nucleus accumbens, lateral septum, HIP, and cerebellum. Results show a complete loss of TRPC1 protein in all of these areas (Figure 5.1). To examine possible compensation by other TRPC channels in the TRPC1 knock-out mouse line, real-time PCR for TRPC3, 4, 5, and 6 was performed in the PFC of TRPC1-/- and +/+ mice. Levels of TRPC3, 4, 5, and 6 were unchanged in the TRPC1-/- compared to the TRPC1+/+ mice. Despite the complete loss of TRPC1 protein, levels of TRPC1 mRNA were reduced only ~50% in the PFC (Figure 5.2).

Examination of the dADP in TRPC1 knock-out mice

We have shown that TRPC5 channels are necessary for induction of a burst-activated dADP in the PFC. We examined the possibility that TRPC1/5 heteromultimeric complexes are underlying the dADP by examining the dADP in TRPC1-/- mice. Patch-clamp recordings by Ming-

Hu Han show no change in the dADP amplitude in PFC neurons of TRPC1-/- compared to +/+ mice (Figure 5.3).

Cocaine behaviors

The differences in the responses to cocaine in the TRPC5flx-CamKII Cre and TRPC5flx AAV-Cre-GFP injected animals prompted the examination of the TRPC1 mouse line in their responses to cocaine.

A locomotor dose response curve for 5, 10, and 20 mg/kg i.p. injections of cocaine in TRPC1+/+ and -/- mice shows no difference in the ambulations of the two groups at any dose (Figure 5.4). In addition, there was no difference in the preference for the cocaine-paired side following conditioning with 5 mg/kg cocaine in the cocaine conditioned place preference paradigm (Figure 5.4).

Motor coordination

Previous studies had implicated a role for TRPC1 in mediating a slow EPSC in the cerebellum. Based on these studies, we predicted that loss of TRPC1 may disrupt functioning of the cerebellum, a structure involved in motor coordination. To determine if there are any changes in

motor coordination in TRPC1-/- compared to +/+ mice, we used the rotarod motor task.

The TRPC1+/+ and -/- mice exhibited no differences in the latency to fall from the accelerating rotarod during eight trials over two days. There were also no differences in motor learning since both TRPC1+/+ and -/- mice improved in their ability to stay on the rotarod over the two days (Figure 5.5).

Auditory responses

To determine if there are hearing impairments in the TRPC1+/+ and -/- mice, we tested them for their acoustic startle response. A series of randomized tones (0-120 dB) were presented and the startle response to each tone was recorded. Results show an increase in the startle response in the TRPC1-/- compared to the TRPC1+/+ mice (Figure 5.6). Due to these results and the possibility for alterations in hearing in the TRPC1-/- mice, we tested the mice for their auditory evoked responses in collaboration with Dr. Stefan Heller at Stanford University. Dr. Heller found no differences in the auditory evoked responses in TRPC1-/- compared to TRPC1+/+ mice (data not shown).

Anxiety and depression behaviors

Immunoblots for TRPC1 protein show robust expression in the lateral septum, an area involved with aggression and anxiety [138]. To examine whether there were any differences in anxiety between TRPC1+/+ and TRPC1-/- mice, we performed open field and elevated plus maze tasks. The open field task measures the frequency and duration of the time of a mouse to enter the center of an open arena over five minutes. An anxious mouse will stay near the walls of the box, resisting the exploratory drive to enter the center of the arena. The results of the open field task show no difference in the duration or frequency to enter the center arena of an open field between TRPC1+/+ and -/- mice (Figure 5.7).

The elevated plus maze consists of a maze with two closed and two open arms. The task measures the amount of time spent in the closed and open arms. An anxious mouse will spend more time in the closed versus the open arms of the maze. Results of the elevated plus maze task show no difference in the amount of time spent in the closed versus the open arms between TRPC1+/+ and -/- mice (Figure 5.8).

Increased anxiety and a decrease in social interaction are indications of depression in mammals; therefore we sought to examine whether the TRPC1-/- mice have a propensity for a depression phenotype.

Depression studies in mice have been difficult due to the complexity of the depressed phenotype; however, recent efforts have been able to develop a behavioral phenotype for depression. In this paradigm, a mouse is physically defeated over the course of several days. Following defeat, a typical mouse will show signs of depression that are measured by social avoidance on a social interaction task, lack of sucrose preference, and increased anxiety on the elevated plus maze task. A pilot study looking at TRPC1 involvement in depressive behavior using TRPC1+/+ and -/- mice showed no difference in the social interaction, time spent in open arms on the elevated plus maze, or sucrose preference in defeated mice (Figure 5.9). There was a trend for the defeated mice in each group to show less social interaction than controls; however the effect was not significant. One interesting finding was the TRPC1+/+ defeated mice showed the expected decrease in time spent in the open arms and in sucrose preference compared to the TRPC1-/- control mice; however, the TRPC1-/- defeated mice showed no difference between the TRPC1-/- control mice in elevated plus maze or sucrose preference (Figure 5.9). These differences, however, are likely due to the large amount of variability seen in a small group of mice.

