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PAM Heterozygosity Disrupts Amygdalar Neurophysiology And Copper Homeostasis With Behavioral Consequences

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Peptidylglycine α-amidating monooxygenase (PAM) is a secretory granule membrane protein whose luminal enzymatic domains catalyze the Cu-dependent amidation biosynthetic step common to many neuroactive peptides. Aside from its essential role as the sole mammalian amidating enzyme, PAM alters Cu homeostasis, modulates transcription and regulates secretory granule trafficking. The model of PAM haploinsufficiency employs mice heterozygous for the Pam gene (PAM+/-); the mice show anxiety-like disorders and deficiencies in fear learning and memory. We performed whole-cell recordings of pyramidal neurons in the PAM+/- amygdala to elucidate neurophysiological correlates of the fear behavioral phenotypes. Consistent with these observations, thalamic afferent synapses in the PAM+/- lateral nucleus were deficient in long-term potentiation (LTP). This deficit was apparent in the absence and presence of the GABA_A receptor antagonist picrotoxin and was abolished when both GABA_A and GABA_B receptors were blocked. Dietary Cu supplementation rescued the cued learned-fear deficits of PAM+/- mice, with little effect on the behaviors of wildtype mice. Dietary Cu supplementation also corrected the LTP deficit of PAM+/- mice in vitro. Bath application of the extracellular specific Cu chelator bathocuproine disulfonate abolished LTP in wildtype and PAM+/- amygdalae, demonstrating a vital role for Cu in amygdalar synaptic plasticity. Dietary Cu supplementation
had no effect on brain Cu or PAM levels, therefore PAM<sup>+/−</sup> behavioral deficiencies do not result from insufficient Cu and/or PAM. Localization of the major neuronal Cu transporter, ATP7A, was altered in the PAM<sup>+/−</sup> brain. In addition, quantitative PCR revealed region-specific deficits in Atox-1 and ATP7A that may account for the physiological and behavioral defects associated with PAM heterozygosity. These data indicate that Cu is necessary for normal amygdalar synaptic function, suggesting the PAM<sup>+/−</sup> behavioral and physiological phenotype stems from dysregulated Cu secretion. Additional studies include analyses of serum Cu and PAM in a population of frail elderly men and electrophysiological study of hippocampal and nucleus accumbens neuronal membrane and synaptic properties in Kalirin7 null mice. Future directions to test the hypothesis of a direct role for PAM in regulating neuronal Cu secretion and explore the essential role of Cu at amygdala afferent synapses are outlined.
PAM Heterozygosity Disrupts Amygdalar Neurophysiology and Copper Homeostasis with Behavioral Consequences

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B.A. Ithaca College, 2005

Doctoral Dissertation

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APPROVAL PAGE

Doctor of Philosophy Dissertation

PAM Heterozygosity Disrupts Amygdalar Neurophysiology and Copper Homeostasis with Behavioral Consequences

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to and balance between science and education to which these two remarkable people devote themselves everyday. They are by far the most hardworking and supportive faculty at UConn. Dick and Betty have played a central role in almost all of the progress and growth I’ve made as a scientist. Additionally, the many things I’ve learned from them extend well beyond scientific methods and principles to the greater contexts of the university, medicine, career and life. In this way, much of my success, including finishing medical school, matching to a competitive residency program and future challenges I will have, I owe to them. I cannot thank them enough for the knowledge and wisdom they’ve shared with me over the years. I also sincerely appreciate the trust they had in me to represent them at various scientific meetings and conferences. Finally, Dick and Betty are not just invaluable mentors, they are close friends with whom I am so lucky to have shared some of the best and challenging years of my life.

Friends

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I met early in my training and she has been by my side throughout my medical and graduate training. There are so many things I must thank her for. I thank her for her patience as I worked late in the lab or thought through new ideas or experiments out loud at the dinner table. I thank her for her uncompromising, unconditional love and support, through my frustrations of failed experiments as well as celebrating successful ones. I thank her for her understanding and caring enough to understand. I thank her for being a light in my life that has kept me both motivated and focused on becoming the best man I can be. I thank her for saying ‘yes’, and I thank her for sharing her love and her life with me.

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being my inspiration to pursue a career in medicine. When I was a young child, uncle Nashat gave me a plastic painted heart he’d been awarded early in his own medical career. I still don’t know why he chose to give me this gift, but it meant the world to me. This heart has and will continue to sit in the most prominent place on my desk where it has encouraged me to continue studying and working even into the late hours of the night through high school, college, medical school, graduate school and even now as I write this dissertation. Throughout my education his excellent example of what it means to be a capable and compassionate doctor has stood as the motivation and the means for my own success. I thank my aunts and uncles and many cousins, especially Giovanni Rabadi, Tony Rabadi, and Nicolette Rabadi Jaze. These extraordinary people have been like second mothers, fathers and siblings to me growing up. They have taught me innumerable life lessons, to be passionate, inquisitive, wise, good-hearted and generous. Through their very different examples, I have been able to recognized and appreciate the beauty that family brings to our lives.

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I thank my maternal grandparents, Hanna and Hillaleh Rabadi, who immigrated to this country in the early 1970s from Jordan. I have few memories of my grandmother, Hillaleh, who died when I was 2 years old. I have always believed that her spirit looks over me, keeps me safe, and gives me the strength and ability to achieve everything I pursue in life. I hope I make her proud.

My grandfather, whom I call Gidoh, passed away the summer of 2007 when I began my graduate studies in the lab. He has always been and remains the most influential person in my life. Among the first memories I have of him, Gidoh informed me of my destiny to become a doctor. He would remind me of this whenever I’d see him, from my early childhood to adolescence to adulthood. He would always ask me about school – I suppose to check that I was still on track to fulfill this destiny. ‘Good’ was never enough; I had to be ‘Excellent’, ‘Number One in the school’! While I was never the best at anything, Gidoh always let me know how very proud of me he was.

When I was in high school and college I would go to visit him on my own, and Gidoh would share his life’s lessons with me. Sitting next to him on the couch, I’d listen intently to his advice. Often he’d forget which lessons he’d already given, but I never minded hearing them again. The meaning of many of these lessons would not become clear to me until years later, and I am certain I still have much to learn from them. He told me the three most important things to which I must dedicate myself are God, school and family (specifically in that order). He also said one day these priorities will shift to include a wife and my own family, but not until I finish school of course. At the conclusion of every visit, Gidoh would remind me that he thinks about me and prays for me at least three times
daily. He said he didn’t pray for my success, but that the success he knew I would have would come easily. One of the last times I visited with him, I asked him what else I can do in my life to make him proud. He told me to be the best doctor I can, and to raise my children to be smart, good doctors like me.

One of the happiest moments of my life was seeing the smile on Gidoh’s face when I shared the news that I had been accepted to the UConn MD/PhD program. He laughed when I said he must have over-prayed for me because I would receive not one but two doctoral degrees by the end of my training. I told him that he deserves one of those degrees for his role in my accomplishments. In keeping that promise, I hereby dedicate this work and this achievement to him.
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CHAPTER 1: Introduction
Peptides are among the most diverse and primitive signaling molecules in the animal kingdom. In contrast to small molecule neurotransmitters such as glutamate and γ-aminobutyric acid (GABA), peptides are packaged into large dense core vesicles whose release is governed separately from that of small synaptic vesicles (Zupanc, 1996; Salio et al., 2006; Tallent, 2008). Whereas small synaptic vesicle release mediates fast synaptic transmission, large dense core vesicles have higher activity thresholds for release and their contents typically modulate neuronal activity and fast synaptic transmission (Tallent, 2008). Despite their diversity, many peptides share a common biosynthetic pathway. It is estimated that about half of all bioactive peptides are amidated (Eipper and Mains, 1988; Eipper et al., 1992). Peptidylglycine α-amidating monooxygenase (PAM) is the sole source of amidating activity to complete this essential reaction in mammals (Czyzyk et al., 2005a). In many cases, amidation is required to confer full biological activity related to optimal G-protein coupled receptor binding (Eipper et al., 1992).
PAM has two luminal enzymatic domains (peptidylglycine α-hydroxylating monooxygenase, PHM, and peptidyl-α-hydroxyglycine α-amidating lyase, PAL) that act sequentially to catalyze the cleavage of a C-terminal glycine at the N-Cα bond, creating an amide (–NH₂). Two copper (Cu) ions are bound by the PHM enzyme and serve in electron transfer (Prigge et al., 2000). Two ascorbates act as reducing agents and molecular dioxygen contributes to water and glyoxylate products. PAL typically uses one Zn ion for catalysis (although other metals may substitute) and one calcium ion for structure. Various peptides are differentially affected by ascorbate and Cu availability presumably related to tissue-specificity and precursor sequence (Eipper and Mains, 1988; Eipper et al., 1992). The complete reaction is depicted in Fig. 1-1.

PHM and PAL are encoded by separate genes in lower organisms such as Cnidaria and Drosophila (Williamson et al., 2000; Kolhekar et al., 1997). In mammals, including mice and humans, the single PAM gene (located on chromosome 1 in mice and 5 in human) encodes a single protein with both enzymatic domains and is the exclusive source for amidating enzymatic activity in the body (Czyzyk et al., 2005a). Cu is delivered to PHM within the secretory pathway via Menkes disease protein ATP7A, which receives cytosolic Cu from Atox-1 (Fig. 1-2).

Homozygous knockout of the Pam gene (PAM⁻⁻) is lethal at E14.5 in mice (Czyzyk et al.,...
2005a). PAM<sup>−/−</sup> mice die in utero with profound edema, a phenocopy of the mice with genetic deficiency of adrenomedullin (Czyzyk et al., 2005a). This is not surprising since adrenomedullin is an amidated peptide involved in maintaining vascular integrity and development (Czyzyk et al., 2005a; Caron and Smithies, 2001). Mice heterozygous for the *Pam* gene (PAM<sup>+</sup><sup>−</sup>) exhibit half of normal amidating activity and grow and reproduce normally, but exhibit striking physiological and behavioral abnormalities. PAM<sup>+</sup><sup>−</sup> mice display increased adiposity and impaired glucose tolerance at 10 month of age (Czyzyk et al., 2005a). PAM<sup>+</sup><sup>−</sup> mice are unable to maintain their body temperature in the cold (Bousquet-Moore et al., 2009), resulting from a combination of deficiencies in vasoconstriction and shivering (Bousquet-Moore et al., 2010b). Neurologically, PAM<sup>+</sup><sup>−</sup>

![Figure 1-2. PAM schematic.](image)

(left) PAM localizes to the secretory pathway of neuroendocrine cells. It is present in the endoplasmic reticulum (ER), golgi apparatus, and secretory granules that comprise the secretory pathway. PAM requires Cu for its catalytic activity. Cu enters cells through the Cu transporter CTR1. Cu is delivered to PAM by the cytosolic chaperone Atox-1 and the P-type ATPase ATP7A (not shown). The cytosolic domain of PAM is cleaved to yield a soluble fragment (sfCD), which enters the nucleus and can up-regulated expression of Atox-1. (right) Cleavage of PAM-1 can occur between the enzymatic domains PHM and PAL. Sequential cleavage between PAL and the TMD, followed by cleavage within the TMD by γ-secretase yields soluble PHM/PAL and the sfCD. The cytosolic domain of PAM is multiply phosphorylated.
mice are hyper-sensitive to the GABA_A receptor antagonist pentylenetetrazol, displaying more severe seizure behaviors at lower doses than their wildtype littermates. They also display more anxiety-like behaviors in the elevated-zero maze (Bousquet-Moore et al., 2010b).

The PAM^{+/−} phenotype could result from a deficiency in amidated neuropeptides through a reduction in amidating activity, but this is unlikely to be the case. Levels of amidated α-melanocyte stimulating hormone, joining peptide and cholecystokinin are not significantly altered in postnatal or adult PAM^{+/−} hypothalami (Czyzyk et al., 2005a). Even for glycine-extended thyrotropin releasing hormone (TRH-Gly), a particularly poor PHM substrate due to its penultimate proline residue, PAM heterozygosity imposes only a modest increase in TRH-Gly levels (Bousquet-Moore et al., 2010b). This suggests that the mechanism of the PAM^{+/−} phenotype likely results from alterations outside of amidating activity.

Indeed PAM plays functional roles outside of amidation that involve Cu homeostasis [for review see (Bousquet-Moore et al., 2010a)]. Over-expression of PAM in a pituitary corticotropic cell line reorganizes the actin cytoskeleton, blocks regulated secretion of even non-amidated peptides and impairs normal secretory granule maturation and localization (Ciccotosto et al., 1999). These effects are mediated through the cytosolic domain of PAM (Alam et al., 2001) which can be cleaved to yield a soluble fragment (sfCD) that localizes to the nucleus (Fig. 1-2) (Rajagopal et al., 2009; Francone et al., 2010). PAM over-expression in a corticotropic cell line increases transcription of Atox-1 (Francone et al., 2010) and Atox-1 mRNA is reduced in the PAM^{+/−} pituitary (Bousquet-Moore et al., 2010b). Cu can regulate the cleavage and recycling of PAM (De et al., 2007) and could therefore have important effects on sfCD signaling. High Cu reduces
the cleavage and secretion of PHM, and increases the degradation of endocytosed PAM to the secretory pathway (De et al., 2007). This bidirectional interaction clearly demonstrates a non-enzymatic role for PAM in Cu homeostasis.

It was originally speculated that any deficit in amidating activity associated with PAM heterozygosity could be improved by supplementing Cu or recapitulated in wildtype mice by restricting Cu through the diet and thus decreasing PAM activity (Bousquet-Moore et al., 2010b). While our hypothesis for how PAM heterozygosity mediates its effects on physiology and behavior have since diverged from amidation, the experiments testing PAM+/− rescue and wildtype recapitulation of the PAM+/− phenotype yielded intriguing results. For dietary Cu supplementation, mice were given 300 parts per million (ppm; 1.2 mM) of CuSO₄ in the drinking water for 8-14 days. Despite the fact that amidation was not dramatically reduced in PAM+/− mice or influenced by dietary Cu supplementation, dietary Cu supplementation rescued temperature regulation, vasoconstriction impairments and anxiety-like behaviors in PAM+/− mice with little effect on wildtype mice. However, dietary Cu supplementation had no influence on the increased seizure susceptibility of PAM+/− mice. For dietary Cu restriction, mice were placed on specialized food with 25-fold less Cu than control food. Wildtype temperature regulation, vasoconstriction, anxiety-like behavior and seizure susceptibility all declined with dietary Cu restriction, whereas PAM+/− performance was relatively unaffected. Moreover, the hypothesis of PAM+/− physiological and behavioral impairment rescue with dietary Cu supplementation and recapitulation in wildtype mice with dietary Cu restriction was confirmed. If the mechanisms for the impairments exhibited by PAM+/− mice do not involve amidation, these data strongly suggest they involve Cu.

When I began my graduate work, a set of preliminary data from the laboratory of our...
collaborator, Dr. William C. Wetsel at Duke University, was completed on wildtype and PAM*/- mice sent from our lab. These data indicated that PAM*/- mice display increased anxiety-like behaviors and a marked deficit in learned fear behaviors. This paradox in altered fear expression intrigued me, so I took on the task of elucidating the neural basis for the PAM*/- fear phenotype primarily using electrophysiology as the main focus of my thesis project.
The amygdaloid complex

The expression of anxiety-like behaviors and fear associated learning and memory both involve the amygdala – a group of limbic nuclei situated in the medial temporal lobe of the mammalian brain [for review see (Sah et al., 2003; Ledoux, 2000; Maren and Quirk, 2004; Pare et al., 2004; LeDoux, 2007; Perez de la et al., 2008)]. Research concerning the amygdala is rapidly expanding in the fields of neuroscience and psychiatry. Despite the complexity and heterogeneity of its structure, the amygdaloid complex and the nuclei that comprise it house the simplest and most accessible neural correlate of learning and memory to date (LeDoux, 2007; Sah et al., 2008).

The vast majority of nuclei and subnuclei are conserved throughout mammalian species (Moreno and Gonzalez, 2007), making the amygdala a very attractive region for brain-behavior study applicable to human disease (Sah et al., 2008). The basolateral amygdaloid complex, comprised of the lateral (LA) and basolateral (BLA) nuclei, receives afferents from many different cortical and subcortical (thalamic) regions and is thus considered the input region of the amygdala (Fig. 1-3). The central amygdala group (CeA) receives glutamatergic fibers from LA and BLA principal neurons and acts as the major output region of the amygdala. GABAergic projections from CeA extend to hypothalamic and brain stem nuclei to drive fear and anxiety-like behaviors. The freezing behavior relevant to fear conditioning is mediated through GABAergic projections from the CeA to the periaqueductal gray in the midbrain (Pare et al., 2004). Local interneuronal and paracapsular GABAergic cells mediate tight feed-forward and feedback inhibition of excitatory signals through the amydaloid complex (Marowsky et al., 2005; Jungling et al., 2008). While a solid framework of amygdala circuitry has been established, it is unclear how the physiology underlying the inputs onto and
interconnections between amygdala nuclei mediate fear and anxiety-like behaviors.

Plasticity at glutamatergic afferent synapses onto LA pyramidal neurons is widely thought to underlie discrete associative fear learning [for review see (Ledoux, 2000; Maren and Quirk, 2004; Kim and Jung, 2006; Sigurdsson et al., 2007; Sah et al., 2008)]. Fear conditioning is a form of classical Pavlovian learning wherein a neutral, conditioned stimulus, acquires emotional valence following its temporal pairing with an aversive, unconditioned stimulus. During training, strong excitatory neural signals reaching the LA convey information concerning the conditioned and unconditioned stimuli. It is thought that these signals trigger synaptic changes while eliciting fear.
behavior during training. Moreover, whereas the conditioned stimulus was formerly unable to drive fearful behavior before training, subsequent presentations are sufficient to elicit fear responses due to specific enhancement in synapses representing the conditioned stimulus. Synaptic changes elicited in the LA are thought to mediate cue-dependent fear while changes in the BLA are thought to mediate context-, hippocampal-dependent fear (Calandreau et al., 2005; Reijmers et al., 2007). Due to the relative simplicity of the circuitry involved, auditory cue-dependent fear conditioning in the LA is more widely studied. In this case, auditory conditioned stimulus information from the auditory thalamus reaches the LA through the fibers passing through the internal capsule and from auditory cortex through the external capsule (Weisskopf et al., 1999; Huang and Kandel, 1998). Neural signals conveying the unconditioned stimulus come from the posterior intralaminar nucleus and the medial geniculate body of the thalamus (Lanuza et al., 2008). Both conditioned and unconditioned stimuli pathways are carried within thalamic afferents. The synapses contributed by thalamic versus cortical afferents impinging on LA pyramidal neuronal dendrites are morphologically, biochemically and functionally different (Humeau et al., 2005; Humeau and Luthi, 2007). Collaterals from cortical and thalamic afferents also impinge on the same population of local GABAergic interneurons within the LA (Szinyei et al., 2000) (Fig. 1-3), with thalamic inputs conveying stronger excitatory signals (Doyere et al., 2003; Shin et al., 2006).

Understanding the mechanisms of synaptic plasticity at amygdala afferent synapses holds great potential for elucidating the pathophysiology underlying psychiatric disorders of fear and anxiety (LeDoux, 2007; Mathew et al., 2008). Moreover, elucidating the synaptic basis for how fear memories are formed and stored is highly relevant to post-traumatic stress disorder (Liberzon and Sripada, 2008). PAM mRNA is present in the
rodent amygdala at high levels as assessed by *in situ* hybridization (Schafer et al., 1992a). In the context of the PAM\(^{+/−}\) fear phenotype, investigating the role of PAM and Cu in amygdala function is likely to be important to this rapidly growing field of research. To date, there is no published work investigating the synaptic/physiological or behavioral roles of Cu in the amygdala.
Cu is a necessity for eukaryotic life and tightly regulated in complex organisms

Copper (Cu) is an essential trace element whose ability to donate and accept electrons gives it great utility in multicellular life. Cu serves many functions in biology through oxidation-reduction reactions and its ability to bind and activate dioxygen. Cu is primarily found in nature in its cupric state (Cu$^{2+}$) and can switch to the cuprous state (Cu$^{1+}$) and back by accepting and donating an electron, respectively. It is believed that microorganisms evolved the ability to utilize Cu when oxygen became more prevalent in the atmosphere (Crichton and Pierre, 2001; Ridge et al., 2008). In this newly-formed oxidative environment, previously insoluble Cu$^{1+}$ was converted to bioavailable Cu$^{2+}$. Cleavage of dioxygen through reduction via Cu allowed these organisms to extract energy from fuels more efficiently. This critical evolutionary juncture coincided with the development of multicellular life (Decker and Terwilliger, 2000; Crichton and Pierre, 2001). In addition, many Cu-containing enzymes have higher redox potentials than those of enzymes containing metals such as iron. An association between Cu and oxygen-utilizing enzymatic reactions accompanied the appearance of Cu in biological systems.

Cu-interacting proteins generally comprise less than 1% of an organism’s proteome (Andreini et al., 2008). These Cu-interacting proteins are divided into cuproenzymes, enzymes that bind and require Cu for their catalytic activity, and Cu-binding proteins. Transmembrane Cu transporters, chaperones that deliver Cu to cuproenzymes, and proteins that simply bind and sequester Cu are much more numerous than cuproenzymes. Only about a dozen cuproenzymes are encoded by the mammalian genome. This number pales in comparison to the number of enzymes that require other metals, like iron or zinc (Zn). The oldest cuproenzyme, cytochrome oxidase, evolved in bacteria and archaea and is involved in energy production (Ridge et al., 2008),
specifically in aerobic respiration which requires dioxygen. Many cuproenzymes are functionally and structurally conserved from yeast and worm to much more complicated organisms like mice and humans (Andreini et al., 2008). Interestingly, mammalian cuproenzymes span many biological functions, and most are not directly related to aerobic metabolism. For example, both PAM and dopamine β-monooxygenase (DβM) are biosynthetic cuproenzymes for signaling molecules and they appear only in multicellular organisms.

However, the advantages of using Cu as an enzymatic co-factor come at a price. The same reactive properties that give Cu its utility pose a large risk for the generation of reactive oxygen species and subsequent oxidative cellular damage in uncontrolled settings (Uriu-Adams and Keen, 2005). Therefore, primitive organisms faced the challenge of getting Cu inside the cell while simultaneously limiting free/reactive Cu$^{1+}$ levels. To meet this challenge, an intricate and highly refined network of binding proteins and transporters evolved, and its components and their interactions have yet to be fully characterized (Banci et al., 2010; Lutsenko, 2010).

Kim and colleagues (Kim et al., 2010a) recently probed the breadth of the extracellular/inter-organ components of this network using a murine model for genetic cardiac-specific ablation of CTR1, a plasma membrane Cu transporter required for cellular Cu import. Cardiac tissue in these mice is Cu deficient, and the mice exhibit cardiac deficiencies like those seen in systemic Cu deficiency. Interestingly, there are also profound compensatory changes in the gut and liver, presumably aimed at increasing Cu uptake and liberating stored Cu. Kim and colleagues elegantly showed that these system-wide changes are mediated through an unidentified soluble factor released into the circulation. This study clearly demonstrates the presence and
physiological relevance of a multi-organ Cu regulatory pathway. While this is a ground-breaking and very informative study, its clearest message is that the major mechanisms through which Cu status is communicated between organs and within a single cell are unclear and require further exploration.
Components of Cu homeostasis and specific cuproenzymes

Cu uptake into the body through the gut is mediated primarily through CTR1 and, to a lesser extent, divalent metal transporter 1 (DMT1) (Lutsenko, 2010; Kaler, 2011). CTR1 is located on the brush border of the small intestine where it imports Cu from various ingested food sources including legumes, liver, shellfish, nuts and chocolate (Linder and Hazegh-Azam, 1996; Ma and Betts, 2000). CTR1 is also a key molecule in the rest of the body involved in cellular Cu uptake into the cytosol. Cu then binds one of several cytosolic chaperones; each chaperone has a designated delivery destination for the Cu it carries. For example, antioxidant-1 (Atox-1, also called HA1) is a chaperone that delivers Cu to the transporters that allow entry into the secretory pathway (Hamza et al., 2001). In the case of enterocytes of the small intestine that take Cu up from the gut, Atox-1 is required for Cu entry into the portal circulation and delivery to the liver. Atox-1 delivers Cu directly to P-type ATPase transmembrane transporters called ATP7A and ATP7B (Hamza et al., 2001; Lutsenko et al., 2007). The liver acts as the main storage site for Cu, and Cu can be mobilized to various organs in the body or eliminated by entry into the bile duct for subsequent excretion (Wijmenga and Klomp, 2004). Cu exported from the liver is typically loaded into newly synthesized ceruloplasmin, a Cu-dependent ferroxidase, and secreted into the circulation. Each ceruloplasmin molecule binds 6 Cu ions, and 75-95% of the Cu in the blood is bound to ceruloplasmin (Hellman and Gitlin, 2002; Bielli and Calabrese, 2002). The remaining non-ceruloplasmin-bound Cu is bound to albumin or free amino acids such that very little free Cu is present in the circulation.

Cu enters the brain through two routes, both involving CTR1 and ATP7A. The first route is through the blood-brain barrier, which serves as a regulated junction between the blood and the extracellular fluid of the brain. The second is through the blood-cerebral
spinal fluid (CSF) barrier, which serves as the junction between the blood and the CSF. The CSF fills the ventricles and subarachnoid space of the central nervous system. Both CTR1 and ATP7A are expressed by the endothelial cells that line the blood brain barrier and the epithelial cells of the choroid plexus (Choi and Zheng, 2009; Kaler, 2011).

The cuproenzyme required for aerobic metabolism mentioned earlier is cytochrome c oxidase (COX). Also known as Complex IV of the electron transport chain, COX is comprised of 13 protein subunits in mammals, including the Cu-binding subunit Cox11. COX is located in the inner mitochondrial membrane and transfers 4 electrons, delivered from upstream Complexes I-III, to 2 protons and dioxygen to form 2 water molecules. In the process, COX pumps 4 protons across the inner mitochondrial membrane to contribute to the gradient that drives ATP biosynthesis and generation of heat. To accomplish this, each COX complex requires 2 molecules of Cu, which shuttle electrons between substrates. COX receives Cu from cytosolic Cu chaperones Cox17, Cox11 and Sco1 (Fig. 1-4).

Superoxide dismutases (SODs) convert superoxide into oxygen and hydrogen peroxide. Intracellular (SOD1 and SOD2) and extracellular (SOD3) forms are encoded by 3 separate genes in mammals. Cytosolic Cu/Zn SOD1 is a heterodimer with one component containing Cu and the other Zn. Although other metals may substitute for Zn, no other metals can substitute for Cu (Harris, 1992). Both metals interact to convert superoxide (\( \text{O}_2^- \)) to hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), reducing damaging oxidative potential. Unlike SOD1 and SOD3 which use Cu and Zn, SOD2 contains manganese at its active site. SOD1 is the only cytosolic cuproenzyme, and SOD2 is found in the intermembrane space of mitochondria. Extracellular SOD3 is secreted through a regulated pathway (Jeney et al., 2005). As such SOD1 and SOD3 receive Cu through separate intracellular
pathways (Fig. 1-4); SOD1 receives Cu from the Cu chaperone for SOD (CCS) while SOD3 receives Cu through Atox-1 and the P-type ATPase transmembrane Cu transporters ATP7A and ATP7B (Jeney et al., 2005). Mutations in Cu/Zn SOD1 have been linked with familial forms of Amyotrophic Lateral Sclerosis, a progressive neurodegenerative disease specifically affecting motor neurons. Interestingly, these mutations do not impair SOD1 enzymatic function [for review see (Rothstein, 2009; Vucic and Kiernan, 2009)].

Lysyl oxidase, another essential cuproenzyme, uses molecular oxygen to catalyze oxidative deamination of lysine and hydroxylysine, facilitating the cross-linking of collagen and elastin. As might be inferred from its enzymatic function, lysyl oxidase is very important in connective tissue construction and maintenance, especially in skin,
bone and cardiovascular tissues (Kagan and Li, 2003; Lucero and Kagan, 2006). Much like SOD3, the lysyl oxidase proenzyme receives Cu through Atox-1 and ATP7A (Fig. 1-4). Upon secretion, the proenzyme is cleaved by a metalloproteinase yielding a 32 kDa mature, active enzyme and a smaller propeptide (Trackman et al., 1992; Smith-Mungo and Kagan, 1998). The reaction for which lysyl oxidase is responsible takes place extracellularly and requires 1 Cu ion, for which no other metal can substitute (Smith-Mungo and Kagan, 1998). Lysyl oxidase also requires a covalently bound carbonyl cofactor, lysyltyrosine quinine, which is formed through autocatalytic hydroxylation and oxidation.