Hippocampal-dependent behaviors

Immunoblots for TRPC1 protein show robust expression in the HIP, an area that involved with spatial learning and contextual fear conditioning. To examine whether there are differences in spatial learning, TRPC1+/+ and -/- mice were tested in their ability to interact with an object in a novel location versus familiar location in the novel object location task. In this task, mice were allowed to interact with two objects in set positions in an arena with spatial cues on the walls over several training trials. The amount of time spent interacting with each object was recorded in the last training trial. For the test trial, one object was moved to a novel location and the time spent interacting with each object is measured. Typically, mice will interact more with the object in the novel location versus the familiar location. Results of the novel object location task show no difference in the ability of the TRPC1+/+ and -/- to distinguish the object placed in the novel location (Figure 5.10).

Another form of HIP dependent spatial learning is contextual fear conditioning, which tests the ability to form context dependent associations. In this paradigm, a negative stimulus (foot shock) is repeatedly paired with a context until that context itself will illicit a conditioned response (freezing). If the HIP is lesioned, animals are unable to respond to contextual fear conditioning, yet cue learned fear

conditioning remains intact [139-141]. In addition, fear conditioning extinction where the animal is repeatedly exposed to the context until the conditioned response is extinguished has been shown to be both HIP and PFC dependent [142, 143], both areas showing high TRPC1 expression. Our results indicate that contextual fear conditioning acquisition and extinction are intact in TRPC1-/- and +/+ animals, suggesting that TRPC1 is not involved in modulating these behaviors (Figure 5.11).

Discussion

In these studies, we used a TRPC1-/- mouse line to examine the role of TRPC1 in modulating neuronal behaviors. We confirmed that TRPC1 protein is eliminated in the brain of KO mice, and showed that there is high expression of TRPC1 in normal mice, especially in corticolimbic areas, such as the PFC, LS, and HIP, suggesting that TRPC1 may be involved in modulating neuronal learning and behavioral responses to cocaine.

A study by Kim *et al* (2003) reported that TRPC1 was responsible for the slow EPSC seen in the cerebellum following mGluR1 activation [73]. This study showed that an mGluR1-mediated slow EPSC in the

cerebellum is reduced following *in vitro* expression a TRPC1 mutant channel and application of a TRPC1 blocking antibody [73]. They also show that expression of TRPC1 in mGluR1 stably expressing CHO cells results in a DHPG-evoked slow activating inward current that is blocked by addition of the TRPC1 blocking peptide. In contrast, no significant inward current was evoked in mGluR1 stably expressing CHO cells that were transfected with TRPC3 [73]. Due to the reported involvement of TRPC1 in modulating cerebellar excitability, we hypothesized that loss of TRPC1 in the cerebellum may result in a deficiency in cerebellar dependent behaviors such as motor skills. To our surprise we found no reduction in the ability of TRPC1-/- to perform on an accelerating rotarod task. This suggests the following: 1) Cerebellar neurons that express TRPC1 channels and respond to mGluR1 activation are not involved in motor learning; 2) There was some compensation in the TRPC1 KO mice that diminished the necessity of TRPC1 channels in mediating the slow EPSC; or 3) The paper reporting TRPC1 receptors as responsible for the slow EPSC was flawed and some other TRPC channel is responsible.

Recently, a paper by Hartmann *et al* (2008) has shown definitively that TRPC3 rather than TRPC1 channels are responsible for the mGluR1-evoked slow EPSC in the cerebellum [114]. Their study showed that TRPC3-/- mice lack the mGluR1-mediated slow EPSC in the cerebellum

and that TRPC1-/- mice show normal synaptic transmission in the cerebellum [114]. They go further to show that TRPC3-/- mice have the expected deficit in motor skills, and confirm our result showing the lack of motor deficits in the TRPC1-/- mice [114]. Hartmann *et al* suggest that the conflicting results in these two studies may be due to the developmental differences in expression of TRPC1 and 3 [114]. TRPC3 has much higher expression than TRPC1 during adulthood, whereas TRPC1 and 3 have similar expression levels earlier in development. The Kim *et al* study used cultures slices taken at P10-12, whereas the Hartmann *et al* paper used acute slices from adult mice. Thus, it's possible that TRPC3 has a larger contribution to synaptic transmission in adulthood when it's highly expressed; and that early on in development, TRPC1 also has a contribution. A study looking at synaptic transmission in the cerebellum at different developmental time points of TRPC1 and 3 deficient mice would be interesting. It's also possible that simply the differences in methodology of slice preparation (slice culture vs. acute slices) could account for the different results in the two studies. Interestingly, the Hartmann *et al* paper did not consider compensation by other TRPC channels as a potential factor involved in the conflicted results. It's possible that there is compensation by TRPC3 channels in the TRPC1-/-

mice, which leads to the continued presence of the mGluR1-evoked slow EPSC.

The study by Kim *et al* suggesting the involvement of TRPC1 in the mGluR1-mediated slow EPSC in the cerebellum led us to question whether TRPC1/5 heteromultimeric complexes may be underlying the similar phenomenon we see in the PFC with the mGluR5-mediated dADP. The Kim *et al* paper found that co-expression of TRPC1 and 5 in mGluR1 stably expressing CHO cells resulted in a larger DHPG evoked current compared to when TRPC1 channels were expressed alone [73]. This evidence lends support to the idea that TRPC1 and 5 could be acting in concert in the PFC. However, patch-clamp recordings performed by our laboratory in PFC neurons of TRPC1-/- mouse found no reduction in the dADP. We considered that compensation by other TRPC channels could account for the continued presence of the dADP in TRPC1-/- neurons; however, real-time PCR results show no compensatory increases in mRNA in any of the other TRPC channels in the PFC. It is possible, however, that compensation is occurring at the level of protein. Our results in the PFC suggest that the dADP is mediated by homomultimeric mGluR5 activation of TRPC5 channels, however this has yet to be definitively determined as TRPC5 can also form heteromultimeric complexes with other TRPC channels, such as TRPC4 [54]. We also

suspected that the TRPC1-/- mice may have a similar cocaine phenotype as the PFC TRPC5-knock-out mice, but we found no differences in the locomotor activating or rewarding effects of cocaine in the TRPC-/- mice. It is important to note, however, that the doses used for CPP in the TRPC1 KO mice differ from those used in our TRPC5 KO studies. The TRPC5 KO studies used low (3.5 mg/kg) and moderate-high doses (7.5 mg/kg), whereas the TRPC1 KO studies used only a moderate dose (5 mg/kg). It's possible that a CPP dose response study could reveal a behavioral difference.

One interesting finding is that the TRPC1-/- have an increased startle response to a 120 dB pulse; however they do not appear to have differences in their hearing ability as evidenced by the lack of difference in the auditory evoked responses performed in collaboration with Dr. Heller in TRPC1+/+ and -/- mice. This increase in the acoustic startle response could indicate that the TRPC1-/- mice have a higher level of anxiety, however there were no differences between TRPC1+/+ and -/- mice in open field or elevated plus maze tests. Increases in the acoustic startle response, paired with a decrease in pre-pulse inhibition of startle has been shown to indicate deficits in sensorimotor gating in an Alzheimer's disease mouse model [144]. Sensorimotor gating refers to the ability to inhibit irrelevant data for sensory, motor, and cognitive functions; and deficits in

sensorimotor gating are seen in neurological disorders such as schizophrenia, attention deficit disorder, and obsessive compulsive disorder [145-147]. The best measure for deficits of sensorimotor gating in rodents is prepulse inhibition (PPI) of acoustic startle, which is a method in which a weaker prestimulus precedes a strong stimulus, reducing the startle response to the stimulus. PPI has been shown to be reduced in the aforementioned neurological disorders [148]. Future studies looking at PPI in the TRPC1 knock-out mouse line may reveal changes in sensorimotor gating in TRPC1-/- mice.

Though we were unable to find a definitive role for TRPC1 in regulating neuronal behavior, the robust expression of TRPC1 in the corticolimbic areas of the brain leads us to suspect that TRPC1 has some function in these areas that is yet to be determined. It is still possible that compensation by other TRPC channels is responsible for the lack of behavioral phenotype in the TRPC1-/- mice. We show that there is no compensation at the level of the mRNA by other TRPC channels in the PFC in response to the loss of TRPC1; however it is possible that compensation is occurring through some other mechanism we didn't test such as post-transcriptional compensation. In addition, we did not examine compensation in other brain areas where TRPC1 expression is high, such as the LS and HIP. It's possible that compensation by other

TRPC channels in those areas is occluding the TRPC1-dependent behavioral phenotype. Furthermore, due the proposed ability of TRPC channels to modulate neuronal excitability, it's possible that TRPC1 exerts different functions depending on the area in which it is expressed. This would make global knock-out of TRPC1 in all brain areas problematic. A closer look at knock-out of TRPC1 in specific brain areas where it is highly expressed may be more informative. In addition, the function of TRPC1 in the brain may be intimately connected to its ability to form heteromultimeric complexes with TRPC3, 4, and 5, which have already been implicated in neuronal functions and behaviors [45, 46, 61, 74, 77, 114]. Future studies should focus on dual-knockouts of TRPC1 with TRPC3, 4, or 5.

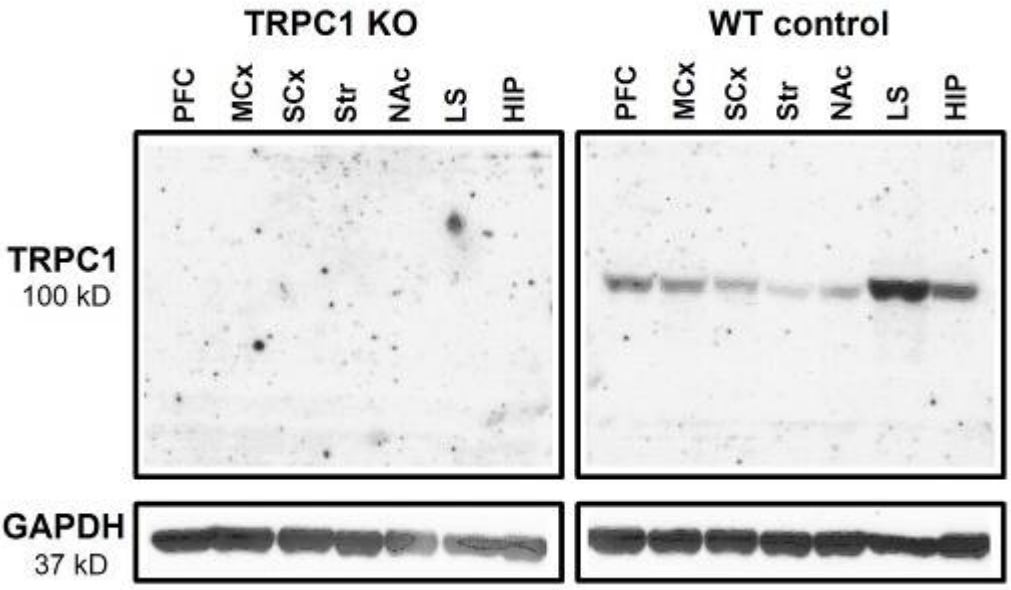

Figure 5.1 Knock-out of TRPC1 in the TRPC1 knock-out mouse line.