Tyrosinase is found ubiquitously throughout nature but is best known for its role in the biosynthesis of melanin pigments, which are found in hair and skin. Tyrosinase is a monophenol monooxygenase which requires 2 Cu ions per enzyme (Olivares and Solano, 2009). Tyrosinase, a membrane glycoprotein whose topology closely resembles that of PAM, receives Cu via ATP7A (Setty et al., 2008). As its name suggests, a major phenol substrate of tyrosinase is L-tyrosine which is converted to L-DOPA and subsequently to L-dopaquinone by a related enzyme. Tyrosinase is expressed in the substantia nigra and locus coeruleus, two catecholaminergic centers located in the mammalian midbrain and brain stem, respectively. This likely accounts for the characteristic pigmentation of these nuclei.

Dopamine β-monooxygenase (DβM) is a hydroxylating enzyme that shares sequence similarity with PHM (Klinman, 2006) and plays a vital role in catecholamine biosynthesis in adrenergic cells. DβM catalyses the hydroxylation of dopamine to make norepinephrine, requiring molecular oxygen and ascorbate as cofactors (Klinman, 2006; Prigge et al., 2000). It is expressed in adrenergic neurons of the locus coeruleus.
and chromaffin cells of the adrenal medulla. It is a membrane-bound tetramer that localizes to synaptic/secretory vesicles \textit{(Fig. 1-4)} (Rush and Geffen, 1980). Norepinephrine is an important neurophysiological and endocrine modulator whose signaling regulates membrane excitability, synaptic plasticity, vascular tone, cardiac contractility, and many other physiological and cognitive functions.

Cuproenzymes clearly play a very large diversity of essential roles that involve multiple inter-dependent organ systems including signaling molecular biosynthesis, structure, metabolism, pigmentation, and protection from oxidative stress (see \textit{Fig. 1-4} for a summary of cuproenzymes). Given the tremendous utility and potential dangers of Cu in biological systems, it is not surprising that evolution has equipped us with such an intricate network of homeostatic mechanisms. Importantly, striving to identify the components of that network and elucidate their interactions is paramount in advancing our understanding of and treatments for a range of diseases in which Cu handling/homeostasis is disrupted.
Genetic disorders of Cu regulation

The necessity for Cu and the potential for Cu toxicity are most clearly demonstrated through the symptoms associated with genetic disorders of Cu transport and dietary Cu deficiency/toxicity. The two major genetic disorders, Menkes and Wilson’s disease, involve dysfunction of the P-type (auto-phosphorylating) ATPase transporters ATP7A and ATP7B, respectively. While each protein can transport Cu into the secretory pathway, the pathophysiologies of these two diseases are quite different, reflecting the distinct roles of the two transporters at cell- and tissue-specific levels.

Menkes disease is an X-linked recessive disorder first characterized by John Hans Menkes in 1962 as an inherited form of Cu deficiency (Menkes et al., 1962)[OMIM 309400; for review see (Kodama et al., 1999;de Bie et al., 2007;Kaler, 2011)]. Identification of the affected gene, ATP7A, was accomplished three decades later by positional cloning (Mercer et al., 1993). Naturally occurring mutations in the human ATP7A gene span the full length of the protein and impair Cu transport function to varying extents (Kaler, 2011). The Menkes gene is a known hot-spot for spontaneous mutations, so about 1/3 of ATP7A mutations arise de novo (Kaler, 1998). Infants born with Menkes disease typically begin to exhibit developmental delay and failure to thrive a few months after birth. Symptoms progress until death around 3 years of age. Symptomology associated with Menkes disease results from Cu deficiency in multiple organs and directly reflects deficient activities of specific cuproenzymes.

Impaired cross-bridging due to lysyl oxidase insufficiency impairs the tensile strength of collagen fibers and the recoil ability of elastin fibers. This manifests as brittle bones, weakened, aneurysm-prone arteries and heart, loose skin and kinky, brittle hair which is characteristic of Menkes disease. Scarcity of melanin due to reduced tyrosinase activity
leads to the steely coloring of hair and pale skin that are also common in Menkes disease presentations. Deficiency in PAM activity may also contribute since α-melanocyte stimulating hormone is an amidated peptide. Reduced COX activity contributes to muscle weakness and hypothermia of Menkes patients through insufficient ATP availability. Impaired function of ceruloplasmin, a ferroxidase, results in iron deficiency and anemia. Deficiency of DβM and PAM activity could both contribute to hypothermia through reductions in catecholamine signaling and impaired biosynthesis of thyrotropin-releasing hormone, respectively.

Seizures are very common and seizure occurrence and severity are typically progressive through the course of the disease (Kodama et al., 1999; de Bie et al., 2007; Kaler, 2011). Deficiencies of both DβM and PAM could contribute to the seizure susceptibility through shifts in excitatory-inhibitory balances caused by deficiencies in catecholaminergic and peptidergic neuromodulators, respectively. Norepinephrine has potent anti-convulsive effects (Mason and Corcoran, 1978; Mason and Corcoran, 1979), and destruction of noradrenergic neurons of the locus coeruleus or genetic deletion of DβM lowers the threshold for epileptogenic stimuli in limbic structures (Mishra et al., 1994; Szot et al., 1999). Alterations in noradrenergic tone likely shift excitatory-inhibitory balances. Disruption of noradrenergic tone could influence the threshold for runaway excitation in a given neural network and lead to epileptiform activity. GABAergic interneurons express and secrete a number of neuropeptides (McDonald and Pearson, 1989; Salio et al., 2006), many of which tend to promote inhibition and/or reduce excitation [for review see (Bajorek et al., 1986; Zadina et al., 1986)]; examples include neuropeptide Y, cholecystokinin, thyrotropin releasing hormone and vasopressin (see Chapter 2 Discussion). Reduced activity of PAM due to Cu insufficiency leads to tissue-specific impairments in amidation, and thus reduced bioactivity, of these inhibitory peptides
(Bousquet-Moore et al., 2010b). Death usually results from opportunistic infection or a devastating vascular event.

Menkes disease-like manifestations have been reported in rodent models of Cu deficiency [for review see (Vonk et al., 2008)]. There are seven mouse lines with mutations in ATP7A that are collectively referred to as mottled mice. Some of the mottled mutations are embryonic lethal while others allow the study of disease pathogenesis beyond the perinatal period. Just as in other ATP7A-related disorders, there is an inverse relationship between disease severity and residual ATP7A activity. The mottled brindled mouse exhibits a phenotype most similar to Menkes disease, including failure to thrive, seizures, hypopigmentation, kinky hair, and early post-natal lethality (Mann et al., 1979). Knockout animal models for CTR1 and Atox-1 are embryonic lethal (Kuo et al., 2001; Lee et al., 2001; Hamza et al., 2001), corroborating the essentiality of Cu cellular import and entrance into the secretory pathway.

Wilson's disease is an autosomal recessive disorder first characterized by Samuel Alexander Kinnier Wilson in 1912 as an inherited degenerative disease of the liver and brain (Compston, 2009). By way of contrast to Menkes disease, manifestations of Wilson's disease result from multiple organ dysfunction secondary to Cu overload and typically arise in adulthood. Dysfunction of the ATP7B protein results in an inability to excrete Cu from hepatocytes into the bile and out of the body. Cu builds up in the liver over time and is thought to periodically leak from the liver into the circulation resulting in its deposition in multiple organs. A striking example of such deposition is Kayser-Fleischer rings, deposits of Cu in the cornea, which are clinically considered pathognomonic for Wilson's disease (de Bie et al., 2007). Pathophysiology in Wilson's disease primarily involves excessive oxidative stress directly resulting from Cu overload.
in the liver and brain [OMIM 277900; for review see (Lutsenko, 2010; de Bie et al., 2007; Pfeiffer, 2007; Fujiwara et al., 2006; Das and Ray, 2006)].

Wilson’s disease typically presents with acute liver failure and its associated symptoms which may include hypoalbuminemia, impaired hemostasis and hepatic encephalopathy (de Bie et al., 2007). Liver cirrhosis with subsequent failure, portal hypertension and hepatocellular carcinoma are common. Hemolytic anemia is also common, likely resulting from episodes of extreme oxidative stress imposed by free Cu leakage into the blood from the liver (Attri et al., 2006). Excessive Cu deposits in the brain result in neurodegeneration, especially of lenticular structures associated with basal ganglia circuitry. As such, movement disorders and psychiatric manifestations are common. Extrapyramidal symptoms including oculomotor defects, ataxia, dysarthria, dystonic posturing and tremor are common (Das and Ray, 2006). Psychiatric disturbances may be severe enough for patients to reach diagnostic criteria for Axis I affective/mood and/or anxiety disorders (Pfeiffer, 2007). Not all psychiatric symptoms are likely to result from neurodegeneration; rather, the many influential effects of Cu on neuronal and synaptic physiologies no doubt contribute to the wide variation of psychiatric manifestations associated with Wilson’s disease.
Non-enzymatic roles of Cu in neurophysiology and behavior

While genetic and dietary disorders of Cu homeostasis support the important role played by Cu as an enzymatic co-factor, Cu itself has recently been shown to play a role in cell signaling, specifically in the nervous system. Cu, like other metals such as Zn and Fe, are concentrated in the brain (Que et al., 2008). Cu concentration in brain tissue has been estimated to be between 80 and 100 µM, about 5-fold higher than that found in blood (Linder and Hazegh-Azam, 1996; Que et al., 2008; Prohaska, 1987). Only the liver and kidney have higher Cu concentrations than the brain, near 100 and 200 µM, respectively (Linder and Hazegh-Azam, 1996). Cu entry into the brain requires both CTR1 and ATP7A; both proteins are expressed in endothelial cells of the blood-brain barrier and on epithelial choroids plexus cells of the blood-CSF barrier (Choi and Zheng, 2009). The intracellular concentration of brain Cu is 2-3 orders of magnitude above the extracellular concentration (Que et al., 2008), and there is 2-fold more Cu found in grey matter than white matter (Prohaska, 1987). Cu content is region-specific with the highest levels in the hypothalamus (Rajan et al., 1976). Subcellularly, Cu is most abundant in cytosolic and mitochondrial subcellular fractions. Cu can be released with neuronal activation in a calcium-dependent manner (Schlief et al., 2006; Dodani et al., 2011), much like glutamate. Cu can reach concentrations of 100-250 µM at synapses (Kardos et al., 1989), although these data are based on experiments run under conditions that were far from physiological. Nevertheless, the reactive biochemical properties of Cu at these concentrations prompts consideration of the many potential effects Cu could have on synaptic function under physiological conditions.

Most of the work exploring the role of Cu in synaptic transmission and plasticity has been conducted in the hippocampus. ATP7A is expressed at high levels in the pyramidal
neurons and interneurons of the neocortex and hippocampus (Schlief et al., 2005; Niciu et al., 2006), where Cu content is relatively high (Rajan et al., 1976; Bakirdere et al., 2010). Cu is released from cultured hippocampal neurons in an NMDA receptor-dependent manner, and stimulation of NMDA receptors results in the trafficking of ATP7A out of the late Golgi into dendrites (Schlief et al., 2005). Since NMDA receptor activity is crucial for different forms of synaptic plasticity and learning and memory (Malenka and Bear, 2004; Yashiro and Philpot, 2008), Cu is likely to be present at synapses during learning. Thus the influences of Cu on synaptic transmission and plasticity are highly relevant to learning and memory. Despite this, few groups have explored the effects of Cu on synaptic transmission in this region.

**Cu and synaptic transmission.** Xie and colleagues (Xie et al., 1993) reported that concentrations of Cu ranging from 100-300 µM enhanced both excitatory and inhibitory synaptic transmission in the CA3 region of the hippocampus. Subsequently, Doreulee and colleagues (Doreulee et al., 1997) bath-applied 1-100 µM CuSO₄ onto hippocampal slices while monitoring synaptic transmission in the Schaffer collateral-CA1 pathway using extracellular recording methods. They found that 10 µM CuSO₄ is needed to depress AMPA receptor-mediated transmission, while NMDA receptor-mediated signals are sensitive to 1 µM. Intracellular recordings confirm these results. They found no effects of Cu on presynaptic glutamate release or GABAergic inhibition, suggesting these effects are mediated post-synaptically. Cu (30 µM) has similar depressing effects on excitatory synaptic transmission in cultured olfactory bulb neurons (Trombley et al., 1998; Horning and Trombley, 2001).

GABAₐ receptors are heteropentameric ionotropic receptors structurally similar to nicotinic acetylcholine receptors (Hevers and Luddens, 1998; Jacob et al., 2008). There
are 18 subunits identified to date, and alternative splicing of those subunits also contributes to GABA<sub>A</sub> receptor diversity. GABA<sub>A</sub> receptors contain 2 α(1-6), 2 β(1-3) and a single γ(1-3), δ, ε(1-3), θ or π subunit (Fig. 1-5A). Importantly, subunit composition of GABA<sub>A</sub> receptors determines localization, activity and physiological function within neurons and networks (Wafford et al., 2004; Rudolph and Mohler, 2006). Each subunit has a large extracellular N-terminal domain (NTD) which includes a short cysteine loop, 4 transmembrane domains (TMD; TMD2 lines the channel pore), and large intracellular loop between TMD3 and TMD4 that acts as a site for protein interactions and post-translational modifications that regulate trafficking and channel activity (Fig. 1-5B) (Jacob et al., 2008). Each GABA<sub>A</sub> receptor binds 2 GABA molecules through NTDs at both α-β interfaces of the receptor, leading to channel pore opening and the passage of Cl<sup>-</sup> ions to inhibit action potential activity. Many endogenous and exogenous compounds bind and regulate GABA<sub>A</sub> receptor activity, including Cu. Compared to Zn, much less is known about the physiological effects of Cu, including those on GABA<sub>A</sub> receptors (Mathie et al., 2006). Nevertheless, the direct effects of Cu on GABA receptors have been demonstrated at physiological Cu concentrations (Ma and Narahashi, 1993; Sharonova et al., 1998; Kim and Macdonald, 2003). Cu and Zn both have inhibitory effects on GABA<sub>A</sub> receptor function (Narahashi et al., 1994; Trombley et al., 1998; Horning and Trombley, 2001) [for review see (Mathie et al., 2006)]. Cu binds different human GABA<sub>A</sub> receptors subtypes with equal affinity at an EC<sub>50</sub> of 2.4 µM (Kim and Macdonald, 2003), unlike Zn which displays subtype preference for binding (α6, EC<sub>50</sub> = 1.9 µM; α1 EC<sub>50</sub> = 10.4 µM) (Fisher and Macdonald, 1998). Cu-mediated inhibition of GABA<sub>A</sub> receptors is reversible (Ma and Narahashi, 1993), but may require removal of Cu from its binding site by using a chelator for example (Sharonova et al., 1998).
Leiva and colleagues used extracellular recordings to investigate the effects of exogenous CuSO₄ on mixed excitatory and inhibitory signals in acute hippocampal slices (Leiva et al., 2000). CuSO₄ (10 µM) application reduced the amplitude and duration of mixed signals. Bicuculline methiodide and picrotoxin, both of which are GABAₐ receptor antagonists, had opposite effects on these mixed signals in the Schaffer-CA1 pathway; bicuculline reduced mixed transmission similarly to CuSO₄, whereas picrotoxin enhanced these signals. These two compounds work through very different mechanisms; bicuculline competitively antagonizes the GABA binding site, whereas picrotoxin acts to block noncompetitively the Cl⁻ channel (Fig. 1-5) (Krishek et al., 1996). Cu (10 µM) had effects similar to GABA to suppress mixed synaptic transmission. Adding Cu with bicuculline produced no additive effect on depression of synaptic transmission, suggesting Cu works through GABA receptors to depress synaptic transmission. By contrast, addition of Cu with picrotoxin remarkably enhanced synaptic transmission, demonstrating clear distinctive interactions between these two GABA antagonists with Cu (Leiva et al., 2000). This likely speaks to the differential pharmacology of these two antagonists. In addition, the plethora of GABAₐ receptor subtypes, with different physiological functions and subunit compositions (Farrant and Nusser, 2005), can be differentially affected by these two antagonists (Zhang et al., 1995). Furthermore, different GABAₐ receptor subtypes are located on different classes of neurons and on different neuronal domains with brain region specificity (see below) (Martina et al., 2001). Confounding factors at this number and level of complexity can more than account for the wide range of results observed across these studies depending on method and small differences in concentrations used. This also makes interpretation of differential effects of these antagonists very difficult even within a single study.
Figure 1-5. Schematic illustrating GABA_A receptor structure and Cu binding. (A) GABA_A receptors are heteropentameric ionotropic receptors permeable to Cl⁻ and thus serve an inhibitory function in the neuronal membrane. GABA_A receptors typically comprise 2 α, 2 β, and γ (or other) subunits, as depicted on the left. On the right, a schematic view of the top of the channel demonstrates the central ion pore (white), 5 subunits (each with 4 transmembrane domains), and approximate binding sites for GABA, Cu, barbiturates and benzodiazepines. (B). Schematic of a single GABA_A receptor subunit. Specific residues important for Cu binding are labeled according to subunit type. Phosphorylation of the loop between TMD3 and TMD4 regulates trafficking.
Competing inhibitory effects of Cu and Zn on GABA\textsubscript{A} receptor function strongly suggests Cu and Zn act through similar mechanisms and may share a binding site (Ma and Narahashi, 1993;Narahashi et al., 1994;Fisher and Macdonald, 1998). Although Cu inhibition of GABA-activated currents follows kinetics similar to competitive antagonists, the site for Cu binding is notably distinct from the GABA binding site (Sharonova et al., 1998;Sharonova et al., 2000). Thus Cu acts to inhibit the effects of GABA-binding on channel gating. Cu binding also influences the binding and activity of other GABA\textsubscript{A} receptor allosteric modulators such as benzodiazepines (Mizuno et al., 1982;Kardos et al., 1984). The residues important for Cu binding include a VR(Q for $\alpha_2$)AECPMH motif which spans the proximal portion of the cystine loop of the $\alpha_1$ NTD (Fig. 5B) (Kim and Macdonald, 2003). Valine 134, arginine/glutamine 135 and histidine 141 are the most important determinants for Cu binding, but the whole motif is required for full Cu effect (Fig. 5B). Additionally, two residues important in Zn binding to $\alpha_6$-containing receptors ($\beta_3$-H267 in TMD2 and $\alpha_6$-H273 in the TMD2-TMD3 loop) are also required for Cu binding (Kim and Macdonald, 2003), but this is not the case for all Zn binding residues (Fisher and Macdonald, 1998). Taken together, these data suggest partial overlapping binding sites and similar mechanisms of action for Cu and Zn on GABA\textsubscript{A} receptor inhibition.

Cu has differential efficacies for various GABA\textsubscript{A} receptor subtypes with stronger effects on $\alpha_1$- and $\alpha_2$-containing receptors compared to $\alpha_4$- and $\alpha_6$-containing receptors (Table 1). (Kim and Macdonald, 2003). By contrast, Zn prefers $\alpha_6$-containing receptors over $\alpha_1$-containing receptors but has similar maximal inhibitory efficacies (Fisher and Macdonald, 1998). This subtype specificity has important functional relevance. $\alpha$(1-3)-containing receptors localize to GABAergic synapses and mediate phasic or fast inhibition, whereas $\alpha$(4-6)-containing receptors are extra-synaptic and mediate tonic or slow inhibition.
(Hevers and Luddens, 1998; Jacob et al., 2008; Farrant and Nusser, 2005). Additionally, synaptic GABA<sub>A</sub> receptor subtypes localize to separate synapses. For example, in pyramidal neurons of the hippocampus, α1-containing and α2-containing receptors localize to parvalbumin-positive and cholecystokinin/vasoactive intestinal peptide-positive axo-somatic and axo-axonic synapses, respectively (Nyiri et al., 2001). α5-containing GABA<sub>A</sub> receptors are also expressed by hippocampal pyramidal neurons and localize to the bases of dendritic spines (Rudolph and Mohler, 2006).

<table>
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<tr>
<th>Subunit</th>
<th>Cu</th>
<th>Zn</th>
<th>Role</th>
<th>Expression</th>
<th>Synapse</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>✓</td>
<td></td>
<td>Fast</td>
<td>Parvalbumin</td>
<td>Axo-somatic</td>
</tr>
<tr>
<td>α2</td>
<td>✓</td>
<td></td>
<td>&quot;</td>
<td>CCK/VIP</td>
<td>Axo-axonic</td>
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<tr>
<td>α5</td>
<td></td>
<td>✓</td>
<td>Slow</td>
<td>Hippo PNs</td>
<td>Axo-dendritic</td>
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<tr>
<td>α6</td>
<td>-</td>
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Table 1-1. GABA<sub>A</sub> receptor α subunit characteristics.

Consistent with findings in vitro, dietary Cu status can affect GABA<sub>A</sub> receptor availability in vivo (Geiger et al., 1984). Additionally, GABA<sub>A</sub> receptors subtypes are thought to be involved in different behavioral pathways (Rudolph and Mohler, 2006). Thus, preference for GABA<sub>A</sub> receptor subtypes can have a profound impact on the complex physiological effects of Cu during neuronal activity. One important example is the induction of synaptic plasticity, which involves the coincident engagement of several neuronal and synaptic receptor types, including GABAergic neurons and receptors.

**Cu and synaptic plasticity.** Fairly consistent effects of Cu have been observed on long-term potentiation (LTP), a form of synaptic plasticity hypothesized to be important for
learning and memory (Malenka, 2003; Lynch, 2004), in the hippocampus. Doreulee and colleagues (Doreulee et al., 1997) found that the depressing effects of Cu on NMDA receptors had consequences for LTP at 1 µM. Goldschmith and colleagues (Goldschmith et al., 2005) confirmed the inhibitory effects of Cu on hippocampal LTP using brain slices generated from rats chronically supplemented with Cu through the diet (8-12 mg/day for 20-25 days) and a similar LTP induction method. Years later, the same groups tested the effects of chronically injected Cu (1 mg/kg, i.p.) on hippocampal LTP with similar results (Leiva et al., 2009). It was reported in both studies that Cu levels were approximately 50- and 15-fold higher in the brains of Cu supplemented rats compared to control rats in each of the two studies, respectively. This startling measurement immediately calls to mind concerns of Cu toxicity in these studies. Nevertheless, given these results it was reasonably concluded that Cu suppresses LTP at Schaffer-CA1 synapses.

This finding is consistent with biochemical evidence of the effect of Cu on key molecules involved in LTP induction. The NMDA receptor acts as a coincidence detector for synaptic activity and post-synaptic excitability. Unlike the majority of their glutamate receptor counterparts, NMDA receptors are permeable to calcium, which triggers a cascade of signaling events that ultimately lead to changes in post-synaptic receptor expression and/or pre-synaptic release probability (Malenka, 2003; Yashiro and Philpot, 2008). Schlief and colleagues (Schlief et al., 2005) found that 200 µM CuCl₂ inhibited NMDA receptor signaling. This effect was later found to depend on nitrosylation of the NMDA receptor (Schlief et al., 2006), which is facilitated in the presence of Cu. Cu also blocks high voltage-activated calcium channels in rat cortical (Castelli et al., 2003) and olfactory bulb neurons (Horning and Trombley, 2001). In particular, L-type voltage-gated calcium channels, which are located post-synaptically on dendrites and dendritic spines
and contribute to calcium influx during LTP induction (Kullmann et al., 2000; Malenka and Bear, 2004), are sensitive to Cu in the low µM range (Korte et al., 2003). Thus Cu could impair LTP through multiple mechanisms (Fig. 1-6).

However, the physiological role of Cu in the hippocampus may not be so simple. To determine the effect of Cu on established LTP, Leiva and colleagues (Leiva et al., 2003) waited until 90 min after LTP induction to bath apply 10 µM Cu, which decreased synaptic efficacy as observed previously. Upon washout, the synaptic response returned at a substantially larger size. Unfortunately, no controls were performed to rule out progressive potentiation as a contributing factor. In a separate set of experiments performed by the same group, paired pulses were applied to Schaffer collaterals at intervals ranging from 20 to 300 ms in hippocampal slices generated from rats on normal or chronic Cu supplemented diets (Goldschmith et al., 2005). Response ratios (second/first) decreased in both groups following LTP induction, as is to be expected with a pre-synaptic component of synaptic potentiation (increased pre-synaptic release probability). However, this change was blunted in Cu supplemented animals, and paired pulse ratios were reduced in Cu supplemented animals at baseline. These findings are consistent with enhanced pre-synaptic release probability at baseline with Cu supplementation and support an alternative interpretation of occluded rather than impaired LTP. Moreover, LTP might have already been established prior to induction in the case of Cu supplemented rats. By contrast, acute application of Cu has no effect on paired pulse responses (Doreulee et al., 1997). Together these results suggest a dual effect of Cu on synaptic plasticity in the hippocampus, where Cu prevents the induction of new LTP and may reinforce LTP that has already been established. Future studies should address a narrower range of hypotheses through more in depth physiological study with more rigorous experimental controls.
Other potential contributing roles of secreted Cu to synaptic plasticity remain to be explored.

Figure 1-6. Schematic summarizing the known roles of Cu in synaptic function.

Cu enters cells through CTR1 and binds to cytosolic chaperones including Atox-1 which delivers Cu to ATP7A. Cu gains entry into the secretory pathway through ATP7A and is stored in LDCVs along with secreted cuproenzymes DBM, SOD3 and PAM. Influx of Ca²⁺ through NMDARs and VGCCs results in LDCV release and Cu secretion. Outside of the cell, Cu has inhibitory effects on NMDARs, VGCCs and GABAARs. Cu binds PrP and APP, which are synaptically localized. Cu stimulates MMP9 which facilitates the activation and release of BDNF along with subsequent binding to TrKB receptors. Abbreviations: Aβ, beta amyloid peptide; AMPAR, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid receptor; APP, amyloid precursor protein; Atox-1, antioxidant-1 (a.k.a. HAH1); ATP7A, Menkes disease protein; BDNF, brain-derived neurotrophic factor; Ca²⁺, calcium; CTR1, Cu transporter 1; DBM, dopamine β-monooxygenase; GABAR, γ-aminobutyric acid A receptor; LDCV, large dense-core vesicle; MMP9, matrix metalloproteinase 9; NMDAR, N-methyl-D-aspartate receptor; PAM, peptidylglycine α-amidating monooxygenase; PrP, prion protein; PSD, post-synaptic density; SOD3, superoxide dismutase 3; TrkBR, tyrosine receptor kinase B; VGCC, voltage-gated calcium channel.
tested. Brain-derived neurotrophic factor (BDNF) is a potent stimulant of neuronal and synaptic growth important in brain development and learning and memory [for review see (Minichiello, 2009)]. BDNF is released upon activity-dependent neuronal stimulation and binds to tropomyosin-related kinase B (TrkB) receptors, inducing activation and autophosphorylation. TrkB receptors expressed on cultured cortical neurons become phosphorylated/activated when exposed to Cu for 15 min (10 µM optimal); Cu is more effective than several other metals, including Zn (Hwang et al., 2007). This effect is mediated through Cu-induced BDNF precursor cleavage and release that depends on the activity of matrix metalloproteinases (MMPs), a class of extracellular Zn-dependent endopeptidases. An interesting aside is that BDNF signaling enhances hippocampal and cortical post-synaptic NMDA receptor activity (Madara and Levine, 2008), which could lead to further Cu release and TrkB activation. Thus, under certain conditions Cu could positively influence LTP through TrkB signaling (Fig. 1-6).

MMPs have been shown to contribute to synaptic plasticity through mechanisms outside of TrkB signaling. MMP-9 activation is activity-dependent in the hippocampus and is necessary and sufficient for hippocampal LTP and learning and memory (Nagy et al., 2006;Bozdagi et al., 2007;Nagy et al., 2007). Moreover, MMP-9 deficient mice lack hippocampal LTP, but respond to LTP induced by bath application of recombinant MMP-9 (Nagy et al., 2006). These results were later corroborated with an MMP-9-dependent increase in dendritic spine volume which typically accompanies increases in synaptic strength (Wang et al., 2008). This effect of MMP-9 is mediated through cleavage of ICAM-5, a post-synaptic integrin and negative regulator of synaptic strength and growth (Conant et al., 2010). Thus, activation of MMP-9 during LTP results in the cleavage of ICAM-5 and disinhibition of synaptic strength and growth. Hwang and colleagues (Hwang et al., 2007) further showed that neuronal, but not glial, MMP-9 is specifically
activated by 10 µM Cu using in situ zymography. It remains to be determined whether this MMP-ICAM-5-LTP pathway requires the release and extracellular availability of Cu (Fig. 1-6).

To add to the many proteins that bind Cu within the cell (Fig. 1-4), other molecules located at the synapse bind Cu, and could therefore influence its non-enzymatic effects. Amyloid precursor protein (APP), whose proteolytic cleavage yields the pathological Aβ peptide, is implicated in Alzheimer's disease pathology. APP contains an extracellular domain that binds Cu with attomolar affinity (Atwood et al., 2000). Cu binding promotes the proteolytic cleavage of APP to yield Aβ, and Aβ-mediated neuronal toxicity is enhanced in the presence of Cu (Huang et al., 1999). The connection between APP and Cu homeostasis is demonstrated by transgenic animals in which knockout of APP increases brain Cu levels (White et al., 1999), and APP over-expression results in a reduction of brain Cu (Maynard et al., 2002). Increasing Cu levels promote cell surface expression of APP and its distribution into neurites. Clearly APP plays a role in Cu homeostasis in normal and pathological conditions, and its localization in dendrites highly implicates an important role for APP in Cu-mediated modulation of synaptic transmission and plasticity (Fig. 1-6).