Immunoblots for TRPC1 and GAPDH protein from microdissections of the PFC, MCx, SCx, NAc, LS, and HIP in TRPC1-/- (left) and +/+ (right) mice.

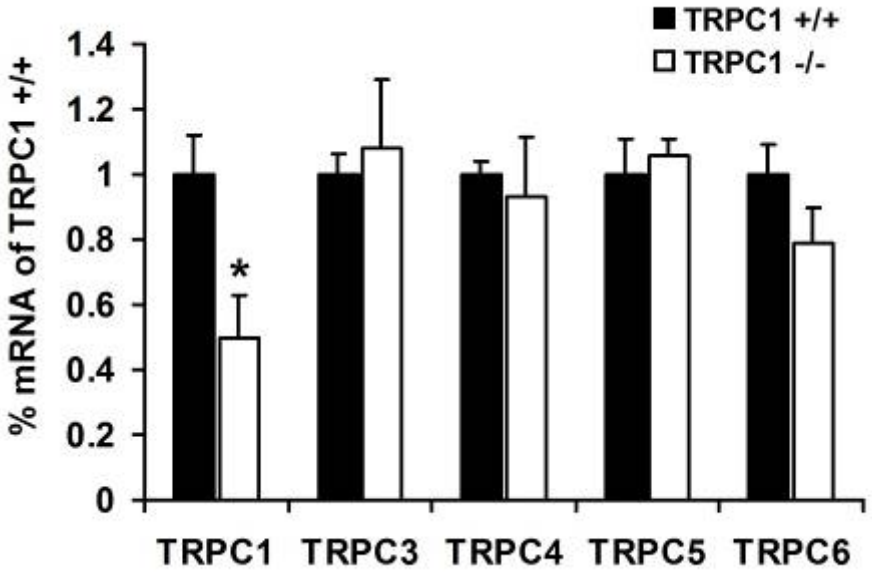

Figure 5.2 Compensation of TRPC channel mRNA expression in the TRPC1 null mouse line.

Quantitative real-time PCR of the TRPC channels expressed in the PFC of TRPC1+/+ and -/- mice (n=5 each; $p<0.05$).

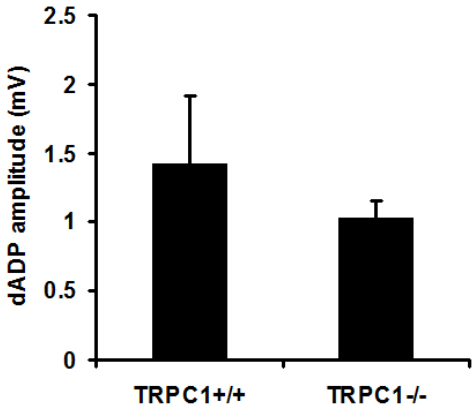

Figure 5.3 Examination of the dADP in the PFC of TRPC1 KO mice.

Graph showing the carbachol-induced (10 μM), burst-triggered dADP amplitude in PFC neurons of TRPC1+/+ (n=5) and TRPC1-/- (n=9) mice.

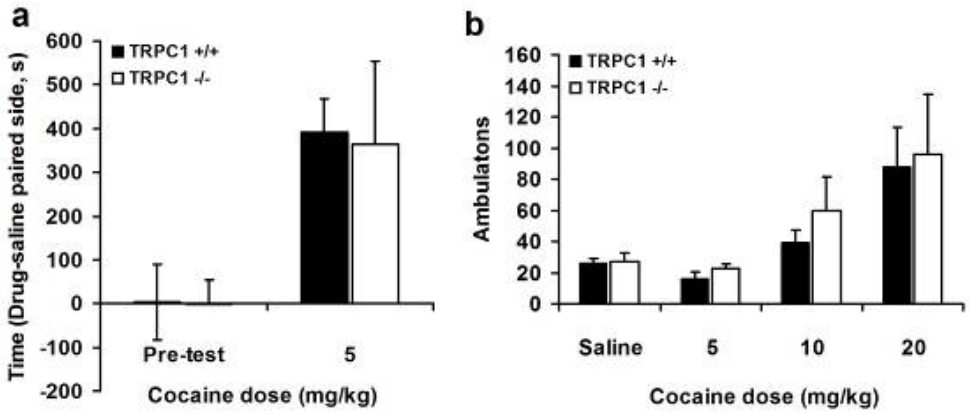

Figure 5.4 Conditioned place preference and locomotor response to cocaine.

(a) Cocaine conditioned place preference at 5 mg/kg of cocaine in TRPC1+/+ (n=10) and -/- (n=13) mice. **(b)** The ambulatory locomotor response to i.p. injections of 5, 10, and 20 mg/kg cocaine in TRPC1+/+ (n=10) and -/- (n=13) mice.

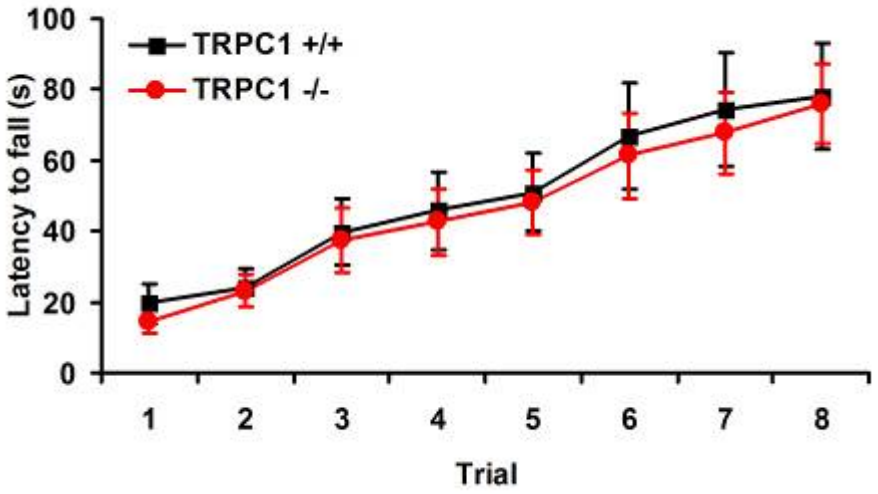

Figure 5.5 Rotarod.

Graph shows the latency to fall (s) from an accelerating rotarod (0-45 rpm over 5 minutes) by TRPC1+/+ (n=12) and -/- (n=14) mice.

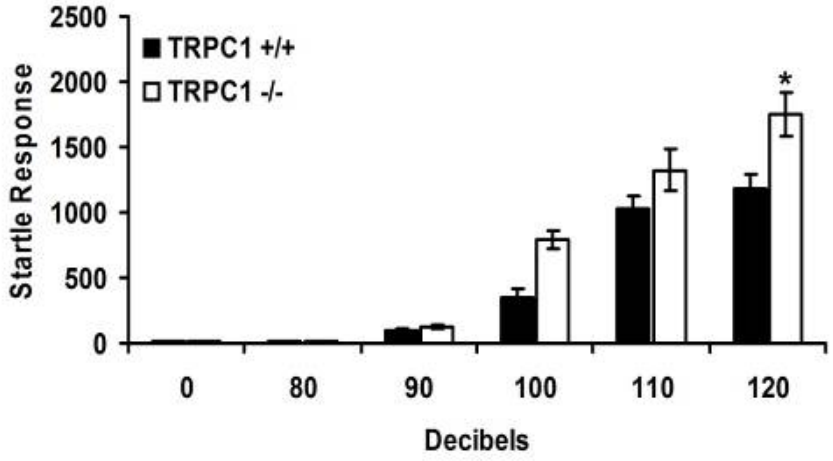

Figure 5.6 Acoustic startle.

Graph shows the startle response to decibel levels from 0-120 dB, presented randomly over 15 minutes. The bars represent the average startle response to each dB level in TRPC1+/+ (n=9) and -/- (n=10) mice ($p<0.05$ for 120 dB).

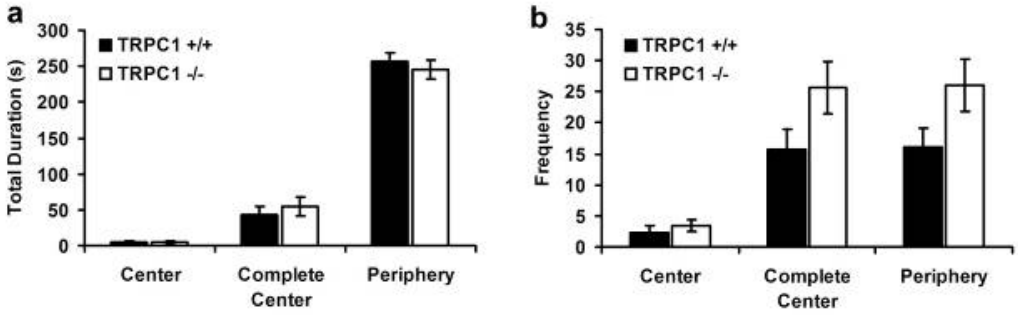

Figure 5.7 Open field.

Graphs show the amount of time spent in **(a)** and frequency to enter **(b)** the center, complete center, and periphery of an open field arena by TRPC1+/+ (n=12) and -/- (n=14) mice.

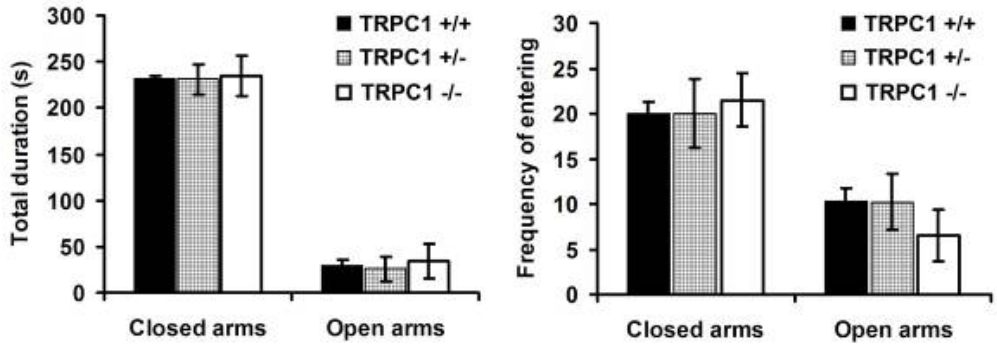

Figure 5.8 Elevated plus maze.