The prion protein (PrP) is another Cu-binding transmembrane protein, which is involved in spongiform encephalopathies such as Creutzfeldt-Jakob disease [for review see (Vassallo and Herms, 2003;Zomosa-Signoret et al., 2008)]. PrP is pre- and post-synaptically expressed in neurons and contains 5 sites that bind Cu with low µM affinity (Fig. 1-6) (Herms et al., 1999;Zomosa-Signoret et al., 2008). Binding of Cu to PrP triggers internalization of the metalloprotein complex. Genetic deficiency of PrP in mice results in a 50% reduction in synaptosomal Cu levels, however total brain Cu levels were
unchanged. The electrophysiological phenotype of these mice was consistent with increased Cu at synapses, including impaired LTP and reduced GABAergic inhibition in the hippocampus (Collinge et al., 1994). Interestingly, the synaptic plasticity deficit of PrP null mice corresponds with impaired spatial learning and memory consolidation (Criado et al., 2005). PrP clearly also plays a role in Cu homeostasis, but the details and significance of that role are not understood (Zomosa-Signoret et al., 2008) (Fig. 1-6). The Cu binding capacity of APP and PrP are not only highly suggestive of their role in Cu homeostatic disease states, but also implicate a pathophysiological role for disrupted Cu homeostasis in Alzheimer’s and prion-related diseases.

_Cu and learning and memory_. Despite these consistent and striking observations made to this point _in vitro_, no group has yet demonstrated a behavioral effect of Cu on hippocampal-dependent learning and memory. Leiva and colleagues 2009 (Leiva et al., 2009) showed that the same chronic Cu injection regimen had no effect on performance in the Morris water maze task, a cognitive behavioral task testing working and long-term spatial (hippocampal-dependent) memory. Another group showed that both dietary Cu supplementation (1 ppm CuSO₄ in the drinking water) and chelation (using 0.1% D-penicillamine dissolved in the drinking water) in rats had no effect on shuttle-box avoidance learning of rats (Fujiwara et al., 2006). Dietary Cu supplementation was anxiolytic and reduced sensorimotor gating performance in the same study. Mild dietary Cu restriction (0.6 ppm food Cu for 9-10 weeks) in mice has an anxiogenic effect in the elevated zero maze (Bousquet-Moore et al., 2010b). Thus the strength of dietary challenge and species differences can weigh heavily on behavioral assessment of the role of Cu in brain function.

The mammalian hippocampus possesses a uniquely high density of NMDA receptors,
specifically at the Schaffer-CA1 synapse (Meoni et al., 1998). As such, LTP at Schaffer-CA1 synapses is characteristically reliant on NMDA receptor-mediated signaling. However, LTP in other brain regions such as the cortex and amygdala does not rely as heavily on NMDA receptors, and may thus respond differently in the presence of Cu. NMDA receptor-independent LTP may also respond differently in the presence of Cu even within the hippocampus. Therefore, the roles of Cu in synaptic plasticity and learning and memory are complex and likely region-specific, which may account for the array of symptoms observed in disorders involving disrupted Cu homeostasis including Alzheimer’s disease, prion disease, and Wilson’s disease [for review see (Uriu-Adams and Keen, 2005)].

Conclusions. The arrival of Cu on the biological scene at such a primitive juncture in evolution suggests the presence of Cu during the evolution of the nervous system. The utility and reactivity of this metal combined with its presence at synapses highly implicate a physiological role for Cu in normal brain function. Studies performed to date support a complicated portrait of interconnected pathways through which Cu signals (Fig. 1-6). However, the number and breadth of these studies are extremely limited. Additionally, several neurological diseases with cognitive and behavioral symptomatology involve pathology in which Cu homeostasis is disrupted. Thus, there is a strong need for further investigation into the complex network of Cu signaling pathways in the brain. Advancement of our understanding of these mechanisms will help identify new molecular targets for neurological and psychiatric diseases, many of which currently have few and poor treatment options, and it will provide great insight into the basic mechanisms through which humans interact with and experience the world.
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CHAPTER 2: Haploinsufficiency in peptidylglycine alpha-amidating monooxygenase leads to altered synaptic transmission in the amygdala and impaired emotional responses

CONTRIBUTIONS

The contents of this chapter were published in the *Journal of Neuroscience* in October of 2010. This work roughly encompasses experimental results from Aims 1 and 2 of my thesis proposal and prospectus. Behavioral analyses were conducted at Duke University by Dr. Ramona Rodriguiz in the laboratory of Dr. William Wetsel using wildtype and PAM heterozygous mice bred, maintained, genotyped and sorted by myself and Dr. Danielle Bousquet-Moore, a former graduate student in our lab. All other experiments were conducted in my hands. I received assistance from Dr. Xin-Ming Ma with immunohistochemistry, and Drs. Srjdan Antic and Eric Levine assisted me with technical issues and experimental guidance with the electrophysiology.
ABSTRACT

The mammalian amygdala expresses various neuropeptides whose signaling has been implicated in emotionality. Many neuropeptides require amidation for full activation by peptidylglycine α-amidating monooxygenase (PAM), a transmembrane vesicular cuproenzyme and regulator of the secretory pathway. Mice heterozygous for the Pam gene (PAM$^{+/−}$) exhibit physiological and behavioral abnormalities related to specific peptidergic pathways. In the present study, we evaluated emotionality and examined molecular and cellular responses that characterize neurophysiological differences in the PAM$^{+/−}$ amygdala. PAM$^{+/−}$ mice presented with anxiety-like behaviors in the zero maze that were alleviated by diazepam. PAM$^{+/−}$ animals were deficient in short- and long-term contextual and cued fear conditioning and required higher shock intensities to establish fear-potentiated startle than their wildtype littermates. Immunohistochemical analysis of the amygdala revealed PAM expression in pyramidal neurons and local interneurons that synthesize γ-aminobutyric acid (GABA). We performed whole-cell recordings of pyramidal neurons in the PAM$^{+/−}$ amygdala to elucidate neurophysiological correlates of the fear behavioral phenotypes. Consistent with these observations, thalamic afferent synapses in the PAM$^{+/−}$ lateral nucleus were deficient in long-term potentiation. This deficit was apparent in the absence and presence of the GABA$_A$ receptor antagonist picrotoxin and was abolished when both GABA$_A$ and GABA$_B$ receptors were blocked. Both evoked and spontaneous excitatory signals were enhanced in the PAM$^{+/−}$ lateral nucleus. Phasic GABAergic signaling was also augmented in the PAM$^{+/−}$ amygdala, and this difference was comprised of activity-independent and activity-dependent components. These physiological findings represent perturbations in the PAM$^{+/−}$ amygdala that may underlie the aberrant emotional responses in the intact animal.
INTRODUCTION

Neuropeptides are the most diverse class of neurotransmitters and many share a common biosynthetic pathway. Peptide α-amidation is a final and often essential step in conferring full biological activity to many neuropeptides (Eipper and Mains, 1988; Eipper et al., 1992). In mammals, this reaction is performed exclusively by a single vesicular transmembrane protein, peptidylglycine α-amidating monooxygenase (PAM) (Eipper et al., 1983; Mains et al., 1991). PAM has two luminal enzymatic domains (peptidylglycine α-hydroxylating monooxygenase, PHM; peptidyl α-hydroxyglycine alpha-amidating lyase, PAL) that act sequentially to catalyze the Cu-dependent cleavage of a C-terminal glycine (Prigge et al., 1997; Prigge et al., 2000). The inherently unstructured cytosolic domain of PAM is multiply-phosphorylated (Steveson et al., 1997; Yun et al., 1995; Rajagopal et al., 2009), can regulate the peptidergic exocytic pathway (Ciccotosto et al., 1999; Alam et al., 2001), and can signal to the nucleus (Rajagopal et al., 2009).

Our group has previously described a model of genetic PAM deficiency (Czyzyk et al., 2005a; Bousquet-Moore et al., 2009). Homozygous deletion of Pam results in embryonic lethality at E14.5 and a phenotype similar to that of mice lacking the precursor of adrenomedullin, an amidated peptide (Caron and Smithies, 2001). Heterozygous mice (PAM^+/−), with half the normal levels of PAM protein and amidating activity, grow normally and reproduce, but exhibit striking physiological and behavioral abnormalities (Bousquet-Moore et al., 2010b; Bousquet-Moore et al., 2009) including impaired vasoconstriction and temperature regulation, a hyper-sensitivity to pentylenetetrazol-induced seizures, augmented anxiety-like behaviors, and disrupted Cu homeostasis. Here we report deficits in fear responses in PAM^+/− mice, prompting our exploration of synaptic transmission in the PAM^+/− amygdala.
The amygdala is a heterogeneous group of limbic nuclei that play a critical role in mediating fear and anxiety-like behaviors (LeDoux, 2007; Sah et al., 2003). The basolateral complex, composed of lateral and basolateral nuclei, receives the majority of inputs to the amygdala. Inhibition of these nuclei by local interneurons is crucial in maintaining low baseline amygdala activity and gating plasticity at excitatory afferent synapses (Marowsky et al., 2005; Shaban et al., 2006; Tully et al., 2007; Muller et al., 2006). Among these afferent pathways are inputs carrying discrete sensory information relevant to Pavlovian fear conditioning, a behavioral model for fear learning and memory (Lanuza et al., 2008; Maren and Quirk, 2004; Pare et al., 2004).

PAM mRNA is present throughout the rodent CNS, including cortex, hippocampus and amygdala (Schafer et al., 1992b). In this study, we show that PAM protein is expressed in pyramidal neurons and interneurons of the basolateral complex. Pyramidal neurons and interneurons secrete neuropeptides in addition to glutamate and γ-aminobutyric acid (GABA) (Salio et al., 2006). Altered function of these neurons, resulting from PAM limitation, could disrupt the excitatory-inhibitory balance of sensitive brain regions and affect behavior. To investigate this hypothesis, we performed electrophysiological studies in the basolateral complex of the PAM +/- amygdala. Impairment of synaptic plasticity at thalamic afferent synapses, increased neural excitability that was GABA_A-dependent, and enhanced activity-dependent GABAergic transmission were evident in the PAM +/- amygdala. Collectively these findings demonstrate that PAM +/- mice provide a unique model for peptidergic disruption with behavioral and physiological perturbations that may be relevant to anxiety-spectrum disorders in humans.
METHODS

Animals
Male and female mice for these studies were generated from PAM"/+ pairings in the University of Connecticut Health Center (UCHC) animal facility. Wildtype and PAM"/+ littermates (>20 generations bred into C57/BL6J background) were weaned between P19 and P21 and group-housed until experiments. Animals were maintained under a 12 h light/dark cycle (lights on at 0700) and were given free access to food and water. The behavioral experiments were conducted at Duke University between 1100-1400 h, where adult male and female wildtype and PAM"/+ mice (12-20 weeks of age) were tested. Since no sex differences were observed, the data were collapsed across this variable. All experiments were conducted with approved protocols from the UCHC and Duke University Institutional Animal Care and Use Committees and in accordance with NIH guidelines for animal care.

Behavior

Drugs. Diazepam (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile water containing less than 0.3% Tween-80 (Sigma-Aldrich) of the total volume. The vehicle or benzodiazepine (Diazepam) was administered (i.p.) 30 min prior to testing.

Neurophysiological screen. The details of the tests used for assessment of general sensory and motor function and spontaneous activity in the open field have been described (Pogorelov et al., 2005; Taylor et al., 2008).

Zero maze. The details of the zero maze have been published (Pogorelov et al., 2005). The maze was illuminated at 50-60 lux and enclosed by black curtains. A video camera was positioned 100 cm above the maze. Naïve mice were placed into a closed quadrant.
and permitted to investigate the maze for 5 min. The videotapes were analyzed with Noldus Observer (Noldus Information Technology, Leesburg, VA) and scored for percent time spent in the open areas, latency to enter the open areas, and number of head-dips and freezing episodes (see below for definition).

Fear conditioning. Details of the fear conditioning tests have been published (Taylor et al., 2008). Mice were assigned to one of four test groups: 1 h context, 24 h context, 1 h cued, and 24 h cued tests. All mice were conditioned with a single 30 s 72 dB tone (CS) and 2 s 0.4 mA scrambled foot-shock (UCS) on day 1. One-half of the animals were examined 1 h after conditioning either in the context or cued test, and the remainder were evaluated separately 24 h later in these two respective tests. Context testing consisted of returning the mouse to the same chamber in which the CS-UCS pairing had been presented. For cued tests the mice were placed into a novel chamber with a different level of illumination, floor, walls, shape and dimensions than the conditioning chamber. All tests were video-taped and the behaviors were later scored using the Noldus Observer by a trained observer who was blind to the genotype of the mice and time-interval of testing. Freezing was defined as the lack of all movement by the animal except for respiration for >1 s (Anagnostaras et al., 2000; Porton et al., 2009).

Fear-potentiated startle. This test has been described in detail elsewhere (Taylor et al., 2008). Testing was conducted in a MedAssociates apparatus (St. Albans, VT) over 5 days. On day 1, baseline startle responses were assessed over 18 trials with 40 ms bursts of white noise at 100, 105 and 110 dB presented in pseudorandom order with an inter-trial interval of 30–90 s. On day 2, one-half of the startle stimuli were administered immediately following a 30 s 12 kHz 70 dB pure tone (CS); the other half were presented without the CS. Twenty-four h later mice were conditioned with 10 CS-UCS pairings.
One-half of the mice were conditioned with 0.25 s 0.4 mA and the remainder with 0.6 mA scrambled shock (UCS). Forty-eight h later, mice were tested for potentiation of their startle responses by the CS using the same procedure described for day 2. Potentiation to the CS was defined as the percent increase in the startle response for the CS+startle-stimulus trials relative to the baseline startle responses on day 1.

**Shock-threshold testing.** This procedure has been described (Grove et al., 2004; Taylor et al., 2008). Briefly, mice were acclimated to a single test chamber (MedAssociates) for 2 min before being presented with five different foot-shock intensities (0, 0.1, 0.2, 0.4, and 0.6 mA) for 2 s. Inter-shock intervals were between 30 and 90 s. Behavioral responses were video-taped and scored subsequently using the Noldus Observer program. Behaviors were given scores of 0-5. A score of 0 denoted the lowest level of response that involved the continuation of activity. A score of 1 signified a low-level response to shock that included freezing, face-wiping, self-grooming, shaking, or rapid forward departures. A score of 2 was a moderate response that could involve retreating from shock or tail-rattling. A score of 3 denoted stationary reactive responses including kicking and vocalization. A score of 4 signified locomotor reactivity such as darting and leaping. A score of 5 was indicative of jumping against the walls or ceiling of the chamber. Behavioral scores were summed for each animal and analyzed as a function of genotype and shock intensity.

**Immunocytochemistry**

Immunohistochemical staining of tissue sections from perfusion-fixed mice has been described previously (Ma et al., 2001; Ma et al., 2002; Ma et al., 2008a). Briefly, male wildtype and PAM+/− littermates were perfused transcardially with 4% formaldehyde/0.1 M sodium phosphate buffer (pH 7.4) under deep ketamine anesthesia. After fixation, brains
were post-fixed in 4% paraformaldehyde for 3 h. Coronal sections were cut (15 µm) through the amygdala using a cryostat and immunostained with rabbit antiserum JH629 to the Exon A (exon 16) region of PAM1 (Maltese and Eipper, 1992); mouse monoclonal IgG to GAD67 (Millipore Biosciences, Billerica, MA); Alexa-488 donkey anti-rabbit (H+L) (Invitrogen, Molecular Probes, Eugene, OR); Cy<sup>TM</sup>3-conjugated AffiniPure F(ab’<sub>2</sub>)-fragment donkey anti-mouse (H+L) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) as described (Ma et al., 2001).

**Electrophysiology**

*Slice preparation.* Male wildtype and PAM<sup>m<sup>mi</sup></sup> littermates 4–7 weeks of age were decapitated and their brains quickly removed into ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 26 NaHCO<sub>3</sub>, 10 glucose, 2.3 KCl, 2 CaCl<sub>2</sub>, 2 MgSO<sub>4</sub>, 1.26 KH<sub>2</sub>PO<sub>4</sub>; aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, pH 7.3, 310 mOsm/kg (Zhou et al., 2008a). Coronal slices, 300 µm thick, were incubated at room temperature for at least 1 h before recordings.

*Whole-cell recordings.* Slices were transferred to a recording chamber heated to 32°C and perfused with aerated aCSF. Recording pipettes had 3-5 MΩ tips. The internal pipette solution used in most experiments was composed of (in mM) 135 K-gluconate, 10 HEPES, 10 P-creatine, 3 Na<sub>2</sub>ATP, 2 MgCl<sub>2</sub>, 0.3 Na<sub>2</sub>GTP, pH 7.3, 285 mOsm/kg (Zhou et al., 2008a). Inhibitory Post-Synaptic Currents (IPSCs) were recorded using pipette solution composed of (in mM) 120 CsCl, 10 HEPES, 1 EGTA, 0.1 CaCl<sub>2</sub>, 1.5 MgCl<sub>2</sub>, 4 Na<sub>2</sub>-ATP, 0.3 Na<sub>2</sub>-GTP, 5 QX-314; pH 7.3, 310 mOsm/kg. Neurons were patched under visual guidance using infrared differential interference contrast optics. Data were collected using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) and analyzed using pClamp 9.2 software (Molecular Devices).
*Evoked synaptic transmission.* Synaptic activity was evoked using 200 μs current pulses elicited with a Master 8 and an ISO-Flex stimulator (A.M.P.I., Jerusalem, Israel) through bipolar tungsten electrodes (World Precision Instruments, Sarasota, FL). Electrode placement in the internal and external capsules preferentially stimulated afferent axons originating in thalamic and cortical areas, respectively (Weisskopf and Ledoux, 1999). Input-output curves for thalamic and cortical inputs were generated from excitatory and inhibitory compound currents recorded at $V_{\text{holding}} = -55$ mV. Only outward currents were sensitive to picrotoxin (not shown). AMPA and NMDA receptor-mediated currents were recorded at $V_{\text{holding}} = -70$ and +50 mV, respectively. NMDA receptor-mediated current amplitude was measured 50 ms after the stimulus to avoid contamination by the AMPA component. For the majority of neurons recorded in these experiments, reversal potentials were assessed by recording currents at voltages between -70 and +50 mV in 10 mV increments. Reversal potential was calculated as the X-intercept of a fit to the linear portion of the I-V curve for each neuron. A 2-term exponential curve was fit to each +50 mV current trace to assess fast and slow decay components of the mixed AMPA- and NMDA-mediated current. To record evoked Inhibitory Post-Synaptic Currents (eIPSCs), placement of the stimulating electrode locally in the amygdala neuropil medial to the recording pipette allowed preferential stimulation of local interneurons (Silberman et al., 2008; Marowsky et al., 2005) and was kept as consistent as possible. The axonal arbors of amygdalar interneurons are extensive, often encompassing the whole basolateral nucleus (Rainnie et al., 2006). In addition, parvalbumin-positive amygdalar interneurons (the most prevalent class of interneuron) are electrically coupled via dendritic and axonal gap junctions (Muller et al., 2005). Therefore, action potential activity could be produced reliably through local stimulation pulses and maximal responses consistently achieved in a given amygdala slice. Paired
pulses were applied, and the ratio of the second eIPSC amplitude to the first was interpreted as an inverse indicator of release probability.

*Synaptic plasticity.* Stimulating electrodes were placed in the internal capsule to evoke thalamic afferents. In whole-cell configuration, experiments were conducted in current clamp mode in the presence and absence of picrotoxin (Tully et al., 2007; Weisskopf et al., 1999). The holding current was adjusted to maintain $V_m = -70$ mV throughout the course of experiments. The 10-90% rise slope of Excitatory Post-Synaptic Potentials (EPSPs) was used as the measure of synaptic efficacy. Stimulation strength was adjusted to produce a 3-6 mV EPSP, and test stimuli were applied at 0.1 Hz. After establishment of a steady baseline, Long-Term Potentiation (LTP) was induced using an action potential pairing induction protocol. This induction paradigm was originally characterized as being L-type voltage-gated calcium channel-dependent (Bauer et al., 2002). Fifteen paired trains were applied at 0.1 Hz; each train consisted of 10 pulses at 30 Hz paired with 2.5 ms current pulses of $\geq +1$ nA to elicit an action potential at a 5 ms delay to the onset of each synaptic event. If 1 nA was not sufficient, then the pulse amplitude was increased by $+50$ pA until action potentials were observed throughout the train. LTP was measured as the normalized fractional difference between the 35-40 min interval post-induction and the 5 min interval prior to induction; neurons were pooled by genotype. Only one neuron was used per slice. For field potential recordings, pulse durations were 100 µs. Recording pipettes (3-5 MΩ) were filled with aCSF and placed in the neuropil of the lateral amygdala. Fast GABAergic transmission was blocked using 100 µM picrotoxin throughout these experiments. Stimulation strength was adjusted to achieve 30-50% of the maximum voltage response for each slice. The same stimulation strength was used for test stimulation and induction. At least 20 min of stable baseline was recorded before applying the induction protocol. Theta Burst Stimulation consisted
of 4 sets at 20 s intervals; each set included 10 bursts at 200 ms intervals; each burst was 4 pulses at 100 Hz. Test stimulation responses for the 10 min prior to induction and between 50 and 60 min after induction were used in the assessment of plasticity.

Membrane properties. A series of negative and positive current steps were applied immediately upon achieving whole-cell configuration (25 x 50 pA steps, −300 to +900 pA; 500 ms steps). The 6 initial negative steps were used to calculate membrane resistance; single exponential curve fits yielded time constant (τ) and capacitance values. Action potential threshold was defined and measured as the point of voltage inflection for the first spike fired in the lowest current step. An action potential was defined as a brief, accelerating depolarization greater than 20 mV in amplitude.

Non-evoked synaptic transmission. All non-evoked synaptic activity was recorded at \( V_{\text{holding}} = -70 \) mV. Synaptic events were filtered at 1 kHz and analyzed using MiniAnalysis (Synaptosoft, Decatur, GA). Events were pooled by experimental condition for statistical comparison. To avoid over-representation of neurons with higher frequencies in our analyses, the smallest number of events recorded from a single neuron in an experiment was used as the number of consecutive event values each neuron contributed to the pool. Spontaneous Excitatory Post-Synaptic Currents (sEPSCs) were quantified using the 5th min of activity after whole-cell conversion for each neuron. For spontaneous and miniature IPSCs (sIPSCs and mIPSCs), the 8th min of activity was analyzed for each neuron to allow equilibration of the pipette solution. Threshold amplitudes were set at 7 pA for sEPSCs and 20 pA for sIPSCs and mIPSCs; these values were based on the root-mean square of the baseline noise from wildtype recordings at multiples of 4 and 5, respectively (Zhang et al., 2009).

Pharmacology. All drugs were applied through the perfusate. Picrotoxin (100 µM; PTX;
Sigma-Aldrich) was used to block fast GABA$_A$ receptor-mediated transmission. CGP35348 (1 µM; Tocris Biosciences, Ellisville, MO) was used to block slow GABA$_B$ receptor-mediated transmission. Tetrodotoxin (1 µM; TTX; Alomone Labs, Jerusalem, Israel) was used to block action potential-dependent activity. 6,7-Dinitroquinoxaline-2,3-dione (10 µM; DNQX; Sigma-Aldrich) or 6-cyano-7-nitroquinoxaline-2,3-dione (10 µM; CNQX; Tocris Biosciences) and 3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid (3 µM; CPP; Tocris Biosciences) were used to block glutamatergic transmission while recording inhibitory synaptic transmission. CNQX and DNQX stocks were prepared in DMSO; the final concentration of DMSO in the recording solution was 0.01%. All other drugs stocks were prepared in water.

**Statistics**

For behavioral data, zero maze responses were analyzed by 2-way ANOVA where genotype and treatment were examined; average percent freezing responses in contextual fear conditioning were evaluated for genotype and test-time. Repeated measures ANOVA (RMANOVA) was used to examine fear conditioning responses over time where within-subject effects were applied to the 1 min observations and between subject effects for genotype and test-time (i.e., 1 or 24 h). RMANOVA was also used to evaluate freezing responses in cued fear conditioning during the pre-CS and CS intervals and for fear-potentiated startle where the within-subject effects were applied to the three startle stimulus intensities and the between subjects effect to the two shock intensities and genotypes. The interactions were decomposed by Bonferroni corrected pair-wise comparisons. In all cases, a $p \leq 0.05$ was considered significant.

For synaptic plasticity experiments, LTP was calculated using averaged values of EPSP rise slope -5 to 0 before and 35 to 40 min after AP pairing or FP rise slope -10 to 0
before and 50-60 min after theta burst stimulation. Successful LTP was defined within a group by Wilcoxon Signed Rank Test comparisons to baseline, and within a neuron as \( \geq 1.15 \) of baseline. Comparisons of proportions of neurons that potentiated between groups employed \( z \)-tests. Comparisons between mean LTP values between groups employed unpaired \( t \)-tests. ANOVA (one- and two-way) were used as indicated for analyses involving genotype (wildtype vs. PAM\(^{+/} \)) and/or treatment (aCSF vs. picrotoxin or mIPSC vs. sIPSC). These include membrane properties, sEPSC, mIPSC/sIPSC. RMANOVA was used to make genotypic comparisons across within-subject treatments (e.g. serial current steps or stimulation intensities). Unpaired \( t \)-tests were used to detect differences for individual treatments. In all cases, a \( p \leq 0.05 \) was considered significant.
RESULTS

PAM\textsuperscript{++} mice present with an anxiety-like phenotype that is alleviated with diazepam.

A general assessment of sensory and motor function revealed the wildtype and PAM\textsuperscript{++} animals to be indistinguishable (not shown). Naïve PAM\textsuperscript{++} mice display anxiety-like behaviors in the elevated zero maze (Bousquet-Moore et al., 2010b), where reduced time in the open areas is the major indicator of this response (Shepherd et al., 1994). To determine whether this behavior was responsive to anxiolytic drugs, wildtype and PAM\textsuperscript{++} animals were given vehicle or diazepam. As reported, vehicle-treated PAM\textsuperscript{++} mice spent less time in the open areas than wildtype controls ($p=0.05$) (Fig. 2-1A). Diazepam dose-dependently increased open area time in both genotypes; however, anxiolytic responses to diazepam were different between genotypes. For wildtype animals, 1 mg/kg diazepam was required to significantly increase open area time ($p<0.001$), whereas both doses were sufficient for PAM\textsuperscript{++} animals ($p$ values $<0.01$). The response in mutants to 0.5 mg/kg diazepam was similar to that of the vehicle wildtype controls; the 1mg/kg dose increased the open area time of wildtype mice more than PAM\textsuperscript{++} mice ($p<0.05$). The latency to first enter an open area revealed wildtype mice to have shorter latencies than PAM\textsuperscript{++} animals ($p<0.01$) (Fig. 2-1B). Diazepam reduced these latencies relative to vehicle for the genotypes at both doses ($p$ values $<0.05$). Both doses of diazepam were less efficacious in PAM\textsuperscript{++} mice than in wildtype controls ($p$ values $<0.05$). Diazepam also dose-dependently increased head-dipping and decreased freezing behaviors in both genotypes (not shown). It also enhanced the numbers of closed-to-open-to-closed area transitions (Wt: 2.00±0.93, 3.00±1.29, 2.30±0.45 and PAM\textsuperscript{++}: 0.00±0.05, 1.00±0.32, 2.10±0.74 transitions to 0, 0.5, and 1 mg/kg diazepam, respectively). Together, these
data demonstrate that PAM\textsuperscript{+/-} mice present with an anxiety-like phenotype that can be alleviated with the anxiolytic drug diazepam.

**PAM\textsuperscript{+/-} mice are deficient in short- and long-term conditioned fear responses.**

Naïve PAM mice were examined in fear conditioning as described (Taylor et al., 2008; Porton et al., 2009), where a 2 s 0.4 mA scrambled foot-shock (UCS) was paired once with a 12 kHz tone (CS). Contextual and cued testing occurred 1 or 24 h after conditioning. During conditioning, no genotypic differences were observed before or during presentation of the CS, or following the CS-UCS pairing (not shown). At both the 1 and 24 h contextual tests, freezing in PAM\textsuperscript{+/-} mice was decreased relative to that for
wildtype littermates (p values<0.01) (Fig. 2-2A-B). This effect was also evident when freezing was collapsed over the 5 min interval (p<0.001) (Fig. 2-2E). Although freezing in the context tests for both genotypes increased during the 24 h relative to the 1 h test (p values<0.001), freezing was reduced at both times in PAM+/− mice relative to the wildtype controls (p values<0.001). Hence, the PAM+/− animals are deficient in contextual fear conditioning at both 1 and 24 h.