Graphs show the duration spent in **(a)** and frequency to enter **(b)** the closed and open arms of an elevated plus maze in TRPC1+/+ (n=10), +/- (n=5), and -/- (n=13) mice.

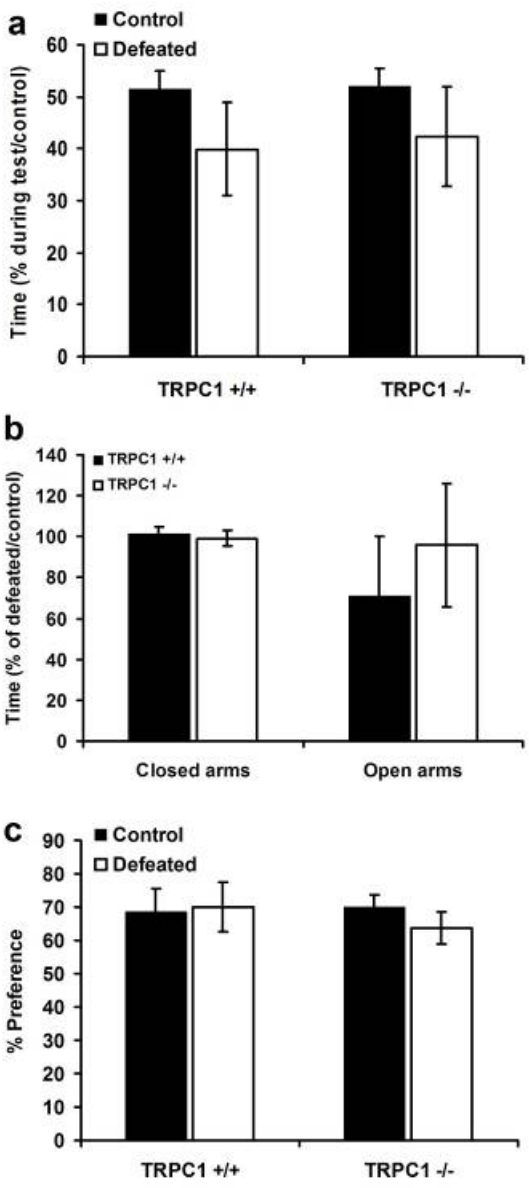

Figure 5.9 Social defeat.

(a) Graph shows the % of time spent interacting with an unfamiliar CD1 mouse by control TRPC1+/+ (n=8) and -/- (n=8) mice, and defeated TRPC1+/+ (n=6) and -/- (n=6) mice. **(b)** Graph showing the percent time the defeated mice in (a) spent in the closed and open arms of an elevated plus maze compared to the control mice in (a). **(c)** Graph showing the percent preference of sucrose over water in the two days following defeats of the mice in (a).

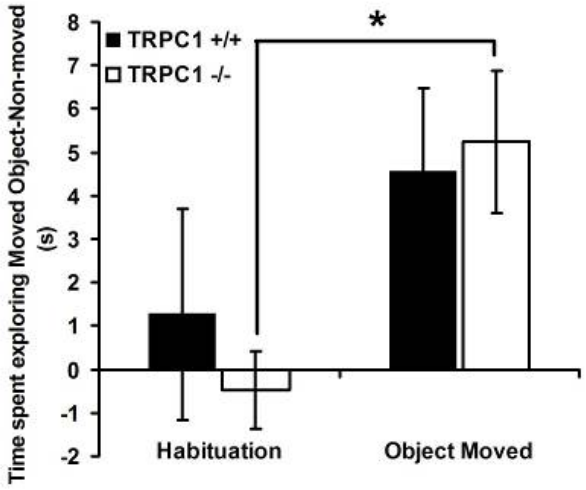

Figure 5.10 Novel object location discrimination.

Graph shows the time spent exploring an object moved to a novel location following five habituation trials in the original location in TRPC1+/+ (n=12) and -/- (n=14) mice ($p<0.05$ in TRPC1-/- mice).

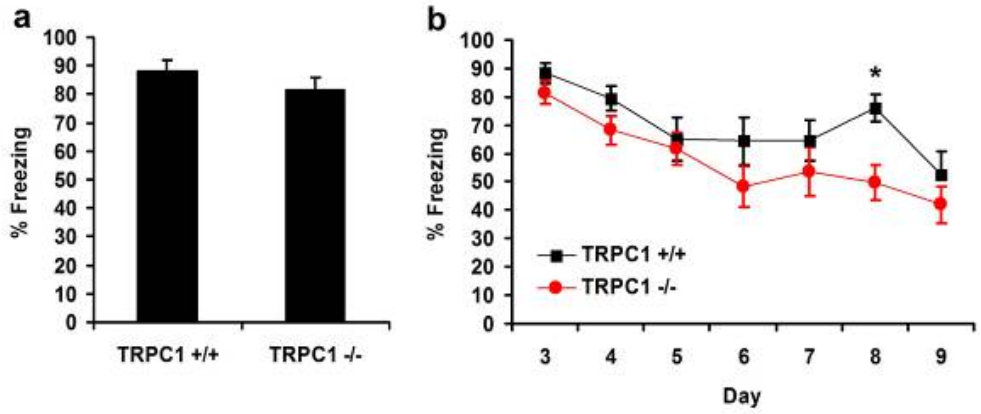

Figure 5.11 Contextual fear conditioning.