Similar results were observed for cued testing (Fig. 2-2C-D). Freezing prior to presentation of the CS was not different between genotypes at 1 (Fig. 2-2C; left) or 24 h (Fig. 2-2D; left). During the 3 min CS, freezing in wildtype mice was higher than that for PAM+/− animals during both the 1 and 24 h tests (p values<0.001). Freezing in wildtype mice was stable across the 3 min period of CS presentation and was higher at 24 than at 1 h (p values<0.001). By comparison, freezing in PAM+/− animals was only enhanced during the 24 h test over the last min compared to that during the 1 h test (p<0.01).

Analysis of collapsed data as mean percent freezing (Fig. 2-2F) demonstrated that both genotypes engaged in more freezing during the CS than the pre-CS interval (p values<0.001). Additionally, freezing by wildtype mice during the CS presentation was higher at 24 h than at 1 h (p<0.001); this increase was not observed in the mutants. Collectively, these data demonstrate that PAM+/− mice are markedly impaired in short- and long-term contextual and cued fear conditioning. Because amygdala lesions depress freezing responses in both the contextual and cued tests whereas lesions of the hippocampus only decrease freezing in context but leave cued freezing intact (Kim and Fanselow, 1992; Phillips and Ledoux, 1992), our results suggest that PAM+/− mice may have some amygdala dysfunction.
Figure 2-2. PAM^{+/−} mice are deficient in fear conditioning. Wt and PAM^{+/−} littermates were conditioned and tested 1 or 24 h later in context- and cue-dependent fear conditioning. (A, B) Percent time freezing during context testing 1 (A) or 24 h (B) after conditioning. Animals were tested in the same chamber in which they had been conditioned but in the absence of the CS and UCS. (C, D) Percent time freezing during cued testing 1 (C) or 24 h (D) after conditioning. In cued testing, animals were tested in a novel chamber and after 2 min the CS was presented in the absence of the UCS. (E) Mean percent time freezing by Wt and PAM^{+/−} mice over the 5 min context test, conducted 1 and 24 h after conditioning. (F) Mean percent time freezing by PAM^{+/−} mice during cued testing at 1 (left) and 24 h (right) after conditioning; the pre-CS results are averaged over the first 2 min interval and the CS results are over final 3 min of testing during CS presentation. *p<0.05, compared to Wt mice; +p<0.05, compared to freezing responses at 1 h; ^p<0.05, compared to the pre-CS interval in the cued test (ANOVA, RMANOVA, Bonferroni corrected pair-wise comparisons). n = 10 mice/genotype/test-condition and time.
PAM$^{+/−}$ mice display aberrant fear-potentiated startle responses.

Fear-potentiated startle was used to examine amygdala function in PAM$^{+/−}$ mice (Taylor et al., 2008; Porton et al., 2009). Mice were conditioned at one of two shock intensities (0.4 and 0.6 mA) and tested for potentiation at three different startle intensities (100, 105, and 110 dB) 48 h after the CS-UCS pairing. Startle responses on test days 1 and 2 did not differ among genotypes to the different UCS conditions (not shown). Hence, baseline startle reactivities and potentiation of responses to a pure tone were similar between wildtype and PAM$^{+/−}$ mice. Forty-eight h after the CS-UCS pairing, animals were examined for fear-potentiated startle. When conditioned with 0.4 mA shock (Fig. 2-3A; left), PAM$^{+/−}$ mice showed significantly less potentiation of their startle responses than

![Figure 2-3. PAM$^{+/−}$ mice show abnormal fear-potentiated startle.](image)

**Figure 2-3.** PAM$^{+/−}$ mice show abnormal fear-potentiated startle. Wt and PAM$^{+/−}$ littermates were conditioned with two different intensities of shock (0.4 and 0.6 mA). (A) Percent potentiation by Wt and PAM$^{+/−}$ mice to a 12 kHz 70 dB tone preceded by a 100, 105 or 110 dB white-noise startle stimulus; animals were conditioned with 0.4 (left) or 0.6 (right) mA shock. (B) Sensitivity of Wt and PAM$^{+/−}$ mice to varying intensities of scrambled foot-shock; behavioral responses are represented by composite scores during the application of the shock. *p<0.05, compared to Wt mice; +p<0.05, compared to responses to the 100 dB startle stimulus; #p<0.05, compared to responses to the 105 dB startle stimulus; ^p<0.05, compared to responses following 0.4 mA shock (ANOVA, RMANOVA, Bonferroni corrected pair-wise comparisons). n = 9-10 mice/genotype/shock-intensity for fear-potentiated startle, n = 10 mice/genotype for shock-threshold testing.
wildtype controls \((p<0.01)\); however, when the shock intensity was increased to 0.6 mA potentiation to the startle stimuli was similar between genotypes (Fig. 2-3A; right). Overall, these findings are particularly noteworthy since in shock-threshold testing (Fig. 2-3B), no genotype differences were discerned across different intensities of foot-shock – including those used in the fear conditioning or fear-potentiated startle tests. Hence the deficiencies of the PAM\(^{+/-}\) mice in fear conditioning and fear-potentiated startle cannot be attributed to genotype differences in response to shock. Together with the fear conditioning results, these findings for fear-potentiated startle strongly suggest that the amygdala is dysfunctional in PAM\(^{+/-}\) mice.

**Pyramidal neurons and interneurons of the basolateral amygdala express PAM.**

To determine how a change in PAM expression causes these behavioral outcomes, we compared the cellular expression patterns of PAM in the basolateral complex of wildtype and PAM\(^{+/-}\) amygdala. There were no gross differences in the structures of PAM\(^{+/-}\) and wildtype amygdalae. In agreement with haploinsufficiency of the \(Pam\) gene (Czyzyk et al., 2005a), PAM immunoreactivity was consistently less intense in PAM\(^{+/-}\) compared to wildtype amygdala (Fig. 2-4A, B). GAD67 immunoreactivity was localized to GABAergic presynaptic terminals and the soma of amygdala interneurons (Muller et al., 2006; Muller et al., 2003; Rainnie et al., 2006). In the basolateral nuclei, GAD67 immunoreactivity in both genotypes was consistently stronger than in the lateral nucleus (Fig. 2-5A).
When examined at higher magnification, PAM immunoreactivity was apparent in the soma of both wildtype and PAM^+/^- lateral amygdala neurons (Fig. 2-4C, D; green). Although present in secretory granules, PAM is concentrated in the trans-Golgi network and endocytic vesicles and is accumulated in the perinuclear region of the cell soma (Niciu et al., 2006). While PAM was present in both GAD67-negative pyramidal neurons
(marked by asterisks) and GAD67-positive interneurons (marked by arrows), PAM immunoreactivity was more intense in interneurons. This finding is consistent with earlier *in situ* hybridization analyses of PAM expression in the rat cerebral cortex and hippocampus (Schafer et al., 1992b). Interneurons of the basolateral nucleus also exhibited stronger PAM immunoreactivities than pyramidal neurons in both genotypes (*Fig. 2-5A*; arrows).

In both lateral and basolateral nuclei, intense GAD67 immunoreactive puncta, representing axo-somatic GABAergic synapses, surrounded the somata of pyramidal neurons (*Fig. 2-4C, D* and *Fig. 2-5A*; red). These synapses are likely contributed by local interneurons and paracapsular neurons, which mediate the tight feed-forward and feed-back inhibition described in these areas (Sah et al., 2003; Marowsky et al., 2005; Szinyei et al., 2000). We combined electrophysiological and pharmacological approaches to study the function of these synapses in regulating excitability and plasticity in the wildtype and PAM⁺⁻ amygdala.
Lateral paracapsular GABAergic neurons, which form the lateral intercalated cell masses, were identified as clusters of GAD67-positive cell bodies along the medial border of the external capsule and lateral border of the lateral nucleus (Fig. 2-4A, B;
Unlike local interneurons, paracapsular neurons were devoid of PAM staining (Fig. 2-5B). This finding is consistent with the fact that no peptide co-transmitter has been identified in these neurons (Marowsky et al., 2005). Strong PAM immunoreactivity was evident in lateral intercalated regions in the form of puncta surrounding the paracapsular cell somata (Fig. 2-5B) (Perez de la et al., 2007). This finding supports the lack of peptidergic expression and abundance of peptidergic innervation of lateral paracapsular cells.

The modulation of other GABAergic neurons that innervate amygdala pyramidal neurons could be affected in PAM\(^{+/−}\) amygdala. Intense immunoreactivity of PAM in the neuropil surrounding lateral paracapsular GABAergic neurons of the intercalated cell masses (Fig. 2-5B) closely resembles that for the D1 receptor, as well as for cholecystokinin (CCK) and gastrin (Perez de la et al., 2007), both of which are amidated neuropeptides (Eipper et al., 1992). Therefore, disrupted peptidergic modulation of paracapsular cells could contribute to altered GABAergic inhibition in the PAM\(^{+/−}\) amygdala.

To this point, Western and enzymatic assays have been used to quantify brain PAM levels at half of normal (Bousquet-Moore et al., 2010b;Bousquet-Moore et al., 2009;Czyzyk et al., 2005a). This is the first study to characterize PAM protein expression in the brain at a cellular level. We found a global reduction in neuronal PAM expression in the PAM\(^{+/−}\) amygdala (Figs. 4, 5), as opposed to a complete loss of PAM expression in half of neurons. This finding allowed us to conduct cellular electrophysiological experiments in the PAM\(^{+/−}\) amygdala in which single neurons were patched later in this study.

**Deficient synaptic plasticity at PAM\(^{+/−}\) thalamic amygdala afferents.**
To investigate the neurophysiological basis for the behavioral abnormalities displayed by
PAM⁺⁻ mice (Figs. 1-3), we performed several electrophysiological experiments in the PAM⁺⁻ amygdala. Extensive experiments have demonstrated that the lateral, basolateral, and central nuclei of the amygdala are critical to fear conditioning (Davis, 1990; Kim and Jung, 2006; Ledoux, 2000). Auditory and somatosensory inputs, which carry relevant information about the CS and UCS, terminate primarily in the lateral nucleus which is essential to normal cued conditioning (Ledoux et al., 1990). Thalamic inputs to the lateral nuclei are also important at the time of the CS–UCS pairing, and play a more critical role in amygdala plasticity and learning than cortical pathways and their corresponding inputs (Quirk et al., 1997).

Figure 2-6. PAM⁺⁻ mice exhibit a deficiency in thalamic afferent long-term potentiation (LTP) that relies upon GABAergic transmission. Thalamic afferents were stimulated and pyramidal neuron membrane responses were recorded in whole-cell mode in the lateral nuclei of Wt and PAM⁺⁻ amygdala slices. Insets above each plot depict representative averaged traces for each genotype prior to (solid) and after (dashed) LTP induction as indicated (baseline and post-induction). (A-D) Time-course (1 min bins) of averaged LTP experiments using an action potential pairing (AP pairing) induction paradigm. Synaptic efficacy was assessed using the rise slope of EPSPs that were normalized for genotypic comparisons. Experiments were conducted in aCSF control solution (panel A; n = 10 Wt; 11 PAM⁺⁻), aCSF with 100 µM picrotoxin (PTX) (panel B; n = 9 Wt; 7 PAM⁺⁻), aCSF with cGP35348 (GABA B receptor antagonist; 1 µM) (panel C; n = 9 Wt; 9 PAM⁺⁻), or aCSF with PTX and cGP35348 (panel D; n = 12 Wt; 10 PAM⁺⁻). (E) Values of LTP assessed at 40 min post-induction for individual pyramidal neurons are plotted by experimental condition (data from panels A-D). Bars depict mean values and error bars depict the standard error of the mean. *p<0.05, compared to baseline; Ns Not significant compared to baseline (Wilcoxon Signed Rank Test).
Since PAM\textsuperscript{+/−} mice were most deficient in cued fear conditioning (Fig. 2-2), we assessed the status of glutamatergic synaptic plasticity at thalamic afferent synapses (Fig. 2-6). Long-term potentiation (LTP) at this synapse is the strongest neural correlate to fear learning and memory to date (Maren and Quirk, 2004; Pare et al., 2004; Sah et al., 2008; Sigurdsson et al., 2007). We stimulated thalamic afferents and recorded excitatory post-synaptic potentials (EPSPs) in wildtype and PAM\textsuperscript{+/−} pyramidal neurons from the lateral nucleus. LTP was induced by synchronized trains of afferent stimulation with post-synaptically induced action potentials. In control solution, both wildtype and PAM\textsuperscript{+/−} neurons failed to potentiate consistently above baseline, and there was no difference in synaptic efficacy was assessed using FP rise slopes, which were normalized to make genotypic comparisons (n = 6 Wt; 6 PAM\textsuperscript{+/−}).

As was found using whole-cell recordings and action potential pairing, slices from Wt mice displayed reliable LTP (1.42 ± 0.17; p<0.05), whereas slices from PAM\textsuperscript{+/−} mice did not (1.04 ± 0.06). This finding provides further support for the LTP deficit in PAM\textsuperscript{+/−} mice we observed using action potential pairing in whole-cell mode. Experiments were performed in the presence of 100 µM picrotoxin. \#p<0.05 compared to baseline (Wilcoxon Signed Rank Test).

**Figure 2-7.** Field potential recordings in the PAM\textsuperscript{+/−} lateral nucleus demonstrate the deficiency in thalamic afferent LTP using a theta-burst stimulation induction paradigm. Thalamic afferents were stimulated and recordings were made in the lateral nuclei of Wt and PAM\textsuperscript{+/−} amygdala slices. Time courses are plotted in 1 min bins. Insets above plots depict representative averaged field potential (FP) traces for each genotype prior to (solid) and after (dashed) LTP induction as indicated (baseline and post-induction). Time-course of averaged LTP experiments using a theta-burst stimulation induction paradigm (4 sets at 20 sec intervals; set = 10 bursts at 200 ms intervals; burst = 4 pulses at 100 Hz). Synaptic efficacy was assessed using FP rise slopes, which were normalized to make genotypic comparisons (n = 6 Wt; 6 PAM\textsuperscript{+/−}). As was found using whole-cell recordings and action potential pairing, slices from Wt mice displayed reliable LTP (1.42 ± 0.17; p<0.05), whereas slices from PAM\textsuperscript{+/−} mice did not (1.04 ± 0.06). This finding provides further support for the LTP deficit in PAM\textsuperscript{+/−} mice we observed using action potential pairing in whole-cell mode. Experiments were performed in the presence of 100 µM picrotoxin. \#p<0.05 compared to baseline (Wilcoxon Signed Rank Test).
mean LTP values between genotypes (Fig. 2-6A). However, a larger proportion of wildtype neurons could be stimulated to induce LTP than PAM^+/^- neurons (wildtype 7 of 10; PAM^+/^- 1 of 11; \( p < 0.05 \), z-test). Since previous LTP studies have shown that limiting fast GABAergic transmission is necessary for LTP induction in acute slices (Tully et al., 2007; Bauer et al., 2002), we used the same induction paradigm in the presence of the GABA_A receptor antagonist picrotoxin (PTX; 100 µM) (Fig. 2-6B). Wildtype neurons reliably displayed LTP under these conditions (\( p < 0.01 \)), while PAM^+/^- neurons failed to potentiate. We replicated this finding using a theta-burst stimulation induction paradigm, which is reliant on NMDA receptor-mediated post-synaptic calcium signaling (Pan et al., 2009), and field potential recordings (Fig. 2-7).

Many forms of LTP at thalamic afferent synapses are dependent upon NMDA receptor signaling (Bauer and Ledoux, 2004; Bauer et al., 2002; Sigurdsson et al., 2007; Weisskopf and Ledoux, 1999). Any differences in NMDA receptors could contribute to the PAM^+/^- deficit in LTP at amygdalar afferent synapses. To isolate AMPA- and mixed AMPA/NMDA receptor-mediated currents, we measured thalamic synaptic currents in the presence of PTX using 3 different stimulation strengths at holding potentials of -70 and +50 mV (Fig. 2-8). Comparison of AMPA:NMDA ratios revealed no significant differences between genotypes. Thus, the NMDA receptor contribution to glutamatergic transmission is normal at PAM^+/^- thalamic afferent synapses. Moreover, the deficit in thalamic afferent LTP cannot be explained by inherent differences in NMDA receptor signaling.
GABAergic transmission can also suppress glutamatergic LTP induction through GABA_B.
receptors. GABA\textsubscript{B} receptors are expressed on the dendrites of amygdalar pyramidal neurons and the glutamatergic terminals that innervate them (McDonald et al., 2004). In addition, GABA\textsubscript{B} activation suppresses glutamate release from thalamic and cortical afferent terminals (Pan et al., 2009). To test whether GABA\textsubscript{B} receptors were involved in the PAM\textsuperscript{+/-} LTP deficit, we induced LTP using the same action potential pairing paradigm described above in the presence of the GABA\textsubscript{B} receptor antagonist CGP35348 (1 \textmu M) (Fig. 2-6C). LTP significantly greater than baseline was not achieved in either genotype, suggesting limitation of slow inhibition alone is not sufficient to gate LTP at thalamic afferent synapses. Next, we asked whether blockade of both GABA\textsubscript{A} and GABA\textsubscript{B} receptors could affect the PAM\textsuperscript{+/-} LTP deficit by inducing LTP in the presence of both picrotoxin and CGP (Fig. 2-6D). Under these conditions, thalamic afferent synapses were potentiated in wildtype and PAM\textsuperscript{+/-} amygdala neurons to similar extents (p values<0.01). Collectively, these data provide clear evidence for a synaptic plasticity...
deficit at amygdalar afferent synapses that is reliant on GABAergic transmission and correlates with the fear learning and memory deficit of PAM\(^{+/-}\) mice.

We also tested whether LTP deficit of PAM\(^{+/-}\) mice extends to cortical afferent synapses in the lateral nucleus. We performed the experiment under identical conditions to the aforementioned LTP experiment except cortical afferents were stimulated rather than thalamic (Fig. 2-9). In the presence of PTX, wildtype neurons consistently potentiated in response to AP pairing \((p<0.05)\), but PAM\(^{+/-}\) neurons failed to potentiate significantly above baseline on average. Therefore, the LTP deficit of PAM\(^{+/-}\) mice applies to both cortical and thalamic afferent synapses, suggesting the underlying mechanism for PAM\(^{+/-}\) synaptic plasticity dysfunction is likely to be intrinsic to the amygdala.

There was considerable overlap among neurons regarding their responsiveness to LTP

Figure 2-9. Cortical afferent amygdala LTP is disrupted in PAM\(^{+/-}\) mice. Cortical afferents were stimulated and pyramidal neuron membrane responses were recorded in whole-cell mode in the lateral nuclei of Wt and PAM\(^{+/-}\) amygdala slices. (A) Insets above depict representative averaged traces for each genotype prior to (solid) and after (dashed) LTP induction as indicated (baseline and post-induction). (A-D) Time-course (1 min bins) of averaged LTP experiments using an AP pairing induction paradigm. Synaptic efficacy was assessed using the rise slope of EPSPs that were normalized for genotypic comparisons. Experiments were conducted in aCSF with 100 \(\mu\)M picrotoxin (PTX) \((n = 8\) Wt; 9 PAM\(^{+/-}\)). (B) Values of LTP assessed at 40 min post-induction for individual pyramidal neurons are plotted for thalamic and cortical afferent experiments. Bars depict mean values and error bars depict the standard error of the mean. \(^{#}p<0.05\), compared to baseline; \(^{NS}\)Not significant compared to baseline (Wilcoxon Signed Rank Test).
induction between genotypes (Fig. 2-6D), where some \( \text{PAM}^{+/−} \) neurons responded at wildtype levels. The difference in average LTP between genotypes resulted from a shift in the distribution of LTP magnitude rather than a change to all-or-none responding. This effect was evident in each pharmacological condition tested, supporting the notion of heterogeneity of pyramidal neurons in the lateral nucleus and their responsiveness to LTP induction that was independent of GABAergic inhibition. Collectively, these data provide substantial support for a deficit in amygdalar afferent synaptic plasticity that correlates with the deficits in fear conditioning and fear-potentiated startle in \( \text{PAM}^{+/−} \) animals.
Input-specific synaptic enhancement in the PAM⁺/⁻ amygdala.

We assessed the balance of excitation and inhibition at pathway-specific afferent synapses in the amygdala by stimulating thalamic or cortical inputs and recording current responses in pyramidal neurons of the lateral nucleus. Interneurons of the amygdala mediate di-synaptic feed-forward inhibition (Sah et al., 2003; Bissiere et al., 2003; Bauer and Ledoux, 2004; Shin et al., 2006; Szinyei et al., 2000) and contribute a GABAergic outward current that follows the mono-synaptic glutamatergic inward current. We adjusted the holding potential to -55 mV to observe both of these currents in a single trace and generated input-output curves for thalamic and cortical input pathways (Fig. 2-10). Both inward and outward current amplitudes increased with stimulation strength in both genotypes. For thalamic inputs, PAM⁺/⁻ inward current amplitudes were enhanced compared to wildtype at stimulation intensities between 25 and 45 µA (Fig. 2-10A-C). PAM⁺/⁻ outward currents were significantly larger over an even wider range of stimulation intensities. For cortical inputs, wildtype and PAM⁺/⁻ inward current amplitudes were identical over the full stimulation ladder, and PAM⁺/⁻ outward currents were significantly larger at only one stimulation intensity (Fig. 2-10D-F).
RMANOVA was used to analyze baseline synaptic efficacy at amygdala afferent synapses. No effect of genotype on inward current responses across stimulation intensities was evident, but there was an effect on outward currents \( F_{(1,18)} = 5.017; \)
$p<0.05$ (Fig. 2-10A-C). This may reflect enhancement of interneuronal network activity that contributes to feed-forward inhibition; whereas the enhancement of thalamic glutamatergic afferent was not strong enough to achieve statistical significance by RMANOVA, contribution of enhanced interneuronal network activity could explain the genotype effect found for di-synaptic outward currents. When current responses to cortical afferent stimulation were analyzed, no effects for genotype were observed for inward or outward currents (Fig. 2-10D-F). These data demonstrate enhancement of afferent synaptic efficacy and feed-forward inhibition in the PAM$^{+/−}$ lateral amygdala that is specific to the thalamic pathway.

The increase in baseline efficacy at PAM$^{+/−}$ thalamic afferents synapses may reflect compensation for enhanced inhibition. Thalamic afferents display stronger feed-forward inhibition than cortical afferents (Bauer and Ledoux, 2004; Humeau et al., 2005), and may require compensatory enhancement as a result. However, our data show larger di-synaptic outward currents for cortical versus thalamic afferents in wildtype neurons (Fig. 2-10C, F). This discrepancy may stem from the inclusion of lateral paracapsular neurons in addition to interneurons in feed-forward inhibition of cortical inputs, or unequal distances of stimulating electrode placements in thalamic versus cortical experiments.

Thalamic and cortical synapses are morphologically, molecularly and physiologically distinct (Weisskopf and Ledoux, 1999; Doyere et al., 2003; Humeau et al., 2005; Humeau and Luthi, 2007; Humeau et al., 2007; Ota et al., 2008). Input specificity of the PAM$^{+/−}$ phenotype may reflect the differential importance of PAM in thalamic versus cortical afferent synapses.

**Subtle signs of hyperexcitability in the PAM$^{+/−}$ amygdala also implicate GABA.** Alterations in the firing properties or synaptic organization of amygdalar neurons could
influence post-synaptic and pre-synaptic responses to LTP induction, thus contributing to the LTP deficit observed in PAM^{−/−} mice. The low dose effect of diazepam on anxiety-like behavior in PAM^{−/−} mice (Fig. 2-1) and the hyper-sensitivity of PAM^{−/−} mice to pentylenetetrazol (Bousquet-Moore et al., 2010b) suggested that GABAergic inhibition was abnormal. With this in mind, we recorded the membrane and synaptic activities of pyramidal neurons in the lateral and basolateral nuclei of the amygdala in aCSF in the presence and absence of PTX.

We began these studies by measuring passive and active membrane properties. In

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<th>Passive Membrane Properties</th>
<th>Active Membrane Properties</th>
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<tr>
<td></td>
<td>RMP (mV)</td>
<td>Ri (MΩ)</td>
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<tr>
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<tr>
<td>Wt aCSF</td>
<td>-72.1 ± 1.23</td>
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<tr>
<td>PAM^{−/−} aCSF</td>
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<td>170.1 ± 6.58</td>
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<tr>
<td>Wt PTX</td>
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<td>153.4 ± 6.36</td>
</tr>
<tr>
<td>PAM^{−/−} PTX</td>
<td>-70.2 ± 1.14</td>
<td>156.0 ± 6.43</td>
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Table 2-1. Membrane properties of wildtype and PAM^{−/−} lateral nucleus pyramidal neurons. Passive and active membrane property parameters for lateral amygdala pyramidal neurons were derived from voltage responses to hyperpolarizing and depolarizing current steps. Wt and PAM^{−/−} neurons were recorded in control solution (aCSF) or aCSF containing 100 µM picrotoxin (PTX). Passive properties pertain to the kinetics of the voltage response to the current step onset (n = 39 Wt aCSF; 43 PAM^{−/−} aCSF; 45 Wt PTX; 45 PAM^{−/−} PTX). Two-way ANOVA revealed no significant effects. Active property parameters pertain to the first action potential fired by a neuron during positive current steps (n = 17 Wt aCSF; 22 PAM^{−/−} aCSF; 22 Wt PTX; 24 PAM^{−/−} PTX). ANOVA found significant main effects of PTX on action potential threshold (F(1,81) = 4.412; p<0.039) and half-width (F(1,81) = 12.731; p<0.001). Bonferroni corrected pair-wise comparisons showed only an individual effect of PTX on PAM^{−/−} threshold (p<0.020). Individual effects of PTX on half-width for both wildtype (p<0.007) and PAM^{−/−} (p<0.027) neurons were evident. + indicates p< 0.05, compared to aCSF within genotype (Bonferroni corrected pair-wise comparisons).
current clamp mode, wildtype and PAM\textsuperscript{+/−} amygdala neurons were injected with a series of negative and positive current steps. Passive membrane properties were derived from voltage responses to negative current steps and yielded no significant genotypic differences (Tables 2-1 and 2-2). Positive current steps induced action potentials with similar kinetics in wildtype and PAM\textsuperscript{+/−} amygdalar pyramidal neurons (Tables 2-1 and 2-2). Current steps of increasing amplitude were applied until the numbers of action potentials declined due to depolarization-induced inactivation of voltage-gated Na\textsuperscript{+} channels. The maximum numbers of action potentials fired in response to a single current step by wildtype and PAM\textsuperscript{+/−} neurons in aCSF were similar in the lateral nucleus

<table>
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<th>Basolateral nucleus</th>
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<tbody>
<tr>
<td></td>
<td>RMP (mV)</td>
<td>R\textsubscript{i} (MO)</td>
</tr>
<tr>
<td>Wt aCSF</td>
<td>-72.7 ± 0.89</td>
<td>94.7 ± 3.87</td>
</tr>
<tr>
<td>PAM\textsuperscript{+/−} aCSF</td>
<td>-72.0 ± 0.83</td>
<td>96.0 ± 3.57</td>
</tr>
<tr>
<td>Wt PTX</td>
<td>-72.0 ± 1.11</td>
<td>97.2 ± 4.81</td>
</tr>
<tr>
<td>PAM\textsuperscript{+/−} PTX</td>
<td>-73.7 ± 0.96</td>
<td>94.0 ± 4.19</td>
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Table 2-2. Membrane properties of wildtype and PAM\textsuperscript{+/−} basolateral nucleus pyramidal neurons. Data for passive (n = 34 Wt aCSF; 40 PAM\textsuperscript{+/−} aCSF; 22 Wt PTX; 29 PAM\textsuperscript{+/−} PTX) and active (n = 24 Wt aCSF; 26 PAM\textsuperscript{+/−} aCSF; 22 Wt PTX; 27 PAM\textsuperscript{+/−} PTX) membrane properties of pyramidal neurons in the basolateral nucleus were obtained as described for Table 1 for lateral nucleus pyramidal neurons. Two-way ANOVA revealed significant interactions between genotype and treatment condition for tau ($F$\textsubscript{(1,95)} = 4.350; $p$<0.039) and capacitance ($F$\textsubscript{(1,95)} = 3.978; $p$<0.048). Bonferroni corrected pair-wise comparisons showed that PTX increased tau ($p$<0.015) and capacitance ($p$<0.034) in Wt, but not PAM\textsuperscript{+/−} neurons. ANOVA also revealed a significant main effect of PTX on action potential threshold ($F$\textsubscript{(1,95)} = 4.350; $p$<0.018) and half-width ($F$\textsubscript{(1,95)} = 7.689; $p$<0.007). Bonferroni corrected pair-wise comparisons revealed individual effects of PTX on Wt AP threshold ($p$<0.022) and half-width ($p$<0.038).
PTX increased the maximum number of action potentials similarly in wildtype and PAM<sup>−/−</sup> neurons; ANOVA showed a significant main effect of PTX on maximum action potential number ($F_{(1,81)} = 14.715; p<0.001$). Bonferroni corrected pairwise comparisons revealed individual effects of PTX for wildtype ($p<0.05$) and PAM<sup>−/−</sup> ($p<0.001$) neurons (Fig. 2-11B). However, this enhancement occurred over a broader range of current steps in PAM<sup>−/−</sup> neurons compared to wildtype (Fig. 2-12A, B). In the basolateral nucleus, the number of action potentials fired was about 2-fold higher than in the lateral nucleus for both genotypes in aCSF (Fig. 2-11D, E), and addition of PTX did not alter the maximum number of action potentials fired in either genotype; no significant effects of PTX or genotype on maximum action potential number were observed. Under aCSF conditions, PAM<sup>−/−</sup> neurons tended to inactivate earlier than their wildtype controls, and this difference was abolished in the presence of PTX (Fig. 2-13A, B).
Upon termination of the current step, an after-hyperpolarization (AHP) of the membrane potential was often observed (Fig. 2-12E, 2-13E; arrow). In the lateral nucleus under control conditions, PAM\textsuperscript{+/-} neurons displayed larger maximum AHP amplitudes than wildtype neurons (p<0.05) (Fig. 2-11C). With the addition of PTX, PAM\textsuperscript{+/-} AHPs were significantly reduced while wildtype AHPs remained unchanged (p<0.001). ANOVA for maximal AHP amplitude revealed significant effects of PTX (F(1,81) = 6.696; p<0.05) and an interaction between genotype and PTX (F(1,81) = 5.080; p<0.05) (Fig. 2-11C). In the basolateral nucleus, no differences were observed between genotypes or treatment conditions (Fig. 2-11F). However, AHP values plotted as a function of current step amplitude revealed a 2-fold enhancement of the AHP in wildtype neurons with PTX, and this effect of PTX was not observed in PAM\textsuperscript{+/-} neurons (Fig. 2-13C, D). Collectively, these data identify subtle but significant signs of membrane hyperexcitability, including enhanced action potential firing and impaired AHP, in both the lateral and basolateral nuclei of PAM\textsuperscript{+/-} mice.
The current underlying the slow AHP is generated primarily by Ca$$^{2+}$$-activated K$$^+$$ channels such as BK and SK channels (Meis and Pape, 1997; Faber and Sah, 2002). This current is strongly modulated by catecholaminergic (Faber et al., 2008; Tully et al., 2007) and cholinergic inputs (Womble and Moises, 1993; Faber et al., 2005), which are dense in the basolateral amygdala. Over-expression of SK channels in the basolateral amygdala can have behavioral and physiological consequences including reduced anxiety-like behavior assessed in the elevated plus maze (Mitra et al., 2009).

**Figure 2-12.** Active membrane properties of lateral nucleus pyramidal neurons. (A-D) Mean values of action potential (AP) number and after-hyperpolarization peak amplitude (AHP) are plotted as a function of current step amplitude in aCSF (panels A, C; n = 17 Wt; 22 PAM$$^{+/−}$$) and PTX conditions (panels B, D; 22 Wt; 24 PAM$$^{+/−}$$). Error bars represent standard error of the mean. RMANOVA found no effects of genotype for these parameters. (E) Representative voltage response of a Wt lateral nucleus neuron to a +400 pA current step (inset); arrow indicates the peak of the AHP. *p<0.05, compared to Wt within treatment. +p<0.05, compared to aCSF within genotype (unpaired t-tests at individual current step amplitudes).
We next examined the status of fast glutamatergic signaling in PAM+/− amygdala neurons (Fig. 2-14). Using the same design as in the previous experiment, we compared spontaneous excitatory post-synaptic currents (sEPSC) in wildtype and PAM+/− amygdala neurons in control and PTX conditions. Under control conditions, sEPSC frequency was higher in PAM+/− than wildtype neurons (p<0.05) (Fig. 2-14A). PTX reduced the frequency and amplitude of sEPSCs in the lateral nucleus and abolished the PAM+/− enhancement in frequency (Fig. 2-14B). ANOVA for sEPSC frequency recorded in the lateral nucleus revealed a main effect of PTX (F_{(1,3416)} = 46.037; p<0.001) and a
significant interaction between genotype and PTX ($F_{(1,3416)} = 8.671; p<0.01$). ANOVA of sEPSC amplitude also showed a significant main effect of PTX ($F_{(1,3511)} = 10.160; p<0.001$), and no genotypic effects. The opposite was observed in the basolateral nucleus wherein PTX increased the sEPSC frequency ($p$ values<0.001) and amplitude ($p$ values<0.05) similarly in wildtype and PAM$^{+/-}$ neurons (Fig. 2-14C, D). ANOVA of sEPSC frequency in the basolateral nucleus revealed a significant main effect of PTX ($F_{(1,5391)} = 38.574; p<0.001$) and no genotypic effects. ANOVA of sEPSC amplitude also revealed a significant main effect of PTX ($F_{(1,5414)} = 15.953; p<0.001$). These data suggest that the membrane and baseline excitatory synaptic properties of PAM$^{+/-}$ amygdala neurons are largely intact with signs of hyperexcitability in PAM$^{+/-}$ lateral nucleus neurons that are GABA-dependent.
We were surprised to find that PTX decreased sEPSC frequency and amplitude in the lateral nucleus. Spontaneous excitatory post-synaptic currents (sEPSC) from Wt and PAM+/- lateral and basolateral nucleus pyramidal neurons were recorded at V_holding = -70 mV in the presence and absence of picrotoxin (PTX; 100 µM). Traces are representative examples of sEPSC activity for each genotype and treatment condition. (A, C) sEPSC activity from Wt and PAM+/- pyramidal neurons in the lateral (panel A; n = 25 Wt; 25 PAM+/-; 38 events/neuron) and basolateral (panel C; n = 20 Wt; 24 PAM+/-; 63 events/neuron) nucleus recorded in control solution (aCSF). Frequency and amplitude of sEPSC events are plotted for each genotype (right). (B, D) sEPSC activity recorded in the presence of PTX. Data are from Wt and PAM+/- pyramidal neurons in the lateral (panel B; n = 24 Wt; 21 PAM+/-) and basolateral (panel D; n = 19 Wt; 23 PAM+/-) nucleus. *p<0.05, compared to Wt; +p< 0.05, compared to aCSF within genotype (Two-way ANOVA; Bonferroni corrected pair-wise comparisons).
lateral nucleus (Fig. 2-14). We expected PTX would increase both parameters as was observed in the basolateral nucleus. This finding may reflect enhanced GABA<sub>B</sub> receptor-mediated transmission under PTX conditions that decreased spontaneous glutamatergic activity in this region. GABA<sub>B</sub> receptors localize to pre-synaptic glutamatergic terminals and post-synaptic dendritic domains of lateral nucleus pyramidal neurons (McDonald et al., 2004), and their activation could account for the PTX-induced reductions in sEPSC frequency and amplitude, respectively. Moreover, our sEPSC data support enhanced GABA<sub>B</sub> receptor activation in the lateral amygdala in the presence of PTX.

**GABAergic transmission is enhanced in the PAM<sup>+/−</sup> amygdala.**

Enhanced behavioral sensitivity to low dose diazepam, and the effects of PTX on amygdala neuronal input and output strongly suggest that GABAergic inhibition is perturbed in the PAM<sup>+/−</sup> brain. To test this hypothesis directly, we recorded GABAergic activity in wildtype and PAM<sup>+/−</sup> lateral nucleus neurons (Fig. 2-15). Addition of tetrodotoxin (1 µM) blocked action potential-mediated GABAergic activity and left only activity-independent, “miniature” inhibitory currents (mIPSCs) (Fig. 2-16A). mIPSCs recorded from PAM<sup>+/−</sup> neurons displayed increased frequencies (p< 0.001) and amplitudes (p< 0.001) compared to those recorded from the wildtype lateral nucleus (Fig. 2-15A). Enhanced frequency of PAM<sup>+/−</sup> activity-independent GABAergic events in the lateral nucleus could result from an increase in release probability or synapse number. To distinguish these possibilities, we evoked inhibitory signals via local stimulation (Marowsky et al., 2005) and increased the stimulation strength to achieve maximal responses (Fig. 2-15B). The charges of evoked synaptic responses were significantly larger than wildtype responses across the full range of stimulation intensities and at their maximal values. RMANOVA revealed a main effect of genotype on evoked IPSC charge (F<sub>1,35</sub> = 11.851; p<0.01) (Fig. 2-15B). This difference in charge was
Figure 2-15. GABAergic signaling is enhanced in the PAM<sup>−/−</sup> lateral nucleus. Wt and PAM<sup>−/−</sup> pyramidal neurons in the lateral nucleus were dialyzed with a high [Cl<sup>-</sup>]-containing pipette solution and inhibitory post-synaptic currents (IPSC) were recorded under glutamatergic blockade. (A) “Miniature” IPSCs (mIPSCs) were recorded in the presence of 1 µM tetrodotoxin. Representative current traces depicting mIPSCs recorded from Wt and PAM<sup>−/−</sup> pyramidal neurons (left). Mean mIPSC frequency and amplitude are plotted for both genotypes (right; n = 28 Wt; 25 PAM<sup>−/−</sup>; 37 events/neuron). (B) Local stimulation elicited “evoked” IPSCs (eIPSCs) in Wt and PAM<sup>−/−</sup> neurons only in the absence of tetrodoxin. Two superimposed representative current traces from each genotype in response to a 20 µA single stimulus are shown (left). Charge (the integral of current) was used as a measure of synaptic strength and is plotted as a function of stimulation intensity (right; n = 21 Wt; 16 PAM<sup>−/−</sup>). *p<0.05, compared to Wt (unpaired t-tests at individual stimulus intensities). (C) Paired pulses were applied under the same conditions as in B. Averaged traces for each genotype depict representative current responses to paired pulses evoked at an intensity that elicited half-maximal responses for individual neurons (left). Ratios for both genotypes are plotted for 25, 50 and 100 ms intervals (right; n = 16 Wt; 13 PAM<sup>−/−</sup>). (D) “Spontaneous” IPSCs (sIPSCs) were recorded from Wt and PAM<sup>−/−</sup> neurons in the absence of tetrodoxin and local stimulation. Representative current traces depicting sIPSCs recorded from Wt and PAM<sup>−/−</sup> pyramidal neurons (left). Arrows indicate putative action potential-dependent GABAergic events. sIPSC frequency and amplitude are plotted for both genotypes (right; n = 25 Wt; 21 PAM<sup>−/−</sup>; 36 events/neuron). *p<0.05, compared to Wt; +p<0.05, compared to mIPSC value within genotype (ANOVA; Bonferroni correct pair-wise comparisons for mIPSC and sIPSC data).

evidently contributed by a combination of peak current amplitude and fast decay time
Figure 2-16. Parameters of evoked GABAergic transmission in the PAM\textsuperscript{+/−} lateral nucleus contribute to enhanced charge. A bipolar stimulating electrode was placed into the neuropil of the lateral amygdalar nucleus and evoked inhibitory post-synaptic currents (eIPSCs) were recorded. (A) A single current trace recorded from a Wt lateral nucleus pyramidal neuron depicting spontaneous inhibitory post-synaptic currents (sIPSCs) and eIPSCs. Stimulations were applied at 0.1 Hz via local stimulation and continued through the course of the trace. Tetrodotoxin (TTX; 1µM) was added after 3 min of baseline recording (top line). (B-E) eIPSCs were recorded during a series of graded stimulations in the neuropil. Parameters of eIPSCs plotted as a function of local stimulation strength (n = 21 Wt; 16 PAM\textsuperscript{+/−}). Peak amplitude (B), 10-90% rise slope (C), 90-10% decay slope (D), and the fast component of the decay time constant (E) are plotted as a function of stimulation intensity for both genotypes. RMANOVA found no effects of genotype for these parameters. *\(p<0.05\), compared to Wt (unpaired \(t\)-tests at individual current step amplitudes).
differences. Charge signified the absolute number of Cl\textsuperscript{−} ions that pass in response to a given stimulus, and its enhancement in the PAM\textsuperscript{+/−} lateral nucleus supports either a greater number of inhibitory synapses or enhanced release probability.

We then adjusted the stimulation strength to that which evoked a half-maximal response and recorded current responses to paired pulses (Fig. 2-15C). There was no statistical difference in paired-pulse ratio between wildtype and PAM\textsuperscript{+/−} at any of the 3 inter-pulse intervals tested, suggesting that the enhanced GABAergic activity observed in the PAM\textsuperscript{+/−} lateral amygdala does not result from a difference in release probability, but rather from an increase in the number of GABAergic synapses. RMANOVA for paired pulse ratios revealed a significant interaction between genotype and inter-pulse interval ($F_{(1,27)} = 7.127; p<0.05$). Thus, response to paired pulses, as a function of inter-pulse interval, depended on genotype. The complicated circuitry of the interneuronal network makes specific interpretation of this result difficult, but it is clear that activity-dependent inhibitory signals from the interneuronal network are perturbed in PAM\textsuperscript{+/−} mice.

To determine whether there is an activity-dependent component of GABAergic enhancement in the PAM\textsuperscript{+/−} amygdala, we recorded “spontaneous” inhibitory post-synaptic currents (sIPSCs) in the absence of tetrodotoxin. We kept glutamatergic transmission blocked to ensure isolation of interneuronal network activity.

As expected, sIPSC frequency and amplitude were significantly increased compared to those of mIPSCs due to the addition of large activity-dependent GABAergic events (Fig. 2-15D; arrows). While PAM\textsuperscript{+/−} neurons appeared to display a larger increase in frequency than wildtype neurons, ANOVA found no significant interaction between tetrodotoxin condition and genotype. In the basolateral nucleus, sIPSC frequency was 3-fold higher than in the lateral nucleus (Fig. 2-17). These results correlate with the substantial
increase in GAD67 immunoreactivity, presumably representing GABAergic synapses, between the lateral and basolateral nuclei (Figs. 2-4, 2-5). sIPSC recorded from PAM⁺/⁻ neurons displayed enhanced frequencies (p<0.05) and reduced amplitudes (p<0.001). Collectively, these data provide clear evidence for enhanced GABAergic transmission in the PAM⁺/⁻ amygdala.
DISCUSSION

This study identifies neurophysiological correlates in the amygdala that may contribute to the behavioral abnormalities observed in PAM deficient animals. We have previously shown that half the normal amount of PAM is insufficient to support normal physiology and that heterozygous mice exhibit an anxiety-like phenotype (Czyzyk et al., 2005a; Bousquet-Moore et al., 2010b; Bousquet-Moore et al., 2009). Data presented here expand the PAM\(\text{+/-}\) behavioral phenotype to include learned fear, characterize cellular PAM expression in the amygdala, and demonstrate signs of enhanced inhibition that likely contribute to the observed deficit in amygdalar afferent synaptic plasticity. Our data indicate that PAM heterozygosity affects several neuronal properties and neurotransmitter systems in the amygdala (Fig. 2-18), which is consistent with the multiple modulatory/signaling pathways in which neuropeptides play a role. Our results emphasize the importance and sensitivity of peptidergic pathways in mammalian amygdala-dependent emotions and behaviors.

Integrating the PAM\(\text{+/-}\) behavioral and physiological phenotypes

Anxiety and fear responses. Our results show that PAM\(\text{+/-}\) mice display anxiety-like behaviors and are deficient in contextual and cued fear conditioning. These findings were unexpected because “anxious” mice often show enhanced fear responses (Korte and De Boer, 2003). Over the years, evidence has accrued that projections from the lateral bed nucleus of stria terminalis mediate certain types of anxiety and stress responses, whereas the medial central nucleus of the amygdala controls phasic fear (Iwata et al., 1986; Ledoux et al., 1988; Hitchcock and Davis, 1991). Recent studies indicate that anxiety and learned fear may differ in a number of attributes that include behavioral responses, anatomical substrates, and transmitter/signaling mechanisms.
The consequences of PAM haploinsufficiency may differentially affect these pathways/systems, resulting in the observed dissociated fear phenotype. Indeed there are neuropeptide receptor knockout models with dissociated fear phenotypes (Zeng et al., 2007; Shumyatsky et al., 2002). Thus, the PAM+/- fear phenotype may signify distinct neuropeptidergic systems in innate and learned fear.
pathways.

**Fear learning/memory and LTP.** The disruption of synaptic plasticity observed at the thalamic inputs to the amygdala strongly correlates with the fear learning and memory phenotype of PAM\(^{+/−}\) mice, and it may serve as the neurophysiological basis for these behavioral impairments. Deficiencies at both short- (1 hr) and long (24 hr) time-points in both cued and contextual testing demonstrate impaired formation and/or consolidation of associative emotional memory. It is clear, however, that PAM\(^{+/−}\) mice are capable of learning; deficiencies in fear-potentiated startle were observed at the low and not the high shock intensity. This result is quite striking since both genotypes display similar levels of behavioral reactivities and sensitivities to a wide range of foot-shock intensities that included those used in conditionning. One explanation is that enhanced GABAergic inhibition in the PAM\(^{+/−}\) amygdala sets a higher threshold for plasticity induction during training. This notion is supported by evidence for enhanced GABAergic activity in the PAM\(^{+/−}\) amygdala and the ability of complete GABAergic blockade to eliminate the LTP deficit in PAM\(^{+/−}\) mice. Future experiments will focus upon determining how specific enhancements in GABAergic inhibition may contribute to the fear learning and memory deficit of PAM\(^{+/−}\) mice.

**Anxiety-like behaviors and GABA.** PAM\(^{+/−}\) mice display anxiety-like behaviors in the zero maze and the alleviation of these responses with diazepam provides pharmacological validation for the behavior. The anxiety-like responses of the heterozygotes may be due to a reduction in bioactivity of one or several PAM-requiring neuropeptides that normally exert anxiolytic actions. Many neuropeptides modulate the excitatory-inhibitory balance by regulating pyramidal neuron and interneuron membrane activities (Fig. 2-18). The prominent enhancement in GABAergic activity in the PAM\(^{+/−}\)
amygdala signifies disruption of that balance. Augmented GABAergic tone may render the PAM$^{+/−}$ brain susceptible to inhibitory limitation during stressful behavioral tasks such as the elevated-zero maze (Stork et al., 2002; Martijena et al., 2002) by endogenous hormones/neuromodulators (e.g. catecholamines, glucocorticoids) (Tully et al., 2007; Duvarci and Pare, 2007; Marowsky et al., 2005). This is also consistent with hypersensitivity of PAM$^{+/−}$ mice to diazepam in the elevated-zero maze and pentylenetetrazol during seizure induction (Bousquet-Moore et al., 2010b). Alternatively, changes in the functional expression of GABA and/or benzodiazepine receptors were not assessed in these experiments and could contribute to the anxiety-like phenotype of PAM$^{+/−}$ mice. It should be emphasized that anxiety is a complex condition that is mediated by a variety of brain regions that at least include the frontal cortex, bed nucleus of stria terminalis, and the amygdala (Maren and Quirk, 2004; Pare et al., 2004; Kim and Jung, 2006; LeDoux, 2000; LeDoux, 2007; Davis et al., 2010). Therefore, we cannot draw any definitive conclusions regarding the physiological basis for the PAM$^{+/−}$ anxiety-like phenotype and sensitivity to diazepam from these studies alone.

**PAM$^{+/−}$ as a model for insufficient amidation**

Approximately half of all neuropeptides are amidated, and amidation can be rate-limiting under physiological conditions (Merkler, 1994) and in PAM haploinsufficiency (Bousquet-Moore et al., 2009). Insufficient amidation and the resulting reduction in bioactive anxiolytic peptides would be the simplest explanation for how PAM haploinsufficiency might affect behavior. The prototypical example is neuropeptide Y (NPY), one of the most abundant neuropeptides in the mammalian brain. NPY, which has a C-terminal tyrosine amide (Eipper et al., 1992), suppresses anxiety-like behavior and learned fear responses when injected directly into the basolateral amygdala (Gutman et al., 2008).
However, relative levels of amidated NPY are unchanged in the PAM+/− brain (Czyzyk et al., 2005a) and direct physiological effectors of NPY signaling (e.g. passive membrane properties of pyramidal neurons) (Sosulina et al., 2008) are unaltered in the PAM+/− amygdala (Fig. 2-18; right). Therefore, it is unlikely that changes in NPY signaling dominate the PAM+/− phenotype.

Different Gly-extended precursors have varied affinities for the PAM catalytic core. For example, thyrotropin releasing hormone (TRH) terminates with proline amide (Eipper et al., 1992), and its Gly-extended precursor (TRH-Gly) is a particularly poor substrate for amidation. Consistent with this, there is an abundance of TRH-Gly in the amygdala compared with high PAM expressing areas like the hypothalamus (PAM+/− mice are euthyroid) (Bousquet-Moore et al., 2009), and PAM heterozygosity further impairs amidation of TRH in the amygdala (E.A. Nillni, unpublished). Since TRH signaling in the amygdala is involved in mediating anxiety-like behavior (Thompson and Rosen, 2000; Yarbrough et al., 2007; Zeng et al., 2007), particularly in the elevated-plus maze (Gutierrez-Mariscal et al., 2008; Zeng et al., 2007), the mechanism how TRH may influence amygdala physiology is an important avenue for future study. For example, TRH enhances GABA_α receptor-mediated transmission in the basolateral complex (Gaier et al, unpublished) (Fig. 2-18), which may underlie its reported anxiolytic effects and relate to the genotypic differences we observed. However, TRH signaling has no effect on learned fear responses (Thompson and Rosen, 2000; Zeng et al., 2007), making it unlikely that deficient TRH signaling could solely account for the PAM+/− phenotype.

Non-enzymatic roles of PAM

Regulating the secretory pathway. PAM heterozygosity could have effects outside of a
reduction in amidating activity. Over-expression of PAM in a pituitary corticotropic cell line reorganizes the actin cytoskeleton, impairs regulated secretion of amidated and non-modified peptides and impairs normal secretory granule maturation and localization (Ciccotosto et al., 1999). These effects are mediated through the cytosolic domain of PAM (Alam et al., 2001). Therefore, it is plausible that limitations in PAM could have the opposite effect and enhance stimulus-evoked secretion. In this sense, increased anxiogenic peptide signaling would account for the PAM\textsuperscript{+/−} phenotypes. Potential candidates include cholecystokinin (CCK) (Chen et al., 2006; Fendt et al., 1995) and corticotropin releasing hormone/factor (CRH/CRF; synthesized in the central nucleus) (Bale and Vale, 2004), both of whose signaling increases GABAergic transmission in the amygdala (Chung and Moore, 2007; Chung and Moore, 2009; Nie et al., 2004). Regulation of GABAergic transmission is clearly a major modulatory target of amygdalar neuropeptides (Fig. 2-18; left). Our findings in the PAM\textsuperscript{+/−} amygdala suggest net enhancement of that signaling, which supports a non-amidation-dependent mechanism for PAM haploinsufficiency.

Gastrin-releasing peptide (GRP), another amidated peptide (Baldwin et al., 2007), is expressed and secreted by pyramidal neurons of the basolateral complex (Fig. 2-18). GRP signaling suppresses LTP by enhancing the excitability of and GABA release from local interneurons on which the GRP receptor is expressed (Shumyatsky et al., 2002). However, contradictory evidence employing GRP receptor antagonist infusion into the amygdala of rats (Mountney et al., 2008; Mountney et al., 2006; Roesler et al., 2004; Bedard et al., 2007) makes it difficult to predict the role of GRP signaling in modulating amygdala neurophysiology and amygdala-dependent behaviors. Thus, altered GRP signaling in the PAM\textsuperscript{+/−} brain may contribute to physiological and behavioral differences we observed.
**Copper metabolism.** It is clear that copper metabolism is intricately tied with PAM function and vice versa. Copper can regulate the cleavage and recycling of PAM (De et al., 2007) and could therefore have important effects on nuclear signaling. Some PAM\(^{+/−}\) behavioral phenotypes can be rescued with dietary copper supplementation of PAM\(^{+/−}\) mice and mimicked with mild copper depletion in wildtype mice (Bousquet-Moore et al., 2010b; Bousquet-Moore et al., 2009). Anxiety-like behavior falls into this category while seizure sensitivity did not. On the other hand, PAM heterozygosity could mediate its effects by altering copper signaling. Copper is secreted by neurons and can suppress NMDA receptor function through a nitric oxide-dependent mechanism (Schlief et al., 2006). Nitric oxide signaling is also required for auditory fear conditioning and LTP at thalamic afferent amygdalar synapses (Schafe et al., 2005; Ota et al., 2008). Therefore, copper signaling may play a role in limiting synaptic plasticity in the PAM\(^{+/−}\) amygdala.

PAM\(^{+/−}\) animals exhibit impairments involving several inter-dependent organ systems, and there is evidence for both amidation- and non-amidation-related mechanisms in explaining their behavioral and physiological deficits. These layers of complexity likely reflect the multi-faceted regulation of peptide signaling and the plethora of roles peptides play in physiology and pathophysiology. Clearly, peptidergic signaling relies on normal PAM function. Peptides are a promising avenue of novel therapeutics in psychiatric treatments of anxiety disorders (Mathew et al., 2008). Pursuing the study of how dietary, pharmacological and genetic influencers of PAM function can alter physiology and behavior brings us closer to identifying how peptidergic signaling pathways can serve as targets for clinical intervention.
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CHAPTER 3: In vivo and in vitro analysis of amygdalar function following loss of peptide amidating monooxygenase reveals essential role for copper
CONTRIBUTIONS

This work roughly encompasses experimental results from Aim 3 of my thesis proposal and prospectus and beyond. Behavioral analyses were conducted at Duke University by Dr. Ramona Rodriguiz in the laboratory of Dr. William Wetsel using wildtype and PAM heterozygous mice bred, maintained, genotyped and sorted by myself and Dr. Danielle Bousquet-Moore, a former graduate student in our lab. I received assistance from Drs. Srjdan Antic and Eric Levine who assisted me with technical issues in electrophysiology. Dr. Richard Mains assisted me with performing qPCR analyses and Dr. Elizabeth Eipper guided me through the PSD preparations. Dr. Mains and Eipper also provided feedback for this written chapter which will be submitted as a manuscript in the very near future to the Journal of Neuroscience.
Peptidylglycine α-amidating monooxygenase (PAM) is a secretory granule membrane protein whose luminal enzymatic domains catalyze the Cu-dependent amidation biosynthetic step common to many neuroactive peptides. Aside from its essential role as the sole mammalian amidating enzyme, PAM alters Cu homeostasis, modulates transcription and regulates secretory granule trafficking. The model of PAM haploinsufficiency employs mice heterozygous for the Pam gene (PAM^+/−); the mice show anxiety-like disorders and deficiencies in fear learning and memory that coincide with deficient long-term potentiation (LTP) at excitatory thalamic afferent synapses. To study the interaction between PAM and Cu in vivo, we supplemented the drinking water of wildtype and PAM^+/− mice. Cu supplementation rescued the cued learned-fear deficits of PAM^+/− mice, with little effect on the behaviors of wildtype mice. Dietary Cu supplementation also corrected the LTP deficit of PAM^+/− mice in vitro. Bath application of the extracellular specific Cu chelator bathocuproine disulfonate abolished LTP in wildtype and PAM^+/− amygdalae, demonstrating a vital role for Cu in amygdalar synaptic plasticity. Dietary Cu supplementation had no effect on brain Cu or PAM protein levels, therefore PAM^+/− behavioral deficiencies do not result from insufficient Cu levels and/or amidating activity. Localization of the major neuronal Cu transporter, ATP7A, was altered in the PAM^+/− brain. In addition, quantitative PCR revealed region-specific deficits in Atox-1 and ATP7A that may account for the physiological and behavioral defects associated with PAM heterozygosity. These data indicate that Cu is necessary for normal amygdalar synaptic function, suggesting the PAM^+/− behavioral and physiological phenotype stems from dysregulated Cu secretion.
INTRODUCTION

Peptidylglycine \( \alpha \)-amidating monooxygenase (PAM), a Cu-dependent monooxygenase, is necessary for the biosynthesis of many neuropeptides (Czyzyk et al., 2005; Eipper et al., 1992). Mice heterozygous for PAM (PAM\(^{+/-}\)) exhibit limited impairments including inability to maintain body temperature in the cold, increased susceptibility to pentylenetetrazol-induced seizures, and increased anxiety-like behavior (Gaier et al., 2010; Bousquet-Moore et al., 2009; Bousquet-Moore et al., 2010b). A subset of these deficits can be rescued with dietary Cu supplementation and mimicked by dietary Cu restriction of wildtype mice (Bousquet-Moore et al., 2010b; Bousquet-Moore et al., 2009). The ability of dietary Cu supplementation to rescue deficits associated with PAM heterozygosity is striking, since neuropeptide amidation is only modestly diminished in PAM\(^{+/-}\) mice (Bousquet-Moore et al., 2010b). In the present set of studies, we use the PAM\(^{+/-}\) amygdala to study the role Cu in synaptic plasticity and associated behaviors.