(a) Acquisition of the fear response in TRPC1+/+ (n=7) and -/- (n=8) mice.
(b) Extinction of contextual fear over 9 days in animals in (a)

CHAPTER SIX: CONCLUSIONS

Drug addiction is a disease characterized by the compulsion to seek drugs despite adverse consequences. Both genetic traits and environmental factors influence the development of addiction in humans [3, 4]. These factors can cause alterations in excitability in the brain reward circuitry, which is important for mediating the rewarding effects of both naturally occurring rewards (i.e. food and sex) and pyschostimulant drugs [7]. The PFC is critical for modulating the behavioral responses to addictive drugs, and changes in PFC excitability mediate the uncontrollable urges for drug seeking associated with drug addiction [6]. In addition, normal PFC functioning, such as attention and working memory are disrupted following repeated psychostimulant use [34]. Understanding how PFC excitability and functioning influence the behavioral responses to psychostimulants is fundamental to understanding addiction. In these studies, we implicate the TRPC5 channels as important for modulating PFC excitability and show that loss of TRPC5 results in susceptibility to cocaine's psychostimulant and rewarding effects.

Chronic cocaine use has been shown to disrupt PFC processes, such as attention and working memory. The mechanism responsible for

PFC working memory is unclear. Recordings during working memory tasks show persistent action potential output in PFC neurons in the delay period between the presentation of a stimulus and the opportunity to make a behavioral response. It is thought that persistent activity is a mechanism for neurons to retain salient information necessary for making a behavioral response. In these studies, we identify an intrinsic mechanism for persistent activity in the PFC which is modulated by chronic cocaine and dopamine 1 receptor (D1R) stimulation. We show that layer 5 pyramidal neurons in the PFC exhibit a delayed after-depolarization (dADP) following brief bursts of action potentials coupled with the activation of G_q-coupled receptors, such as mGluRs or muscarinic acetylcholine receptors. This prolonged depolarizing response results in an increase in the firing frequency of the cell for several seconds, which may enable the neuron to convert sub-threshold inputs into persistent firing output as a way to store information briefly. The dADP is modulated by dopamine (DA) through a D1R/PKA pathway. Application of DA or a D1R agonist reduces the dADP amplitude; yet, activation of D2Rs has no effect. This reduction in the dADP may serve to bias or focus neurons towards very strong inputs, such as those associated with drug cues, while preventing the neuron from responding to smaller, sub-threshold inputs. Dopamine may thus bias PFC-dependent attentional processes toward powerful drug-related

cues. We sought to identify the non-selective cation channel responsible for the dADP in PFC neurons and the role of the dADP in modulating cocaine-induced behavior.

Our suspicion that TRPC5 channels were involved in mediating the dADP in the PFC sprouted from pharmacological and expression data. Induction of the dADP is dependent upon intracellular Ca^{2+} and on IP_3R activation. Furthermore, the dADP is blocked following application of a non-selective cation channel blocker, flufanemic acid. The TRPC channels are a family of non-selective cation channels that are shown to be activated following G_q-coupled receptor signaling and are dependent upon activation of the PLC-IP_3R pathway and intracellular Ca^{2+}. The application of a TRPC3/6/7 blocker, La^{3+}, failed to block the dADP; and application of La^{3+} alone was able to induce a small dADP, presumably through its ability to potentiate TRPC4/5 channels. These results suggest TRPC4 or 5 channels as possible candidate channels for mediating the dADP.

We sought to examine the expression of the TRPC channels in the rodent brain in order to provide evidence for their potential role in underlying the dADP seen in the PFC. At the start of this project, there were few expression studies examining the distribution of the TRPC channels in the mammalian brain. We provided an extensive

characterization of the distribution of the TRPC channels that remains the most thorough study of the expression of these channels in the rodent brain published to date. These studies found that the TRPC4 and 5 channels have the highest expression in the rat brain. A closer look reveals that the TRPC4 and 5 channels are also the highest expressed channels in the areas where we see the dADP. The TRPC5 channels, in particular, showed dense expression specifically in the pyramidal cell layers of the PFC which is consistent with their potential involvement in mediating the dADP. The pharmacological and expression data presented, however, was merely correlative, so our next step was to use knock-out studies to definitively show that TRPC5 is necessary for the induction of the dADP in the PFC.