Copper is an essential trace element required by aerobic, multicellular organisms. Its redox potential and electron accepting capacity make Cu essential for several enzymes that consume molecular oxygen (Decker and Terwilliger, 2000; Crichton and Pierre, 2001). Only about a dozen cuproenzymes (requiring Cu for activity) are encoded in the mammalian genome (Linder and Hazegh-Azam, 1996; Crichton and Pierre, 2001), including cytochrome c oxidase, lysyl oxidase, dopamine \( \beta \)-monooxygenase and PAM. Delivery of Cu to these enzymes involves a larger number of proteins dedicated to transport, binding and chaperoning of Cu. Cu is known to have direct inhibitory effects on several synaptic receptors and ion channels including GABA\(_A\) receptors (Mathie et al., 2006). The function of copper bound to other proteins like Prion Protein (Vassallo and Herms, 2003; Zomosa-Signoret et al., 2008) and Amyloid Precursor Protein (Atwood...
et al., 2000; Lin et al., 2010) is not well understood.

The importance of Cu to normal nervous system function is demonstrated by the severe phenotype of Menkes disease, an X-linked genetic disorder of Cu transport. The Menkes protein, ATP7A, is a P-type ATPase responsible for Cu uptake from the gut and from blood into the brain (Lutsenko et al., 2007; Kaler, 2011). Some of the pathophysiological features of Menkes disease reflect deficits in cuproenzyme function. Notable neurological features include severe seizures, neurodegeneration and death, typically in early childhood (Kaler, 2011). However, some of these deficits could reflect deficient direct Cu signaling.

Lack of Ctr1, a plasma membrane Cu transporter, is incompatible with life (Kuo et al., 2001; Lee et al., 2001); mice lacking Ctr1 expression in the heart exhibit a profound increase in ATP7A expression in liver and intestine (Kim et al., 2010a). Cells exposed to serum from these mice express increased levels of ATP7A, revealing homeostatic controls responsive to tissue-specific deficits in Cu availability. Manipulation of Cu availability in vitro alters the proteolytic processing and endocytic trafficking of PAM (De et al., 2007). In vivo, manipulation of dietary Cu alters PAM secretion and genetic alteration of PAM affects Cu handling in multiple organs (Bousquet-Moore et al., 2010b). This bidirectional interaction suggests a non-enzymatic role for PAM in Cu homeostasis. The γ-secretase-mediated cleavage of PAM releases a soluble fragment, which accumulates in the nucleus and alters gene expression (Ciccotosto et al., 1999; Rajagopal et al., 2009). This may be part of a pathway by which PAM regulates Cu homeostasis.

Since the neural pathways involved in fear learning and memory are well understood (Ledoux et al., 1990; Ledoux, 2000; Maren and Quirk, 2004; Pare et al., 2004; Kim and
Jung, 2006), we first asked whether the amygdala-related physiological deficits observed in PAM\(^{+/-}\) mice could be rescued by dietary Cu supplementation. Since they were, we next asked whether similar effects could be detected in slice preparations. Finally, we probed the effects of Cu on normal amygdalar synaptic function, testing how dysfunction of normal Cu signaling could account for the deficits seen in PAM\(^{+/-}\) mice.
METHODS

Animals
Male and female mice were generated from PAM\textsuperscript{+/-} pairings in the University of Connecticut Health Center (UCHC) animal facility. Wildtype and PAM\textsuperscript{+/-} littermates (>20 generations bred into C57/BL6J background) were weaned between P19 and P21 and group-housed until experiments. Animals were maintained under a 12 h light/dark cycle (lights on at 0700) and were given free access to food and water. The behavioral experiments were conducted at Duke University between 1100-1400 h, where adult male and female wildtype and PAM\textsuperscript{+/-} mice (12-20 weeks of age) were tested. Since no sex differences were observed, data were pooled. Dietary Cu supplementation consisted of the addition of 300 ppm (1.2 mM) CuSO\textsubscript{4} to standard reverse-osmosis drinking water for 10-14 days prior to experiments. This was not a toxic dose of Cu as mice supplemented with dietary Cu appeared and behaved normally; no mice treated with dietary Cu supplementation died prematurely. All experiments were conducted with approved protocols from the UCHC and Duke University Institutional Animal Care and Use Committees and in accordance with NIH guidelines for animal care.

Behavior

Fear conditioning. Details of the fear conditioning tests have been published (Taylor et al., 2008). Wildtype and PAM\textsuperscript{+/-} mice were tested in either context or cued fear conditioning 24 h after training. All mice were conditioned with a single 30 s 72 dB tone (CS) and 2 s 0.4 mA scrambled foot-shock (US) on day 1. Context testing consisted of returning the mouse to the same chamber in which the CS-US pairing had been presented. For cued tests the mice were placed into a novel chamber with a different level of illumination, floor, walls, shape and dimensions than the conditioning chamber.
All tests were video-taped and the behaviors were later scored using the Noldus Observer by a trained observer who was blind to the genotype and dietary condition of the mice. Freezing was defined as the lack of all non-respiratory movement by the animal for >1 s (Anagnostaras et al., 2000; Porton et al., 2009).

_Fear-potentiated startle_. This test has been described in detail (Taylor et al., 2008). Testing was conducted in a MedAssociates apparatus (St. Albans, VT) over 5 days. On day 1, baseline startle responses were assessed over 18 trials with 40 ms bursts of white noise at 100, 105 and 110 dB presented in pseudorandom order with an inter-trial interval of 30–90 s. On day 2, one-half of the startle stimuli were administered immediately following a 30 s 12 kHz 70 dB pure tone (CS); the other half were presented without the CS. Twenty-four h later mice were conditioned with 10 CS-US (0.25 s 0.4 mA scrambled shock) pairings. Forty-eight h later, mice were tested for potentiation of their startle responses by the CS using the same procedure described for day 2. Potentiation to the CS was defined as the percent increase in the startle response for the CS+startle-stimulus trials relative to the baseline startle responses on day 1.

**Electrophysiology**

_Slice preparation_. Male wildtype and PAM<sup>+/−</sup> littermates 4–7 weeks of age were decapitated and their brains quickly removed into ice-cold artificial cerebrospinal fluid (aCSF) aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, pH 7.3, 310 mOsm/kg, containing (in mM): 125 NaCl, 26 NaHCO<sub>3</sub>, 10 glucose, 2.3 KCl, 2 CaCl<sub>2</sub>, 2 MgSO<sub>4</sub>, 1.26 KH<sub>2</sub>PO<sub>4</sub> (Zhou et al., 2008a). Coronal slices, 300 µm thick, were incubated at room temperature for at least 1 h before recordings.

_Whole-cell recordings_. Slices were transferred to a recording chamber heated to 32°C and perfused with aerated aCSF. Recording pipettes had 3-5 MΩ tips. The internal
pipette solution contained (in mM) 135 K-gluconate, 10 HEPES, 10 P-creatine, 3
Na<sub>2</sub>ATP, 2 MgCl<sub>2</sub>, 0.3 Na<sub>2</sub>GTP, pH 7.3, 285 mOsm/kg (Zhou et al., 2008a). Neurons
were patched under visual guidance using infrared differential interference contrast
optics. Data were collected using a Multiclamp 700B amplifier (Molecular Devices,
Sunnyvale, CA) and analyzed using pClamp 9.2 software (Molecular Devices). A series
of negative and positive current steps were applied immediately upon achieving whole-
cell configuration (25 x 50 pA steps, –300 to +900 pA; 500 ms steps) and the firing
pattern observed was used to verify pyramidal cell type. Series resistance was
continuously monitored throughout each experiment using either a 5 mV voltage or 25
pA current step. Neurons were eliminated from analysis if series resistance changed by
more than 20%. Bath-application of CuSO<sub>4</sub> did not result in a consistent change in series
resistance.

_Evoked synaptic transmission._ Synaptic activity was evoked using 200 µs current pulses
elicited at 0.1 Hz with a Master 8 and an ISO-Flex stimulator (A.M.P.I., Jerusalem,
Israel) through bipolar tungsten electrodes (World Precision Instruments, Sarasota, FL).
Electrode placement in the internal capsule preferentially stimulated thalamic afferent
axons (Weisskopf and Ledoux, 1999). For Cu wash-in experiments, neurons were
clamped at V<sub>Holding</sub> = -35 mV to simultaneously monitor excitatory and feed-forward
inhibitory transmission. A 5 mV, 25 ms hyperpolarizing step was applied 800 ms after
each synaptic response to monitor passive membrane properties. The perfusate was
switched during continuous recording to aCSF containing 10 µM CuSO<sub>4</sub>.

_Synaptic plasticity._ In whole-cell configuration, experiments were conducted in current
clamp mode in the presence of 100 µM picrotoxin (PTX) (Tully et al., 2007; Weisskopf et
al., 1999). The holding current was adjusted to maintain V<sub>m</sub> = -70 mV throughout the
course of experiments. The 10-90% rise slope of Excitatory Post-Synaptic Potentials (EPSPs) was used as the measure of synaptic efficacy. Stimulation strength was adjusted to produce a 3-6 mV EPSP. Paired pulses were applied at a 50 ms interval, and the ratio of the second EPSP 10-90% rise slope to that of the first was interpreted as an inverse indicator of release probability. After establishment of a steady baseline, Long-Term Potentiation (LTP) was induced using an action potential pairing induction protocol. This induction paradigm was originally characterized as being L-type voltage-gated calcium channel-dependent and NMDA receptor-independent (Bauer et al., 2002). Fifteen paired trains were applied at 0.1 Hz; each train consisted of 10 pulses at 30 Hz paired with 2.5 ms current pulses of ≥ +1 nA to elicit an action potential at a 5 ms delay to the onset of each synaptic event. If 1 nA was not sufficient, then the pulse amplitude was increased by +50 pA until action potentials were observed throughout the train. LTP was measured as the normalized fractional difference between the 5 min baseline and 30-40 min post-induction; neurons were pooled by genotype. Only one neuron was used per slice.

**Pharmacology.** PTX (100 µM; Sigma-Aldrich) was used to block fast GABA_A receptor-mediated transmission. CGP35348 (1 µM; Tocris Biosciences, Ellisville, MO) was used to block slow GABA_B receptor-mediated transmission. The open voltage-gate Na channel blocker QX-314 (3 mM; Sigma-Aldrich) was added to the internal pipette solution when indicated to block action potential activity selectively in the patched neuron.

**Immunocytochemistry**

Immunohistochemical staining of tissue sections from perfusion-fixed mice was described previously (Ma et al., 2001;Ma et al., 2002;Ma et al., 2008a). Briefly, male
wildtype and PAM/+ littermates were perfused transcardially with 4% formaldehyde/0.1 M sodium phosphate buffer (pH 7.4) under deep ketamine/xylazine anesthesia. After fixation, brains were post-fixed in 4% paraformaldehyde for 3 h. Coronal sections were cut (15 µm) through the amygdala using a cryostat and immunostained simultaneously with rabbit antiserum JH629 to the Exon A (exon 16) region of PAM1 (Maltese and Eipper, 1992) and mouse monoclonal IgG to ATP7A (Neuromab, University of California Davis, CA). Alexa-488 donkey anti-rabbit (H+L) antibody (Invitrogen, Molecular Probes, Eugene, OR) and Cy3-conjugated AffiniPure F(ab’)2-fragment donkey anti-mouse (H+L) antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) were then applied. To-Pro3 (Invitrogen-Molecular Probes, Eugene, OR) was added separately as described (Ma et al., 2001). Images are single focus planes captured using an automated Zeiss LSM 510 Meta confocal microscope at the Center for Cell Analysis and Modeling, UCHC. Low power images employed a 20x 0.75 NA air objective, 92 µm pinhole; high power images employed a 63x 1.4 NA oil DIC objective, 92 µm pinhole.

**PSD fractionation and Western blotting**

Subcellular fractionation was used to prepare samples enriched in endoplasmic reticulum/Golgi, cytosol, synaptosomal membranes, synaptic vesicles, and synaptosomal cytosol (Ma et al., 2008a; Huttner et al., 1983). Purified PSDs were prepared using a modification of published procedures (Carlin et al., 1980; Ma et al., 2008a). Samples removed from the interface of the 1.0/1.2 m sucrose layers of an equilibrium gradient were diluted, pelleted and then solubilized by incubation for 30 min at 4°C with 0.5% Triton X-100 (TX-100), 10 mm HEPES, pH 7.4. PSDs were pelleted and TX-100-soluble fraction was saved for analysis. Protein concentrations were determined using bicinchoninic acid (Pierce) with bovine serum albumin as the standard. Polyclonal rabbit antiserum to ATP7A (CT-77) was described previously (Niciu et al.,
Commercial mouse monoclonal antibodies to NR2B (clone N59/20; NeuroMab), GM130 (BD Biosciences, Franklin Lakes, NJ) and βIII-tubulin (Covance, Princeton, NJ) were used.

**Quantitative PCR**

Bilateral 2 mm diameter punches from individual mice containing the amygdalae and hippocampi were isolated from 1 mm coronal sections and immediately transferred from isotonic phosphate buffered solution, pH 7.4, into 500 µl TRIzol. RNA and cDNA were prepared essentially as per the manufacturer's instructions, with minor modifications (Mains et al., 2011). All primers were chosen to have identical melting temperatures and to produce products of 120 ± 5 nt, as listed in Table 3-1. The maximal rate of amplification was 1.9 ± 0.15. All data were calculated with respect to GAPDH and groups of 5-6 mice were averaged.

<table>
<thead>
<tr>
<th>Transcript Alias</th>
<th>Transcript Name</th>
<th>Primer Sequence</th>
<th>T&lt;sub&gt;Melting&lt;/sub&gt; (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td></td>
<td>TTGTCAGCAATGCATCCTGCACC</td>
<td>61</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td>CTGAGTGGGCACTGTGGGATGCC</td>
<td>61</td>
</tr>
<tr>
<td>mPAM</td>
<td></td>
<td>TGCTGCTGCTGCTGGGCTGCT</td>
<td>62</td>
</tr>
<tr>
<td>mPAM</td>
<td></td>
<td>CAAGGCATTCAATTGGGAAATGACATGTGGTATTTCTTT</td>
<td>61</td>
</tr>
<tr>
<td>Atox-1</td>
<td></td>
<td>CACGAGTTCCTCCGTGGACATGACC</td>
<td>61</td>
</tr>
<tr>
<td>Atox-1</td>
<td></td>
<td>GTCTAGGGCAGACTCTTTTGTGGG</td>
<td>61</td>
</tr>
<tr>
<td>ATP7A</td>
<td></td>
<td>GGATGCAATTCAATTACTATCATGCTGAGGG</td>
<td>61</td>
</tr>
<tr>
<td>ATP7A</td>
<td></td>
<td>CTCTAGGAAACTTTAATGTGGATGGACACC</td>
<td>61</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt;</td>
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<td>CCAGTTTCGGACGACTCTTTTACACC</td>
<td>61</td>
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<tr>
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<td>β2</td>
<td>TCTCGGGAATCCCTCCAAGGC</td>
<td>61</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;B&lt;/sub&gt;</td>
<td>β2</td>
<td>CGTTGTCTGAGGGACACTGCC</td>
<td>60</td>
</tr>
<tr>
<td>GAD65</td>
<td></td>
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<tr>
<td>GAD67</td>
<td></td>
<td>GAGCTCTGGGACATGGGCTGGAGG</td>
<td>60</td>
</tr>
</tbody>
</table>
**Statistics**

For behavioral data, freezing responses were analyzed by 2-way ANOVA where genotype and dietary condition were examined. Repeated measures ANOVA (RMANOVA) was used to examine fear-potentiated startle responses over the three startle stimulus intensities between genotype and dietary condition. The interactions were decomposed by Bonferroni corrected pair-wise comparisons. For synaptic plasticity experiments, LTP was calculated using averaged values of EPSP rise slope -5 to 0 min before and 30 to 40 min after AP pairing. Successful LTP was defined within a group by Wilcoxon Signed Rank Test comparisons to baseline. Comparisons between mean LTP values between groups employed unpaired $t$-tests. ANOVA (one- and two-way) were used as indicated for analyses involving genotype and/or treatment. Unpaired $t$-tests were used to detect differences for individual treatments. In all cases, a $p \leq 0.05$ was considered significant.
RESULTS

Cu rescues fear learning and memory deficits in PAM$^{+/c}$ mice

We have previously shown that PAM$^{+/c}$ mice exhibit both short and long-term deficits in fear learning and memory assessed by classical contextual and cued fear conditioning as well as fear potentiated-startle (Gaier et al., 2010). Since other physiological and behavioral deficits of PAM$^{+/c}$ mice could be ameliorated by dietary Cu supplementation (Bousquet-Moore et al., 2009; Bousquet-Moore et al., 2010b), we asked whether the fear learning and memory deficit were sensitive to Cu. Wildtype and PAM$^{+/c}$ mice were placed on diets including either normal reverse-osmosis drinking water (control) or drinking water supplemented with CuSO$_4$ for 10-14 days (Bousquet-Moore et al., 2010b). Mice were trained with a single fear conditioning trial (CS-US pairing) and tested in either contextual or cued fear conditioning 24 h later. Nearly identical shock sensitivities between genotypes were demonstrated previously (Gaier et al., 2010). Dietary Cu supplementation had no effect on wildtype baseline freezing (Fig. 3-1A). The only significant difference in baseline freezing between groups was a reduction displayed by PAM$^{+/c}$ mice on the Cu supplemented diet compared to the control diet, consistent with an anxiolytic effect of dietary Cu supplementation in PAM$^{+/c}$ mice (Bousquet-Moore et al., 2010b). Mice of both genotypes in both dietary conditions displayed increased freezing on the test day compared to their respective training trials, indicating successful learning and memory (Fig. 3-1).
Figure 3-1. Dietary Cu supplementation impairs wildtype contextual and partially rescues PAM^{+/-} cued fear conditioning. Wt and PAM^{+/-} littermates were placed on a diet consisting of either control water (left) or Cu supplemented (right) water prior to conditioning. (A) Mean percent time freezing during 2 min context exposure and subsequent 3 min cue exposure prior to shock on the conditioning day. (B) Mean percent time freezing during 5 min context exposure testing 24 h after conditioning. Animals were tested in the same chamber in which they had been conditioned but in the absence of the CS and UCS. (C) Mean percent time freezing during cue exposure testing 24 h after conditioning. In cued testing, animals were tested in a novel chamber and after 2 min the CS was presented in the absence of the UCS for 3 min; the pre-CS results over the first 2 min are not shown. *p<0.05, compared to Wt mice within dietary condition; #p<0.05, compared to control water within genotype. (ANOVA, Bonferroni corrected pair-wise comparisons). n = 10 mice/genotype/dietary condition.
In agreement with our previous observations, PAM^{+/-} mice drinking control water spent significantly less time freezing in response to the context than wildtype mice receiving control water (Fig. 3-1B). Dietary Cu treatment reduced contextual freezing in wildtype mice but had no effect on freezing in PAM^{+/-} mice. The context-dependent deficit exhibited by PAM^{+/-} mice remained after dietary Cu supplementation. As reported previously, in response to cue exposure, PAM^{+/-} mice on the control diet spent significantly less time freezing than wildtype mice on the control diet (Fig. 3-1C). Dietary Cu supplementation had no significant effect on cue-dependent freezing responses in wildtype mice. However, Cu supplementation significantly increased cued freezing responses in PAM^{+/-} mice, abolishing the genotypic difference in cued fear conditioning. These data indicate that contextual and cued fear conditioning are differentially influenced by dietary Cu supplementation.

Since we previously demonstrated a deficit in PAM^{+/-} fear-potentiated startle (Gaier et al., 2010), we next evaluated the effects of dietary Cu on this response. Wildtype and PAM^{+/-} mice on the control and Cu supplemented diet were tested. On testing day, PAM^{+/-} animals on the control diet displayed impairments in startle response potentiation compared to their wildtype counterparts at each of the 3 startle amplitudes tested (Fig. 3-2A), consistent with previous results (Gaier et al., 2010). Dietary Cu treatment had no effect on wildtype startle response potentiation at any of the 3 startle amplitudes. By contrast, dietary Cu supplementation increased PAM^{+/-} potentiation to levels comparable to wildtype and abolished the genotypic difference at all startle amplitudes (Fig. 3-2A). The only difference in baseline startle observed between genotypes on the control diet was a reduction in response amplitude to the lowest stimulus intensity (Fig. 3-2B); this difference was not observed at the other stimulus intensities or in a previous study (Gaier et al., 2010). Dietary Cu supplementation reduced the baseline startle response
to all three stimulus intensities in wildtype mice, and reduced startle responses to two of three stimulus intensities of PAM\textsuperscript{+/-} mice. This effect may be partially attributable to the anxiolytic effects of dietary Cu supplementation (Bousquet-Moore et al., 2010b). No significant differences were observed in potentiation of any startle responses by the 12 kHz 70 dB tone between genotype or dietary condition (not shown), indicating that the tone does not contribute to startle response potentiation in mice of either genotype.

These data are consistent with results for cued fear conditioning, and together they prompted us to explore the neurophysiological effects of dietary Cu supplementation
using slice preparations.

**Cu rescues synaptic plasticity in PAM\(^{+/\text{-}}\) mouse amygdala**

Based on previous study, the fear learning and memory deficit in PAM\(^{+/\text{-}}\) mice is thought to be caused by an impairment in synaptic plasticity at thalamic afferent synapses in the amygdala (Gaier et al., 2010). Since some aspects of the fear learning and memory deficit were ameliorated with dietary Cu supplementation (Figs. 1,2), we tested whether the LTP deficit at thalamic afferent synapses of PAM\(^{+/\text{-}}\) mice responded to dietary Cu supplementation.

Thalamic afferent fiber bundles were stimulated and excitatory post-synaptic potentials (EPSPs) were recorded post-synaptically from lateral nucleus pyramidal neurons using whole-cell patch clamp methods. Neurons recorded from wildtype mice fed control water achieved statistically significant LTP (Fig. 3-3A). Confirming previous results, neurons from PAM\(^{+/\text{-}}\) mice on the control diet failed to achieve significant LTP (Gaier et al., 2010). Normalized EPSP rise slope was significantly reduced in PAM\(^{+/\text{-}}\) neurons compared to wildtype 30-40 min after LTP induction \((p<0.05)\). Wildtype synaptic potentiation coincided with a reduction in the paired pulse ratio \((p<0.05)\), indicating an enhancement in pre-synaptic release probability (not shown). PAM\(^{+/\text{-}}\) paired pulse ratios remained unchanged following induction, corroborating the lack of change in PAM\(^{+/\text{-}}\) release probability and synaptic efficacy (not shown). There was no difference in paired pulse ratio between genotypes at baseline. These data confirm the previously reported deficit in synaptic plasticity in the PAM\(^{+/\text{-}}\) amygdala.

Neurons from wildtype mice fed Cu supplemented water exhibited LTP that was indistinguishable from LTP in wildtype mice receiving control water (Fig. 3-3B versus Fig.3A). Importantly, dietary Cu supplementation abolished the genotypic difference in
Figure 3-3. Dietary Cu supplementation rescues the PAM<sup>+/−</sup> amygdalar synaptic plasticity deficit. Wt and PAM<sup>+/−</sup> littermates were placed on the + CuSO<sub>4</sub> dietary regimen prior to amygdalar slice preparation. Thalamic afferents were stimulated and pyramidal neuron membrane responses were recorded in whole-cell mode in the lateral nuclei in the presence of 100 µM picrotoxin. (A,B) Time-course (1 min bins) of averaged LTP experiments using an action potential pairing induction paradigm at minute 0 (arrow) of Wt and PAM<sup>+/−</sup> neurons from mice on the control (A; n = 7 Wt; 7 PAM<sup>+/−</sup>) and Cu supplemented (B; n = 7 Wt; 8 PAM<sup>+/−</sup>) diets, normalized rise slopes of the EPSPs (NL EPSP slope). Insets above depict representative averaged traces for each genotype at baseline (solid) and post-induction (dashed). *p < 0.05, compared to Wt mice within dietary condition; †p < 0.05, compared to baseline within genotype and dietary condition; ‡p < 0.05, compared to control diet within genotype; NS Not significant compared to baseline (unpaired t-test; Wilcoxon Signed Rank Test). (C) A series of hyperpolarizing and depolarizing 500 ms current steps were applied to Wt pyramidal neurons acutely isolated from control (left) or Cu supplemented (right) mice. Representative membrane responses demonstrating similar action potential activity are depicted.

LTP observed in wildtype and PAM<sup>+/−</sup> mice receiving control water. Moreover, Cu supplemented PAM<sup>+/−</sup> LTP was significantly higher compared to baseline and PAM<sup>+/−</sup> LTP.
in the control water condition. Dietary Cu had no gross effects on neuronal membrane excitability as assessed by somatic positive current injection (Fig. 3-3C), demonstrating that the effects of dietary Cu on amygdalar synaptic plasticity do not result from changes in intrinsic neuronal membrane properties. Thus 10 to 14 days of dietary Cu supplementation successfully rescued the synaptic plasticity deficit observed in the PAM\(^{+/-}\) amygdala.

The fact that providing PAM\(^{+/-}\) mice with supplementary Cu for 10-14 days can eliminate both their behavioral deficit and lack of LTP diminishes concerns that these differences arise from altered development. Being deficient in PAM from birth, PAM\(^{+/-}\) mice are susceptible to potential impairments resulting from PAM deficiency, but the ability of dietary to reverse these deficits strongly argues against a developmental cause. Alteration in the biosynthesis of amidated neuropeptides is also not likely to be a contributing factor because amidating activity is only mildly affected by PAM heterozygosity, and neuropeptide amidation is unaffected by dietary Cu supplementation (Bousquet-Moore et al., 2010b). PAM\(^{+/-}\) impairments in fear behavior and amygdalar synaptic plasticity are large and striking, so minute changes in neuropeptides in these models are not likely to contribute to these deficits. Therefore, our data support the hypothesis that dietary Cu supplementation ameliorates behavioral and physiological deficits through remediation of impaired Cu homeostasis in PAM\(^{+/-}\) mice. Further analysis of the interactions of PAM and Cu in the amygdala should provide insight into the mechanisms through which they interact.

**Secreted Cu is essential for LTP**

We next wanted to ask whether amygdalar slices from wildtype and PAM\(^{+/-}\) mice differed in their dependence on Cu by removing or adding Cu in vitro. We first tested the role of
endogenous Cu on thalamic afferent LTP in the amygdala. To do this, we induced LTP in wildtype and PAM$^{+/−}$ neurons in the presence of picrotoxin and the cell-impermeable specific Cu chelator bathocuproine disulfonate (BCS). To target secreted, endogenous

![Graph A](image1.png)

![Graph B](image2.png)

**Figure 3-4. Extracellular Cu is essential for LTP at thalamic afferent amygdalar synapses.** Wt and PAM$^{+/−}$ littermates kept on the control diet were used for slice preparation. Thalamic afferents were stimulated and pyramidal neuron membrane responses were recorded in whole-cell mode in the lateral nuclei in the presence of 50 µM bathocuproine disulfonate (BCS). (A,B) Time-course (1 min bins) of averaged LTP experiments using an action potential pairing induction paradigm at minute 0 (arrow) of Wt and PAM$^{+/−}$ neurons from mice in the presence of BCS with picrotoxin (100 µM; PTX) (A; n = 12 Wt; 8 PAM$^{+/−}$) or BCS with PTX and the GABA$_B$ receptor antagonist CGP34358 (1 µM; CGP) (B; n = 11 Wt; 7 PAM$^{+/−}$). Synaptic efficacy was assessed using the normalized rise slopes of the EPSPs (NL EPSP slope) that were normalized for genotypic comparisons. Insets depict representative averaged traces for each genotype at baseline (solid) and post-induction (dashed). **NS** Not significant compared to baseline (Wilcoxon Signed Rank Test).
Cu, perfusion of BCS began no more than 20 min prior to LTP induction. In the context of GABA<sub>A</sub> receptor blockade with picrotoxin, wildtype amygdala neurons potentiate, but PAM<sup>+/−</sup> neurons do not (Fig. 3-3A) (Gaier et al., 2010). Addition of BCS abolished LTP in wildtype neurons and had no effect on PAM<sup>+/−</sup> neurons (Fig. 3-4A). We had shown previously that addition of the GABA<sub>B</sub> antagonist CGP rescues PAM<sup>+/−</sup> LTP (Gaier et al., 2010). Therefore, we tested the effects of BCS on LTP in the context of both GABA<sub>A</sub> and GABA<sub>B</sub> receptor blockade. Again, BCS completely eliminated LTP in both wildtype and PAM<sup>+/−</sup> neurons (Fig. 3-4B). These data suggest an essential role for endogenous, secreted Cu in amygdalar excitatory synaptic plasticity independent of GABAergic inhibition. Additionally, removal of extracellular Cu eliminated the genotypic difference in LTP in both pharmacological conditions, supporting the hypothesis that altered Cu secretion underlies that PAM<sup>+/−</sup> LTP deficit.

Copper modulation of synaptic transmission

The data presented thus far strongly support a disruption in synaptic Cu release. If the alterations in PAM<sup>+/−</sup> synaptic plasticity result from disruption in amygdalar synaptic Cu homeostasis, then Cu could have direct effects on synaptic transmission in this brain region. To elucidate the effects of Cu on synaptic transmission, we patch clamped wildtype lateral nucleus pyramidal neurons and bath-applied 10 µM CuSO<sub>4</sub> (Doreulee et al., 1997; Leiva et al., 2003). We held neurons at V<sub>holding</sub> = -35 mV to simultaneously monitor excitatory and inhibitory transmission during Cu application. Voltage-gated Na channels were blocked exclusively in the clamped cell by including lidocaine (QX-314; 3 mM) in the intracellular pipette solution (Fig. 3-5A). The fact that lidocaine also suppressed slow feed-forward inhibition, which is mediated by GABA<sub>B</sub> metabotropic receptors (Duvarci and Pare, 2007), is an important caveat (Fig. 3-5B). Stimulation of thalamic glutamatergic afferents produced a mono-synaptic excitatory inward current
which was closely followed by a di-synaptic inhibitory outward current (Fig. 3-5C). The di-synaptic outward current represents feed-forward inhibition of thalamic afferent
synapses (Humeau et al., 2005; Gaier et al., 2010). Both currents were sensitive to AMPA receptor antagonism with CNQX (10 μM), whereas only outward currents were sensitive to GABA\textsubscript{A} receptor blockade with 100 μM picrotoxin (not shown). Since the excitatory and inhibitory events partially overlapped in time, we assessed the efficacy of excitatory transmission using the 0-70% rise slope of the inward current and the efficacy of feed-forward inhibition using the peak-to-peak slope.

We recorded these bi-phasic current responses to episodic synaptic stimulations of thalamic afferents while bath applying exogenous CuSO\textsubscript{4} (10 μM) for 10 min followed by a 30 min washout period. Cu application had no effect on excitation but had a profound potentiating effect on inhibition ($p<0.05$) (Fig. 3-5D). This increase in inhibition appeared to be reversible, as outward currents returned to baseline with Cu washout. Cu application also briefly increased the holding current ($p<0.05$) (Fig. 3-5E), representing a hyperpolarizing effect of Cu on the post-synaptic neuron. This effect on the membrane did not correspond to any significant changes in membrane resistance.

Thus far, the data presented indicate that dietary Cu rescues the behavioral synaptic plasticity deficits of PAM\textsuperscript{+/−} mice, and that Cu applied through the perfusate transiently enhances GABAergic inhibition of lateral amygdala pyramidal neurons. Together, these data support a net inhibitory role for Cu in the amygdala.
PAM colocalizes with ATP7A in neurons

ATP7A, also known as the Menkes disease protein, is the major P-type Cu ATPase expressed in brain and delivers Cu to PAM in the secretory pathway (El Meskini et al., 2003; Steveson et al., 2003). To search for a relationship between PAM expression and Cu homeostasis, we visualized PAM and ATP7A in wildtype and PAM+/- brains. We observed co-expression of PAM and ATP7A in neurons throughout the forebrain, including the amygdala (Fig. 3-6) and hippocampus (not shown). Both proteins overlap with the Golgi marker GM130 in these regions (not shown), as reported previously (Niciu et al., 2006). Levels of ATP7A are generally higher in the basolateral nucleus of the amygdala than in the lateral nucleus (Fig. 3-6A,B). Also, PAM is enriched in the central nucleus of the amygdala whereas ATP7A is not. Within the lateral nucleus, PAM and ATP7A are expressed at higher levels in a subset of neurons (Fig. 3-6C). We have shown previously that PAM is expressed at higher levels in amygdalar GABAergic interneurons (Gaier et al., 2010); therefore ATP7A may also be expressed at higher levels in interneurons of the basolateral amygdaloid complex. Both PAM and ATP7A immunoreactivities were also observed in the neuropil of the lateral nucleus, likely representing vesiculuar ATP7A and PAM in the dendrites of neurons. ATP7A is present in the amygdala of PAM+/- mice, and its expression pattern was not altered by PAM heterozygosity (Fig. 3-6B,D). This result provides evidence for co-localization of PAM with the major secretory pathway Cu transporter in the amygdala and demonstrates the potential for a functional interaction between these two molecules.
With PAM and ATP7A in somatic and dendritic compartments of amygdalar neurons, PAM could easily influence Cu homeostasis in this brain region. The heterogeneous expression of these two proteins suggests they are regulated with cell-type specificity. Thus, our data support the hypothesis that PAM heterozygosity mediates its behavioral

Figure 3-6. PAM co-localizes with ATP7A in the amygdala of wildtype and PAM\(^{+/-}\) mice. Single focus plane images were taken from 15 µm slices co-immunostained for ATP7A (red), PAM (green) and nuclei (To-Pro3; blue). Merge is yellow. (A,B) Montage of low power images depicting the amygdaloid complex from Wt (A) and PAM\(^{+/-}\) (B) mice. High power images from the lateral nucleus of Wt (C) and PAM\(^{+/-}\) (D) mice. One high PAM/ATP7A-expressing neuron (putative interneuron) is indicated in each case (arrow). Putative pyramidal neurons were more numerous and examples in each case are indicated (arrow heads). Scale bars: low power, 100 µm; high power 20 µm.

With PAM and ATP7A in somatic and dendritic compartments of amygdalar neurons, PAM could easily influence Cu homeostasis in this brain region. The heterogeneous expression of these two proteins suggests they are regulated with cell-type specificity. Thus, our data support the hypothesis that PAM heterozygosity mediates its behavioral
and physiological effects through alterations in Cu homeostasis (Bousquet-Moore et al., 2010b).

**ATP7A localization is altered in PAM<sup>+/−</sup> mouse amygdala**

ATP7A expression increases and redistributes along with Cu into dendrites in response to neuronal activation (Schlief et al., 2005; Dodani et al., 2011). In addition, Cu is secreted from neurons in an ATP7A-dependent manner (Schlief et al., 2005). Therefore, alterations in ATP7A expression and localization in PAM<sup>+/−</sup> amygdalar neurons could affect Cu secretion. To investigate potential alterations in ATP7A localization, we employed a subcellular fractionation method aimed at isolating synaptosomes and the complex of proteins at post-synaptic densities (PSDs). Bilateral neocortices were acutely isolated from wildtype and PAM<sup>+/−</sup> mice, tissue samples were fractionated in parallel and ATP7A levels in different subcellular fractions were compared between genotypes (**Fig. 3-7A**). Detection of NR2B in PSDs and GM130 in the ER/Golgi fractions validated our fractionation methods in both genotypes. ATP7A was enriched in the ER/Golgi fraction (**Fig. 3-7A**), consistent with previous reports of ATP7A localization using immunohistochemistry (**Fig. 3-6**) (Niciu et al., 2006). The majority of ATP7A present in synaptosomal membranes recovered from the sucrose gradient was Triton-soluble, but a fraction was Triton-insoluble, indicating that it was part of the PSD.

To compare the localization of ATP7A in wildtype and PAM<sup>+/−</sup> mice, levels were normalized to protein for each fraction in each animal (**Fig.3-7B**). The amounts of ATP7A in the input and ER/Golgi fractions appeared greater in PAM<sup>+/−</sup> cortex compared to wildtype; however, these differences did not reach statistical significance. The ratio of ATP7A present in the PSD versus the Triton-soluble fractions was 2.5-fold higher in PAM<sup>+/−</sup> cortex compared to wildtype, demonstrating a shift in ATP7A distribution at
Gene expression is altered in PAM<sup>+/−</sup> mouse amygdala

Another mechanism through which PAM heterozygosity could affect amygdalar function is through alterations in gene expression (Francone et al., 2010). PAM over-expression up-regulates Atox-1 mRNA levels in a corticotropic cell line (Francone et al., 2010), and PAM heterozygosity reduces Atox-1 transcript levels in mouse pituitary (Bousquet-Moore et al., 2010b). To determine whether this effect extends to the amygdala, we used quantitative PCR to probe for differences in Atox-1 and ATP7A mRNAs, two Cu binding proteins required for Cu delivery to PAM in the secretory pathway (Prigge et al., 2000; De et al., 2007; Bousquet-Moore et al., 2010a). Atox-1 is a cytosolic chaperone that delivers Cu to ATP7A (Hamza et al., 2001). Tissue was isolated from bilateral amygdalae and dorsal hippocampus of individual wildtype and PAM<sup>+/−</sup> mice and results were compared...
between genotypes. As with all other tissues collected from PAM\textsuperscript{+/-} mice, amygdala and hippocampus of PAM\textsuperscript{+/-} mice contained reduced levels of PAM mRNA compared to wildtype samples (Fig. 3-8). There was significantly more PAM mRNA in the amygdala compared to the hippocampus of each genotype. As was the case for PAM, more Atox-1 mRNA was present in the amygdala compared to the hippocampus of wildtype but not PAM\textsuperscript{+/-} mice. PAM\textsuperscript{+/-} mice expressed significantly less Atox-1 and ATP7A in the amygdala than wildtype mice (Fig. 3-8A), but expression of these mRNAs in the hippocampus was not different (Fig. 3-8B). Comparable levels of ATP7A were found in the amygdala and hippocampus of both genotypes. These data indicate region-specific reductions in molecules required for Cu delivery to the secretory pathway and suggest potentially decreased delivery of Cu to the secretory pathway in PAM\textsuperscript{+/-} amygdala.

**Figure 3-8.** PAM\textsuperscript{+/-} mice display amygdala-specific deficits in Atox-1 and ATP7A mRNA expression. Wt and PAM\textsuperscript{+/-} bilateral amygdalae and dorsal hippocampi were dissected from individual animals (n = 6 Wt, 5 PAM\textsuperscript{+/-}). Levels of mRNA for each primer set were quantified with respect to (wrt) GAPDH and average values for each genotype are depicted for the amygdala (A) and hippocampus (B). p values are provided for significant comparisons (unpaired t-test).
neurons.

The LTP deficit of PAM−/− mice is GABA-dependent, and PAM+/− mice display enhanced GABAergic inhibition in the basolateral amygdaloid complex (Gaier et al., 2010). To elucidate a molecular basis for these changes, we probed for differences in mRNA encoding GABAergic inhibitory molecular machinery in PAM+/− mice. We compared mRNA expression of the predominant GABA_A receptor subunit (α1), the obligatory GABA_B receptor subunit (2), and both the vesicular and cytosolic forms of glutamic acid decarboxylase (GAD65 and GAD67, respectively). Levels of GABAergic inhibitory molecular machinery were higher in the amygdala compared to the hippocampus in both genotypes with the exception of the PAM+/− GABA_B receptor subunit. Thus PAM+/− mice exhibit a region-specific reduction in GABA_B receptor expression that may contribute to the alterations in GABAergic inhibition in this region. There were no differences between genotypes for the GABA_A receptor α1 subunit in either brain region. However, GABA_B receptor subunit mRNA was significantly and selectively reduced in the PAM+/− amygdala compared to wildtype (Fig. 3-8A); no genotypic difference was observed in the hippocampus (Fig. 3-8B). No differences were observed for either GAD65 or GAD67 between genotypes in either region.
DISCUSSION

In the present study, we demonstrated that dietary Cu supplementation rescues the fear learning and memory impairment and corresponding synaptic plasticity deficit associated with PAM haploinsufficiency. We characterized the inhibitory effect of physiological Cu levels on neuronal and synaptic excitability and demonstrated an essential role for secreted Cu in amygdalar synaptic plasticity. We also provided novel insight into neuronal expression and localization of ATP7A, a key Cu export pump, in limbic structures associated with emotionality and memory formation. Taken together, our data identify a novel role for the interaction between Cu and PAM in amygdalar functions such as emotionality and fear learning and memory. However, the mechanism by which PAM heterozygosity alters neuronal Cu handling remains unclear.

PAM+/− mice have whole brain Cu levels comparable to wildtype mice, and dietary Cu supplementation does not change brain total Cu levels in either genotype (Bousquet-Moore et al., 2010b). However, dietary Cu supplementation has profound effects on the behavioral and physiological deficits of PAM+/− mice. Cu levels vary across brain regions (Rajan et al., 1976; Bakirdere et al., 2010), and we observed region-specific effects of PAM+/− heterozygosity on levels of transcripts encoding Atox1 and ATP7A (Fig. 3-8). The compartmentalization of Cu may differ in PAM+/− mice while total levels of brain Cu are unchanged (Fig. 3-9); dietary supplementation may normalize the subcellular localization of Cu. Regulated delivery of Cu to the secretory pathway is a major means of maintaining Cu homeostasis in high Cu environments (La and Mercer, 2007; Lutsenko et al., 2007). Our identification of ATP7A in purified PSDs suggests a role for this P-type ATPase in the release of Cu into the synapse. In PAM+/− mice, we observed an increase in the level of ATP7A at the PSD. Dietary Cu supplementation might alter ATP7A
localization or activity, restoring wildtype Cu secretion. Region- and cell-type specific expression of PAM and ATP7A is consist with region-specific responses to PAM heterozygosity and to dietary Cu supplementation.

**Cu rescue of PAM^{+/−} fear learning and memory**

Dietary Cu supplementation selectively rescued PAM^{+/−} cued fear conditioning, while wildtype contextual fear conditioning was impaired (Fig. 3-1). This striking discrepancy speaks to the differential role of Cu in these two neural processing pathways. Contextual
fear conditioning requires hippocampal as well as the amygdalar function, whereas cued fear conditioning does not require the hippocampus (Kim and Fanselow, 1992; Phillips and Ledoux, 1992). The hippocampus, a very epileptogenic brain region, has a high density of NMDA receptors (Rafiki et al., 1998; Meoni et al., 1998). Increased seizure sensitivity in PAM +/- mice was the only deficit that was not eliminated by dietary Cu (Bousquet-Moore et al., 2009; Bousquet-Moore et al., 2010b; Gaier et al., 2010). However, mild dietary Cu restriction increased seizure susceptibility and severity in wildtype mice, with no effect on PAM +/- mice (Bousquet-Moore et al., 2010b). 

Our findings suggest that the effects of Cu on hippocampal excitability and function are distinct from its effects on the amygdala.

Cu suppresses NMDA receptor function in hippocampal neurons through a nitrosylation-dependent mechanism (Schlief et al., 2006) and reduces AMPA receptor mediated signaling and LTP in hippocampal slices (Doreulee et al., 1997). An inhibitory effect of chronic Cu treatment on hippocampal LTP was demonstrated through diet and Cu
injections (Goldschmith et al., 2005; Leiva et al., 2009), although the extremely high brain Cu levels created raise worries of Cu toxicity; performance in the Morris water maze, a cognitive behavioral task testing working and long-term spatial (hippocampal-dependent) memory, was not affected (Leiva et al., 2009). Cu supplementation had no effect on shuttle-box avoidance learning of rats, a behavioral test similar to context-dependent fear conditioning (Fujiwara et al., 2006). By contrast, dietary Cu supplementation reversed the effects of dietary Zn supplementation on anxiety-like behavior and Morris water maze performance in rats; the effects of dietary Cu supplementation were only tested following Zn supplementation (Railey et al., 2010). Since Zn and Cu compete for uptake in the gut, Cu deficiency may contribute to effects attributed to Zn supplementation (Oestreicher and Cousins, 1985). Thus, our data are in agreement with previous results, supporting a differential role for Cu in hippocampal and amygdala function in rodents.

**Cu is essential for LTP**

We demonstrated that dietary Cu supplementation rescued the deficit in pre-synaptic thalamic afferent LTP observed in slices from PAM-/- mice (Fig. 3-3). Previous studies focusing on the effects of acute (bath-applied) (Doreulee et al., 1997; Leiva et al., 2003) or chronic (dietary supplementation or injection) (Goldschmith et al., 2005; Leiva et al., 2009) exogenous Cu on hippocampal LTP demonstrated an inhibitory effect of Cu. This is thought to result from the inhibitory effects of Cu on NMDA receptors (Doreulee et al., 1997; Schlief et al., 2006). AMPA receptor-mediated synaptic transmission in the Schaffer collateral-CA1 pathway is inhibited by 10 μM CuSO₄ while inhibition of NMDA receptors is sensitive to 1 μM CuSO₄ (Doreulee et al., 1997). Cu also blocks high voltage-activated calcium channels in rat cortical (Castelli et al., 2003) and olfactory bulb neurons (Horning and Trombley, 2001). Notably, L-type voltage-gated calcium channels,
located post-synaptically on dendritic spines and contributing to calcium influx during LTP induction (Kullmann et al., 2000; Malenka and Bear, 2004), are sensitive to Cu in the low µM range (Korte et al., 2003). Thus Cu could impair hippocampal LTP through multiple mechanisms.

By contrast, we demonstrated that endogenous extracellular (secreted) Cu is necessary for pre-synaptic LTP in the amygdala (Fig. 3-4). It may be that secreted Cu is a vital component of LTP induction in other brain regions as well, including the hippocampus. The action potential pairing LTP induction protocol we employed in this study has been shown previously to be L-type voltage-gated calcium channel-dependent and NMDA receptor-independent at thalamic afferent synapses (Bauer et al., 2002; Weisskopf et al., 1999). A different form of LTP may be spared in the context of endogenous Cu chelation. One caveat of those experiments concerns the selectivity with which Cu limits extracellular Cu availability; while the duration of BCS exposure was kept at a minimum, there may have been some unintended effects on intracellular Cu status. Regardless, the present study clearly demonstrates an essential role for Cu in a form of LTP that tightly correlates with performance in discrete fear learning and memory tasks.

Cu and GABAergic transmission

In normal aCSF, we observed a selective potentiating effect of Cu on feed-forward GABAergic transmission at thalamic inputs to lateral amygdala pyramidal neurons (Fig. 3-5). It is not possible to relate these findings to our LTP data because LTP experiments were conducted in the presence of picrotoxin. Moreover, addition of lidocaine to the intracellular solution and the subsequent blockade of post-synaptic GABA$_B$ receptors may have occluded additional effects of Cu on the post-synaptic neuron. Nevertheless, the effect of Cu we observed on GABAergic transmission was profound and consistent
with most reports in the literature. Leiva and colleagues (Leiva et al., 2000) used extracellular recordings to investigate the interaction between Cu and GABAergic inhibition on excitatory synaptic transmission in the hippocampus. They found depressing effects of Cu (10 µM) on synaptic transmission in the Schaffer-CA1 pathway, suggesting Cu may suppress glutamatergic transmission by enhancing GABAergic inhibition. However, addition of Cu in the presence of picrotoxin remarkably enhanced synaptic transmission, implicating non-GABAergic receptors in the actions of copper on synaptic transmission (Leiva et al., 2000).

Direct effects of Cu on GABA<sub>A</sub> receptors have been demonstrated at physiological Cu concentrations (Ma and Narahashi, 1993;Sharonova et al., 1998;Kim and Macdonald, 2003). Cu concentration in brain tissue has been estimated to be between 80 and 100 µM, about 5-fold higher than that found in blood (Linder and Hazegh-Azam, 1996;Que et al., 2008;Prohaska, 1987). Cu can be released with neuronal activation in a calcium-dependent manner (Schlief et al., 2006;Dodani et al., 2011) and can reach concentrations of 100-250 µM at synapses (Kardos et al., 1989). Cu binds different human GABA<sub>A</sub> receptor subtypes with equal affinity at an EC<sub>50</sub> of 2.4 µM (Kim and Macdonald, 2003), unlike Zn (Fisher and Macdonald, 1998). Cu reduces coupling of the GABA-binding region to channel gating; Cu and Zn both have inhibitory effects on GABA<sub>A</sub> receptor function (Fig. 3-9) (Narahashi et al., 1994;Trombley et al., 1998;Horning and Trombley, 2001) [for review see (Mathie et al., 2006)]. Cu-mediated GABA<sub>A</sub> receptor inhibition is reversible (Fig. 3-5) (Ma and Narahashi, 1993), but may require removal of Cu from its binding site by using a chelator (Sharonova et al., 1998). Cu enhances the binding and activity of other GABA<sub>A</sub> receptor allosteric modulators such as benzodiazepines (Mizuno et al., 1982;Kardos et al., 1984). Interestingly, PAM<sup>−/−</sup> mice are particularly sensitive to the anxiolytic effects of benzodiazepines (Gaier et al., 2010).
residues important for Cu binding include a motif which spans the proximal portion of the cysteine loop of the α1 N-terminal domain (Kim and Macdonald, 2003), and two residues also important in Zn binding to α6-containing receptors are also required for Cu binding (Kim and Macdonald, 2003; Fisher and Macdonald, 1998).

Importantly, subunit composition of GABA_A receptors determines localization, activity and physiological function within neurons and networks (Wafford et al., 2004; Rudolph and Mohler, 2006). Cu has differential efficacies for various GABA_A receptor subtypes, with stronger effects on α1- and α2-containing receptors compared to α4- and α6-containing receptors (Kim and Macdonald, 2003). This subtype specificity is functionally relevant; α(1-3)-containing receptors localize to GABAergic synapses and mediate phasic or fast inhibition, whereas α(4-6)-containing receptors are extra-synaptic and mediate tonic or slow inhibition (Hevers and Luddens, 1998; Jacob et al., 2008; Farrant and Nusser, 2005). In the basolateral amygdaloid complex, parvalbumin-positive interneurons that mediate feed-forward inhibition (Muller et al., 2006) express high levels of the α1 subunit (McDonald and Mascagni, 2004). Inhibition of these α1-containing receptors on interneurons could account for Cu-mediated enhancement of feed-forward inhibition observed in the present study (Fig. 3-9). Additionally, synaptic GABA_A receptor subtypes localize to separate synapses in the hippocampus (Nyiri et al., 2001). GABA_A receptor α1 subunit mRNA expression was not affected by PAM heterozygosity in amygdala or hippocampus, but transcripts encoding the GABA_B receptor were selectively down-regulated in the PAM^{+/-} amygdala (Fig. 3-8). GABA_B receptors are expressed on GABAergic interneurons, pyramidal neuronal dendrites and glutamatergic afferent terminals in the basolateral amygdaloid complex (McDonald et al., 2004). Thus, reduced GABA_B receptor levels could have multiple effects on afferent signaling in the PAM^{+/-} amygdala.
**ATP7A and Cu are present at synapses**

We demonstrated the presence of ATP7A in synaptosomal and PSD fractions isolated from mouse neocortex (Fig. 3-7). ATP7A also co-localized with PAM in the neuropil of the basolateral amygdaloid complex (Fig. 3-6). This puts ATP7A and secretable Cu in a position to affect synaptic transmission; secreted Cu could participate in the synapse specificity of LTP essential to fear memory formation. With this in mind, observing the net effects of exogenous Cu applied through the perfusate may not provide information physiologically relevant to memory formation. Rather, chelation of endogenous Cu secreted following stimulation is likely to reveal the role of Cu in learning and memory.

The distribution of ATP7A is shifted toward the PSD in the cortex of PAM\(^{+/−}\) mice, suggesting increased synaptic Cu secretion in the cortex of these mice; total ATP7A levels were not significantly altered. ATP7A and Atox-1 mRNA levels were reduced in the PAM\(^{+/−}\) amygdala compared to wildtype (Fig. 3-8); it is not clear whether this reflects region-specificity in the influence of PAM heterozygosity on ATP7A gene transcription and/or a disconnect between ATP7A transcript and protein levels. Regardless, our data support the hypothesis that releasable synaptic Cu is reduced in the PAM\(^{+/−}\) amygdala and may contribute to the synaptic plasticity and learning and memory deficits observed in these mice.

Cu homeostasis is tightly regulated, with the roles of different organ systems just beginning to be elucidated (Kim et al., 2010a). The brain contains more Cu than most other organs (Linder and Hazegh-Azam, 1996;Que et al., 2008;Prohaska, 1987) and our data support an essential role for Cu in the modulation of synaptic transmission and in synaptic plasticity. The bidirectional relationship between PAM and Cu homeostasis is reflected by the diminished Atox-1 and ATP7A transcript levels observed in the PAM\(^{+/−}\).
amygdala, and likely speaks to an additional essential role for PAM as a Cu sensor (Fig. 3-9) (Bousquet-Moore et al., 2010a). Other cuproenzymes and Cu-binding proteins translocate into the nucleus and affect transcription of genes relevant to Cu homeostasis (Lutsenko, 2010). Clearly the network of genes involved in homeostasis is much more complex than previously thought. Indeed Cu homeostasis is hypothesized to be disrupted, and in some cases causative, of some neurological disorders affecting emotionality and learning and memory including Alzheimer’s disease (Lin et al., 2010) and Wilson’s disease (Pfeiffer, 2007). This study identifies one small piece of that puzzle and expands our current view of the role Cu in normal and pathological nervous system function.
REFERENCE LIST


CHAPTER 4: Serum Cu and PAM activity in frail elderly men

[High serum Cu and Cu/Zn ratios correlate with impairments in bone density, physical performance and overall health in a population of frail elderly men. Gaier, Eric D; Kleppinger, Alison; Ralle, M; Mains, Richard E; Kenny, Anne M; Eipper, Betty A. J Gerontology. Submitted March, 2011.]
CONTRIBUTIONS

My first summer working in the Mains/Eipper lab, I took on a project measuring PHM activity in serum from human subjects. We hypothesized that single nucleotide polymorphisms in the PAM gene could have functional impacts on serum PHM activity. This was the first study to measure amidating activity in human samples. Those preliminary data lead to a larger funded study of serum metals and PAM activity in serum samples collected from a cohort of 144 frail elderly men as part of a previous study [Kenny AM, Kleppinger A, Annis K, Rathier M, Browner B, Judge JO, McGee D (2010) Effects of transdermal testosterone on bone and muscle in older men with low bioavailable testosterone levels, low bone mass, and physical frailty. J Am Geriatr Soc 58:1134-1143]. This study was conducted in collaboration with Dr. Anne Kenny at the UConn Center on Aging and Dr. Jonathan Covault in the department of Psychiatry. I was involved in organizing the data, collecting relevant literature and background, generating figures and writing a manuscript. This work was recently completed and the manuscript was submitted to the Biological series of the Journal of Gerontology in March, 2011. I also presented this work in poster form at the UConn Center on Aging Research Day on April 18, 2011.
ABSTRACT

Levels of copper (Cu), an essential trace element, are tightly controlled. Serum Cu levels rise with age and high serum Cu levels and Cu/Zinc (Zn) ratios are linked with multiple-cause mortality in the elderly. To explore a functional link for this phenomenon, we assessed serum levels of Cu, Zn and a Cu- and Zn-dependent monooxygenase (PAM), along with measures of bone, physical and overall health in a cohort of 144 frail elderly men. We found strong connections between high serum Cu levels and high Cu/Zn ratios with deficits in femoral bone mineral density, physical performance and hematocrit. High Cu/Zn ratios were also associated with reduced muscle mass and strength and increased reliance on ADL assistance. Serum PAM activity correlated with serum Cu and exhibited similar associations with functional impairments. Our results support studies indicating that serum Cu levels and the Cu/Zn ratio may serve as useful biomarkers in the elderly.
INTRODUCTION

Copper (Cu) is essential to the life of organisms from bacteria to humans; its redox activity contributes to its utility as a co-factor for electron transfer reactions. Only about a dozen cuproenzymes, which require Cu for their catalytic activity, are encoded in the mammalian genome; these enzymes are essential to the maintenance of multiple organ systems (Crichton and Pierre, 2001; Ridge et al., 2008). Among these are cytochrome c oxidase, Cu/Zinc(Zn)-dependent superoxide dismutase (SOD), lysyl oxidase, tyrosinase, dopamine β-hydroxylase and peptidylglycine α-amidating monooxygenase (PAM). Many other proteins bind Cu and play a role in Cu transport and homeostasis. Cu deficiency affects the cardiovascular, musculoskeletal, hematopoietic and nervous systems (Uauy et al., 1998; Uriu-Adams and Keen, 2005); symptomology is thought to reflect inadequate activities of specific cuproenzymes. For example, dysfunction of Cu/Zn SOD results in tissue damage by superoxide and reduced lysyl oxidase cross-linking of collagen contributes to musculoskeletal and cardiovascular deficits (Uauy et al., 1998). Menkes disease, an X-linked recessive genetic disease of Cu transport, results in severe Cu deficiency (Kaler, 2011); reductions in norepinephrine and neuropeptides from impaired dopamine β-hydroxylase and PAM activity may contribute to the seizure susceptibility observed. Moreover, genetic reduction of PAM in a mouse model is associated with increased susceptibility to seizure, anxiety-like phenotype, learning and memory deficits and neuronal hyperexcitability in limbic structures (Bousquet-Moore et al., 2009; Bousquet-Moore et al., 2010b; Gaier et al., 2010). Interestingly, many of these deficits can be reversed with dietary Cu supplementation (Bousquet-Moore et al., 2010b; Bousquet-Moore et al., 2009). It is now clear that a complex regulatory system governs copper homeostasis to keep availability of Cu at optimal levels (Kim et al.,
Copper overload is also detrimental, and is thought to result in oxidative stress through Fenton-like reactions and the generation of free-radicals that contribute to chronic/degenerative diseases and carcinogenesis (Uriu-Adams and Keen, 2005). Higher serum Cu concentrations have been linked with advancement of chronic diseases such as diabetes mellitus and Alzheimer's disease (Uriu-Adams and Keen, 2005; Mezzetti et al., 1998; Arnal et al., 2010; Viktorinova et al., 2009). Zn, another essential element, competes with Cu for uptake in the gut and is thought to serve as a natural antioxidant (Uriu-Adams and Keen, 2005; Malavolta et al., 2010). Previous studies linked high serum Cu and concomitant low serum Zn to mortality in elderly populations (Leone et al., 2006; Malavolta et al., 2010; Reunanen et al., 1996). In several cases, the Cu/Zn ratio proved to be a better predictor of disease severity and/or mortality than Cu levels (Leone et al., 2006; Malavolta et al., 2010; Reunanen et al., 1996; Mezzetti et al., 1998). PAM, which uses both Cu and Zn to catalyze the production of bioactive peptides, is released from nerve endings, peptide secreting endocrine tissues and endothelial cells and can be assayed in serum (Prigge et al., 2000; Kolhekar et al., 2002; Prohaska and Broderius, 2006).