We first obtained a full TRPC5 knock-out mouse from Dr. Lutz Birnbaumer, however, due to a compensatory increase in the expression of TRPC3 mRNA in the TRPC5-/- mouse, we turned to a conditional method for knock-down with AAV-Cre-GFP injections into TRPC5flx mice. This method allowed us temporal and regional control over the knock-down of TRPC5 in the PFC. Viral mediated, AAV-Cre-GFP, knock-down of TRPC5 in the PFC of TRPC5flx mice indicated that TRPC5 channels are necessary for induction of the dADP in pyramidal neurons of the PFC. These studies implicate the TRPC5 channels in modulation of PFC

excitability, and specifically, for induction of persistent activity in the PFC. This ability of the TRPC5 channels to modulate persistent activity in the PFC lends evidence for their role in PFC-dependent processes, such as working memory and attention. Deficits in these processes are associated with a plethora of psychological disorders, such as attention deficit disorder, schizophrenia, and drug addiction [149, 150]. Due to the ability of DA and cocaine to modulate the dADP, we focused on how TRPC5 loss affected behavioral responses to cocaine.

Viral-mediated, PFC knock-down of TRPC5 produced enhanced cocaine locomotor response and reward in a CPP paradigm. A similar approach to knock-down TRPC5 in the HIP failed to increase the rewarding or psychostimulant effects of cocaine, suggesting that the increase in drug seeking is mediated by changes in PFC and not HIP function. To further examine a role for TRPC5 in cocaine mediated behaviors we crossed the TRPC5 floxed mouse line with the CamKII-Cre mouse line to achieve knock-down of TRPC5 in the forebrain areas. Using this line, we show that loss of TRPC5 in the CAMKII expressing neurons results in an increase in the rewarding effects of cocaine in the CPP paradigm. We propose that the reduction in PFC excitability following loss of the TRPC5-mediated dADP is responsible for the increase in the rewarding effects of cocaine by biasing the cell towards the

strong synaptic inputs. The involvement of TRPC5 in modulating cocaine reward suggests that repeated cocaine use may alter TRPC5 function in a way that alters PFC excitability and results in the uncontrollable drive for drug seeking seen in addicts. However, drug addiction has been shown to be dependent upon both genetic and environmental factors. It's possible that genetic changes in TRPC5 expression or function may create susceptibility for drug abuse in humans. It would be interesting to see if levels of TRPC5 expression in the PFC are altered in normal vs. addicted human subjects.

Interestingly, though loss of mGluR5 results in loss of the dADP in the PFC, we do not see an enhancement in the locomotor and rewarding effects of cocaine in mGluR5-/- mice. To the contrary, previous studies show a complete absence of the locomotor activation and rewarding effects of cocaine [119], and we see a reduction in locomotor activation to high doses of cocaine in mGluR5-/- compared to +/+ mice. Though the results of our behavioral studies with mGluR5 differ somewhat a previous study it is clear that loss of mGluR5 does not have the same behavioral effect as loss of TRPC5. However, it is imperative to note that the mGluR5 knock-out line is a full knock-out in all brain areas, whereas we specifically knocked out TRPC5 in the PFC or the forebrain areas. MGluR5 is highly expressed in the NAc and dorsal striatum, which is

involved in mediating the effects of cocaine. We would expect loss of mGluR5 in the NAc to result in a blunted response to cocaine. We propose that mGluR5 exerts oppositional effects on the NAc and the PFC, and that loss of mGluR5 in the NAc would result in a reduced response to cocaine whereas loss in the PFC would result in an enchanced response to cocaine. It would be interesting for future studies to selectively knock-down mGluR5 in the NAc and the PFC to see if loss of mGluR5 in each area would have opposing effects on the rewarding properties of cocaine.

The last study sprouted from reports that TRPC1 and 5 form heteromultimeric complexes in the brain [151], suggesting that TRPC1/5 complexes may be responsible for mediating the dADP in the PFC. However, patch-clamp recordings of the PFC performed by our laboratory in TRPC1-/- mice found no reduction in the dADP (data not shown). The lack of compensation of other TRPC channels mRNA in the PFC of TRPC1-/- mice suggest that the continued presence of the dADP is not due to a compensatory increase in TRPC channel expression. These findings lend evidence that the dADP is mediated by homomeric TRPC5 channel complexes; however, this has yet to be definitively determined.

The dense expression of the TRPC1 channels in the corticolimbic areas led us to hypothesize that TRPC1 may have some modulatory role in neuronal learning and plasticity, as well as on behavioral responses to

cocaine. Our behavioral tests of the TRPC1 knock-out mice did not reveal any striking role for TRPC1 in modulating neuronal behaviors. However, it would be premature to conclude that TRPC1 is not involved in regulating neuronal behaviors because its function may be intimately connected to its ability to form heteromultimeric complexes with TRPC3, 4, and 5. Futures studies looking at dual TRPC1 and TRPC3, 4, or 5 knock-outs could reveal the function of TRPC1 in the mammalian brain.

In conclusion, our studies sought to reveal a novel role for TRPC channels in regulating neuronal excitability and behavior. We showed that TRPC5 channels underlie a burst-activated dADP that could be a mechanism for persistent activity in the PFC underlying transient single-cell memory. Loss of TRPC5 channels in the PFC disrupted PFC excitability and led to an enhanced sensitivity to the locomotor activating and rewarding properties of cocaine. These studies lay the groundwork for future studies looking at how TRPC5 channels may be involved in modulating other PFC-dependent processes, such as attention and working memory. The involvement of TRPC5 in modulating PFC excitability also lends support for future studies looking at the role of TRPC5 in other psychological disorders, such as attention deficit disorder and schizophrenia.

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