Since serum Cu levels increase steadily with age (Malavolta et al., 2010; Mezzetti et al., 1998) as does sensitivity to nutritional and physiological challenges, serum Cu, Zn and Cu/Zn ratios have been explored as biomarkers (Malavolta et al., 2010). Serum PAM activity has not been tested as a biomarker. We assessed the relationship between these parameters and physical and mental measures of well-being in a population of frail elderly men. We found strong negative relationships between serum Cu and the Cu/Zn ratio with bone mineral density, muscle strength and measures of overall health and
independence. Serum PAM activity correlated with Cu levels and shared many of the same associations with bone health, physical performance and overall health as the Cu/Zn ratio. These results identify serum Cu, Cu/Zn ratio and PAM activity as strong associative measures for frailty within elderly populations.
METHODS

Study Population

Frail men aged 60 years or older residing in the community or assisted living were recruited to participate in the study. The individuals were screened for potential participation in a previously reported study to assess testosterone effects on bone and frailty in men (Kenny et al., 2010). The data used in this analysis are baseline assessments, prior to any interventions. Briefly, exclusion criteria were: [1] Diseases or medications known to affect bone or muscle metabolism (i.e. Paget’s disease, osteomalacia, hyperparathyroidism: current use of corticosteroids, calcitonin, heparin, phenytoin, phenobarbital, methotrexane, bisphosphonates, selective estrogen receptor modulator or PTH; [2] Use of estrogen, DHEA or androgen in the preceding year; [3] metastatic or advanced cancer (other than skin cancer); [4] History of prostate cancer; [5] Active cardiac ischemia by history of angina or myocardial infarction in the preceding 6 months; [6] Elevation of PSA [7] History of sleep apnea; [8] Polycythemia. All study participants provided written informed consent. The institutional review board at the University of Connecticut Health Center approved the study.

Evaluations

Baseline evaluation of the following outcomes were performed: basic demographic characteristics; dietary intake using a 3 day food record; serum Cu and Zn levels; bone assessment including bone mineral density (BMD), bone markers, sex and calcium regulating hormone levels, body composition and physical function assessment including frailty evaluation, body composition, strength measures, multiple physical performance measures, self-reported physical activity estimated by Physical Activity Scale for the Elderly (PASE) questionnaire (Washburn et al., 1993), activities of daily living and
hematocrit; and overall health assessment including Folstein Mini-mental status examination, Geriatric Depression scale and lipid analysis. The frailty phenotype evaluation was based on Fried et al. (Fried et al., 2001) (frail =3-5 characteristics, intermediate frail =1-2 characteristics, non-frail= 0 characteristics). Bone mineral density and body composition (Lunar DPX-L, Madison, WI) of the proximal femur, lumbar spine, distal radius, and total body (total lean body mass [LBM (kg)], total fat mass (kg) and total body bone mineral content (kg)) were obtained. The coefficient of variation of BMD measurement at the proximal femur, spine and total body was <1%, 1.5% and 2%, respectively. Appendicular skeletal muscle mass (ASM) was determined by combining the lean tissue mass of the regions of the arms and legs, excluding all other regions from analysis (Wang et al., 1996). We adjusted ASM for height by dividing each by height^2 (m^2). Leg extension strength [1 repetition maximum (Judge et al., 1996); intra- and inter-tester variability <10%] was measured on the Keiser Sitting Leg Press. Physical performance was assessed by the Short Physical Performance Battery [ability to rise from a chair, static balance and the 8 foot walk ](14), supine to stand test (Alexander et al., 1997) and Get Up and Go test (Podsiadlo and Richardson, 1991). Dietary records were analyzed using Food Processor (version8.1; Salem, OR).

Biochemical Analysis

Biochemical analyses of bone turnover markers were previously described (6) and included markers of bone formation [bone specific alkaline phosphatase (BAP), N-terminal type I procollagen peptide (PINP) and osteocalcin (OC)]; markers of bone resorption [crosslinked N-telopeptide (NTX) of type I collagen and free deoxypyridinoline crosslinks (DPD)]; sex hormones [total and bioavailable testosterone, SHBG, estradiol, DHEAS]; calcium
regulating hormones [25OHD and PTH]; overall health measures [cholesterol measurements, and hematocrit].

PHM activity was assayed as described using a trace amount of $^{[125I]}$-Ac-Tyr-Val-Gly, 0.5 µM Ac-Tyr-Val-Gly and 4.0 µM CuSO$_4$; serum samples were diluted 10-fold into 20 mM Na TES, pH 7.4, 10 mM mannitol, 1 mg/ml bovine serum albumin, 1% TX-100 (Surfact-Amps X-100) (Thermo Scientific) and 4 µl (0.4 µl of serum) was assayed in triplicate in 100 mM Na MES, pH 5.5 (Bousquet-Moore et al., 2009). In the absence of exogenous Cu, PHM activity cannot be accurately assessed; the addition of exogenous Cu (4.0 µM CuSO$_4$) yielded maximal levels of PHM activity for serum samples in the upper and lower quintiles. PAL activity was assayed in triplicate from the same dilutions (0.2 µl serum) using a trace amount of $^{[125I]}$-Ac-Tyr-Val-α-hydroxyglycine, 0.5 µM Ac-Tyr-Val-α-hydroxyglycine, 1 mM CdCl$_2$, 0.02% Thesit and 100 mM Na MES, pH 5.5 (Kolhekar et al., 2002). Ceruloplasmin was assayed in duplicate using O-dianisidine dihydrochloride (Prohaska and Broderius, 2006); a linear response was observed with 1.0 to 5.0 µl serum and samples were compared using 2.5 µl serum.

**Measurement of serum Cu and Zn levels**

Inductively coupled plasma mass spectrometry analysis was performed using an Agilent 7700x equipped with an ASX 250 autosampler. The system was operated at a radio frequency power of 1550 W, an argon plasma gas flow rate of 15 L/min, and an Ar carrier gas flow rate of 1.04 L/min. Elements were measured in kinetic energy discrimination (KED) mode using He gas (4.3 mL/min). For the analysis, serum samples were diluted 25-fold into 1% HNO$_3$ (Fisher Scientific). Data were quantified using a 5-point (0, 1, 10, 100, 1000 ppb (ng/g)) calibration curve with external standards for Fe, Cu, and Zn. For each sample, data were acquired in triplicate and averaged. An internal
standard (Er) introduced with the sample was used to monitor for plasma instabilities.

**Statistical Analysis**

Baseline and clinical characteristics were reported using percentages, means and standard deviations. All variables were checked for normal distribution and the impact of outliers. Outliers beyond 3 standard deviations were dropped from the analyses (n=3). Correlation coefficients were used to detect preliminary associations of serum Cu with other measures of interest. Quintiles for serum metals, enzyme activities and ratios were created using the binned function in SPSS software (version 18.0). Four cut points were applied to create approximately five equal proportions of the sample distribution (quintiles). Independent t-tests were used to compare the means of the lowest quintile to the middle or reference quintile. Separate independent t-tests were used to compare the means of the highest quintile to the middle or reference quintile. The outcome measures being compared among the high and low quintile groups were bone mineral density, bone markers, hormones, frailty factors, body composition, physical performance, and overall health factors. Statistical analyses were performed using SPSS version 18.0 (Chicago, IL).
RESULTS

Subject population. One hundred and forty-four men were included in this analysis with a mean age of 77 years and BMI of 27 kg/m². Baseline information on the group appears in Table 4-1. Subjects consumed a mean of 2000 calories/day, 1.21 ± 1.07 mg of Cu and 12.9 ± 10.6 mg of Zn. Dietary intake shared no statistical relationship with serum values (not shown). Bone mineral density, bone turnover markers and sex and calcium regulating hormone information are summarized in Table 4-1. The mean femoral neck t-score was -2.12 ± 0.75 g/cm², indicating relatively low bone mass on average. Bone turnover markers, sex hormone levels and vitamin D and parathyroid hormone (PTH) were above the normal range and typical of those in older men (Ferrini and Barrett-Connor, 1998). Average results for the assessment of body composition and physical function are also outlined; the majority of men (91%) met the criteria for frailty (18%) or prefrailty (72%). Approximately 50% of the men had appendicular skeletal muscle mass (ASM/Ht²) values that met criteria for sarcopenia or low muscle mass associated with aging (Baumgartner et al., 1998). Overall, the men were community dwelling but had some limitation in function, with 20% requiring assistance in instrumental activities of daily living and 12% requiring assistance in activities of daily living such as dressing, toileting and feeding.

Serum Cu, Zn and the Cu/Zn Ratio. Serum Cu and Zn levels were measured by inductively coupled plasma mass spectrometry, and Cu/Zn ratios were calculated for each subject. Serum levels of Cu were 1009 ± 241 ppb, Zn levels were 877 ± 281 ppb and the Cu/Zn ratio was 1.22 ± 0.37 (Table 4-1). Serum Cu, serum Zn, and Cu/Zn ratios were normally distributed among subjects in our sample (Fig. 4-1). For analysis, subjects were divided into quintiles based on serum Cu, Zn and serum Cu/Zn ratios. In
all cases, measures of BMD, physical performance and overall health were compared
between the lowest or highest quintile and the reference (middle) quintile.

**Serum Ceruloplasmin and the Cu/Ceruloplasmin Ratio.** Ceruloplasmin (Cp) is a Cu-
dependent ferroxidase that acts as the major carrier for Cu in the blood. As expected,
serum Cp levels correlated tightly with serum Cu levels (**Fig. 4-2A**). Previous studies

<table>
<thead>
<tr>
<th>Table 4-1. Characteristics of the whole sample</th>
<th>Total Mean ± SD (N=144)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (yrs)</strong></td>
<td>77.1 ± 7.6</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>26.9 ± 4.4</td>
</tr>
<tr>
<td><strong>Nutrition</strong></td>
<td></td>
</tr>
<tr>
<td>Calories</td>
<td>2046 ± 1130</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>898 ± 660</td>
</tr>
<tr>
<td>Vitamin D (IU)</td>
<td>209 ± 276</td>
</tr>
<tr>
<td>Copper (mg)</td>
<td>1.21 ± 1.07</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>94.8 ± 51.5</td>
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<tr>
<td>Magnesium (mg)</td>
<td>285.4 ± 161.9</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>12.9 ± 10.6</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>19.8 ± 12.9</td>
</tr>
<tr>
<td><strong>Serum Levels</strong></td>
<td></td>
</tr>
<tr>
<td>Copper (ppb)</td>
<td>1009 ± 241</td>
</tr>
<tr>
<td>Ceruloplasmin (U/uL)</td>
<td>188.4 ± 46.3</td>
</tr>
<tr>
<td>Zinc (ppb)</td>
<td>877 ± 281</td>
</tr>
<tr>
<td>Iron (ppb)</td>
<td>970 ± 531</td>
</tr>
<tr>
<td>Copper/Zinc Ratio</td>
<td>1.22 ± 0.37</td>
</tr>
<tr>
<td>Copper/Ceruloplasmin Ratio</td>
<td>5.48 ± 1.10</td>
</tr>
<tr>
<td>PHM Activity (nmol/mL/h)</td>
<td>5.56 ± 1.13</td>
</tr>
<tr>
<td>PAL Activity (nmol/mL/h)</td>
<td>19.00 ± 4.46</td>
</tr>
<tr>
<td><strong>Bone Mineral Density</strong></td>
<td></td>
</tr>
<tr>
<td>Femoral Neck BMD (g/cm²)</td>
<td>0.794 ± 0.098</td>
</tr>
<tr>
<td>Femoral Neck (t-score)</td>
<td>-2.12 ± 0.75</td>
</tr>
<tr>
<td>Femoral Total BMD (g/cm²)</td>
<td>0.886 ± 0.113</td>
</tr>
<tr>
<td>Femoral Total (t-score)</td>
<td>-1.56 ± 0.87</td>
</tr>
<tr>
<td>Femoral Trochanter BMD (g/cm²)</td>
<td>0.794 ± 0.121</td>
</tr>
<tr>
<td>Femoral Trochanter (t-score)</td>
<td>-1.24 ± 1.10</td>
</tr>
<tr>
<td>Radius BMD (g/cm²)</td>
<td>0.551 ± 0.072</td>
</tr>
<tr>
<td>Radius (t-score)</td>
<td>-1.11 ± 1.30</td>
</tr>
<tr>
<td>Total body BMD (g/cm²)</td>
<td>1.160 ± 0.094</td>
</tr>
<tr>
<td>Total body (t-score)</td>
<td>-0.75 ± 1.17</td>
</tr>
<tr>
<td><strong>Bone Turnover Markers</strong></td>
<td></td>
</tr>
<tr>
<td>NTX/CR</td>
<td>43.1 ± 41.9</td>
</tr>
<tr>
<td>BAP (U/L)</td>
<td>26.0 ± 15.9</td>
</tr>
<tr>
<td>Osteocalcin (ng/mL)</td>
<td>10.8 ± 6.9</td>
</tr>
<tr>
<td><strong>Sex Hormones and Calcium Regulating Hormones</strong></td>
<td></td>
</tr>
<tr>
<td>DHEA (ug/dL)</td>
<td>55.3 ± 44.9</td>
</tr>
<tr>
<td>Estradiol (pg/mL)</td>
<td>34.2 ± 10.0</td>
</tr>
<tr>
<td>Testosterone (pg/mL)</td>
<td>400 ± 185</td>
</tr>
<tr>
<td>Vitamin D (nmol/L)</td>
<td>86.5 ± 36.2</td>
</tr>
</tbody>
</table>
identified free or unbound Cu as having a stronger association with disease than total Cu or Cp levels (Arnal et al., 2010). Therefore, we calculated the Cu/Cp ratio for each subject as an indicator of unbound Cu in serum and found these values to be normally distributed among subjects in our sample (Fig. 4-2B). Subjects were divided into quintiles based on their serum Cu/Cp ratio, as had been done for the serum metals.

Table 4-1. Characteristics of the whole sample (continued)

<table>
<thead>
<tr>
<th>Total Mean ± SD (N=144)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Composition</strong></td>
</tr>
<tr>
<td><strong>and Physical Function</strong></td>
</tr>
<tr>
<td>Nonfrail % (n)</td>
</tr>
<tr>
<td>Prefrail % (n)</td>
</tr>
<tr>
<td>Frail % (n)</td>
</tr>
<tr>
<td>ASM (Kg)</td>
</tr>
<tr>
<td>ASM/H²°</td>
</tr>
<tr>
<td>Whole body fat mass (Kg)</td>
</tr>
<tr>
<td>Whole body lean mass (Kg)</td>
</tr>
<tr>
<td>Leg Press Strength (Newtons)</td>
</tr>
<tr>
<td>Leg Press Power (Watts)</td>
</tr>
<tr>
<td>Handgrip (Kg)</td>
</tr>
<tr>
<td>Chair rise time (secs)</td>
</tr>
<tr>
<td>Walk best time (m/sec)</td>
</tr>
<tr>
<td>Get up and Go (secs)</td>
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<tr>
<td>PASE score</td>
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<tr>
<td>SPPB score</td>
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<tr>
<td><strong>Overall Health</strong></td>
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<tr>
<td>MMSE score</td>
</tr>
<tr>
<td>CESD score</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
</tr>
<tr>
<td>IADL needs assistance % (n)</td>
</tr>
<tr>
<td>ADL needs assistance % (n)</td>
</tr>
<tr>
<td>HCT (%)</td>
</tr>
<tr>
<td>Heart disease % (n)</td>
</tr>
<tr>
<td>Hypertension % (n)</td>
</tr>
<tr>
<td>On Cholesterol Medication % (n)</td>
</tr>
<tr>
<td>On Hypertension Medication % (n)</td>
</tr>
</tbody>
</table>
Figure 4-1. Serum Metals. Graphs depict frequency histograms for serum Cu (A), serum Zn (B), and the Cu/Zn ratio (C). Values for all three parameters were normally distributed. Reference lines for histograms denote boundaries for quintile groupings.
Serum PAM Activity. Although Cu is essential for the catalytic activity of key enzymes such as mitochondrial Cyt c oxidase, cytosolic Cu/Zn SOD and secreted amine oxidases, it must be bound to transporters and chaperones in order to limit its ability to damage lipids, nucleic acids and proteins. The vesicular PAM enzyme catalyzes the α-amidation reaction essential to the biosynthesis of many neuropeptides and hormones. The first part of this two-step reaction, the α-hydroxylation of peptidylglycine, is accomplished by the peptidylglycine α-hydroxylating monooxygenase (PHM) domain of PAM, which requires Cu. The second step, C-N bond cleavage to yield the final amidated product plus glyoxylate, is accomplished by peptidyl-α-hydroxyglycine α-amidating lyase (PAL), which uses Zn; several other divalent metals can substitute for Zn. We evaluated both activities in serum samples from our subjects. Assays were done after adding exogenous metals and reflect the amount of PHM and PAL protein, not metallation of each enzyme, in serum. Serum PHM and PAL activities were normally distributed in our sample (Fig. 4-3A, B). PHM and PAL activities were strongly correlated
(Fig. 4-3C), as would be expected for enzymes cleaved from the same bifunctional precursor. Pearson correlation analysis revealed significant associations between PHM and PAL activity and serum Cu (Fig. 4-3D,E), but not with serum Zn or the Cu/Zn ratio (not shown). As for serum Cu, serum Zn and the Cu/Zn ratio, PHM and PAL activities were divided into quintiles and measures of BMD, physical performance and overall health were compared between groups. Associations found in the PAL quintile analysis (but not for PHM) typically followed those of the Cu/Zn ratio. This may reflect the fact that individuals in the high Cu/Zn group also had significantly higher serum PAL activities (Fig. 4-3F). Since serum PHM and PAL assays are not widely used, we chose to focus on associations with serum metals.
Figure 4-3. PAM Activity. Graphs depict frequency histograms for PHM (A) and PAL (B) activities. (C) PHM and PAL activities for each subject are positively and linearly correlated. PHM (D) and PAL (E) activities are positively and linearly correlated with serum Cu. (F) Subjects in the high serum Cu/Zn ratio group have significantly higher serum PAL activities than the reference group. Inset depicts scatter plot with reference line boundaries for quintile groupings. * denotes P<0.05 compared to the reference group.
Bone Mineral Density. Mean bone mineral density and factors that contribute to bone mineral density were compared across serum Cu levels and Cu/Zn ratios (Table 4-2).

Bone mineral density measures were lower overall in subjects in the high Cu quintile, reaching significance for the femoral neck (Table 4-2A; Fig. 4-4A) and total body. Markers for bone turnover, including bone alkaline phosphatase, N-telopeptide crosslinks of type I collagen, and osteocalcin, were significantly or near significantly elevated in subjects in the low serum Cu group. However, these individuals did not display any reduction in bone mineral density. No statistically significant differences

Table 4-2a. Copper groupings by bone mineral density and bone turner markers

<table>
<thead>
<tr>
<th></th>
<th>Low</th>
<th>Reference</th>
<th>High</th>
<th>P values</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;836.69 ppb (n=27)</td>
<td>906.44 - 1013.91 ppb (n=28)</td>
<td>&gt;1193.06 ppb (n=22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femoral Neck BMD (g/cm²)</td>
<td>0.814 ± 0.087</td>
<td>0.806 ± 0.070</td>
<td>0.747 ± 0.113</td>
<td>.698</td>
<td>.031</td>
</tr>
<tr>
<td>Femoral Total BMD (g/cm²)</td>
<td>0.891 ± 0.085</td>
<td>0.910 ± 0.096</td>
<td>0.845 ± 0.146</td>
<td>.456</td>
<td>.066</td>
</tr>
<tr>
<td>Femoral Trochanter BMD (g/cm²)</td>
<td>0.788 ± 0.094</td>
<td>0.811 ± 0.110</td>
<td>0.757 ± 0.155</td>
<td>.394</td>
<td>.157</td>
</tr>
<tr>
<td>Radius BMD (g/cm²)</td>
<td>0.535 ± 0.084</td>
<td>0.536 ± 0.062</td>
<td>0.540 ± 0.082</td>
<td>.153</td>
<td>.267</td>
</tr>
<tr>
<td>Total Body BMD (g/cm²)</td>
<td>1.150 ± 0.092</td>
<td>1.188 ± 0.069</td>
<td>1.126 ± 0.114</td>
<td>.087</td>
<td>.022</td>
</tr>
<tr>
<td>NTX/CR</td>
<td>43.4 ± 18.1</td>
<td>33.9 ± 18.4</td>
<td>44.6 ± 38.6</td>
<td>.061</td>
<td>.203</td>
</tr>
<tr>
<td>BAP (U/L)</td>
<td>27.7 ± 8.9</td>
<td>22.6 ± 6.4</td>
<td>23.8 ± 9.0</td>
<td>.017</td>
<td>.581</td>
</tr>
<tr>
<td>Osteocalcin (ng/mL)</td>
<td>11.7 ± 6.3</td>
<td>9.0 ± 5.9</td>
<td>10.2 ± 6.2</td>
<td>.086</td>
<td>.439</td>
</tr>
<tr>
<td>DHEA (µg/dL)</td>
<td>62.8 ± 39.5</td>
<td>53.4 ± 57.0</td>
<td>44.5 ± 24.3</td>
<td>.504</td>
<td>.483</td>
</tr>
<tr>
<td>Estradiol (pg/mL)</td>
<td>34.4 ± 9.2</td>
<td>35.3 ± 7.3</td>
<td>31.6 ± 10.3</td>
<td>.690</td>
<td>.146</td>
</tr>
<tr>
<td>Testosterone (pg/mL)</td>
<td>452.2 ± 174.9</td>
<td>386.7 ± 150.7</td>
<td>344.2 ± 204.4</td>
<td>.142</td>
<td>.406</td>
</tr>
<tr>
<td>Vitamin D (nmol/L)</td>
<td>88.7 ± 34.6</td>
<td>89.6 ± 35.3</td>
<td>78.4 ± 37.6</td>
<td>.922</td>
<td>.281</td>
</tr>
<tr>
<td>DHEA (µg/dL)</td>
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<td>44.5 ± 24.3</td>
<td>.504</td>
<td>.483</td>
</tr>
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<td>31.6 ± 10.3</td>
<td>.690</td>
<td>.146</td>
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<td>.142</td>
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<td>53.4 ± 57.0</td>
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<td>.483</td>
</tr>
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<td>.690</td>
<td>.146</td>
</tr>
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<td>344.2 ± 204.4</td>
<td>.142</td>
<td>.406</td>
</tr>
<tr>
<td>Vitamin D (nmol/L)</td>
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<td>89.6 ± 35.3</td>
<td>78.4 ± 37.6</td>
<td>.922</td>
<td>.281</td>
</tr>
</tbody>
</table>

Bold represents p values < .05. P values were based on independent t-tests comparing mean values between copper level groups.
between sex hormones or calcium regulating hormones were found between Cu groups, although trends toward lower estradiol and vitamin D and higher PTH were found in the high Cu group. Larger significant deficits in BMD, including that of the femoral neck (Fig. 4-4B), were found in individuals in the high Cu/Zn ratio group (Table 4-2B). No differences in bone health measures were found in the low Cu/Zn ratio group. Individuals in both the low and high PAL groups had significantly lower femoral neck and total femur BMD than the reference group (not shown).

PTH and vitamin D are important hormonal regulators of bone mineral density and of serum calcium. While the average PTH value for our sample was above the normal range, as is typical for older individuals, PTH was up significantly in the highest Cu/Zn ratio group (Fig. 4-5A). Vitamin D levels were reduced overall in our sample, as expected in elderly individuals who receive less sun exposure than the general population. Especially low vitamin D levels can result in compensatory increases in PTH to maintain serum calcium levels. To determine whether the elevation in PTH in subjects with high Cu/Zn ratios could be accounted for by especially low vitamin D levels, we...
compared PTH/vitamin D ratios between the Cu/Zn ratio groups (Fig. 4-5B). There was a more prominent increased in the PTH/vitamin D ratio than there was for PTH alone in the high serum Cu/Zn ratio group. Similar trends for higher PTH levels and PTH/vitamin D ratios both approached significance for the high PAL group (not shown). This result suggests that a high serum Cu/Zn ratio is associated with elevated PTH levels, which may be driven by a deficiency in vitamin D in those individuals.
Physical Performance. Measures of physical performance, including those testing frailty, body composition, speed, balance and muscle strength, were also compared across serum metal groups. Interestingly, distinct impairments were observed in the high serum Cu and Cu/Zn ratio groups (Table 4-3). Subjects in the high serum Cu group displayed slower walking speeds and get-up-and-go times (Table 4-3A; Fig. 4-6A). The SPPB incorporates measures of speed, muscle strength and balance. Subjects in the high serum Cu group had body compositions and muscle strengths similar to the reference group. The decrease in SPPB score in the high serum Cu group approached statistical

### Table 4-3a. Copper grouping by frailty and physical performance

<table>
<thead>
<tr>
<th></th>
<th>Low &lt;836.69 ppb</th>
<th>Reference 906.44 - 1013.91 ppb</th>
<th>High &gt;1193.06 ppb</th>
<th>P values</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=26)</td>
<td>(n=27)</td>
<td>(n=20)</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Nonfrail % (n)</td>
<td>7 (2)</td>
<td>4 (1)</td>
<td>14 (3)</td>
<td>.531</td>
<td>.193</td>
</tr>
<tr>
<td>Prefrail % (n)</td>
<td>89 (24)</td>
<td>75 (21)</td>
<td>68 (15)</td>
<td>.182</td>
<td>.697</td>
</tr>
<tr>
<td>Frail % (n)</td>
<td>4 (1)</td>
<td>21 (6)</td>
<td>18 (4)</td>
<td>.049</td>
<td>.776</td>
</tr>
<tr>
<td>ASM (Kg)</td>
<td>23.1 ± 3.5</td>
<td>22.2 ± 2.9</td>
<td>21.8 ± 3.1</td>
<td>.273</td>
<td>.658</td>
</tr>
<tr>
<td>ASM/Ht</td>
<td>7.8 ± 0.8</td>
<td>7.5 ± 1.0</td>
<td>7.6 ± 1.0</td>
<td>.280</td>
<td>.695</td>
</tr>
<tr>
<td>Whole body fat mass (Kg)</td>
<td>29.0 ± 5.7</td>
<td>27.4 ± 7.2</td>
<td>29.3 ± 9.0</td>
<td>.366</td>
<td>.411</td>
</tr>
<tr>
<td>Whole body lean mass (Kg)</td>
<td>54.4 ± 7.1</td>
<td>52.8 ± 6.2</td>
<td>51.1 ± 5.9</td>
<td>.392</td>
<td>.332</td>
</tr>
<tr>
<td>Leg Press Strength (Newton)</td>
<td>742 ± 192</td>
<td>606 ± 139</td>
<td>597 ± 140</td>
<td><strong>.005</strong></td>
<td>.842</td>
</tr>
<tr>
<td>Leg Press Power (Watts)</td>
<td>280 ± 145</td>
<td>256 ± 100</td>
<td>215 ± 108</td>
<td>.492</td>
<td>.186</td>
</tr>
<tr>
<td>Handgrip (Kg)</td>
<td>27.4 ± 6.8</td>
<td>24.8 ± 9.2</td>
<td>24.2 ± 7.9</td>
<td>.287</td>
<td>.824</td>
</tr>
<tr>
<td>Chair rise time (secs)</td>
<td>14.4 ± 4.4</td>
<td>15.0 ± 4.5</td>
<td>17.6 ± 8.2</td>
<td>.681</td>
<td>.172</td>
</tr>
<tr>
<td>Walk best time (m/sec)</td>
<td>0.95 ± 0.17</td>
<td>0.94 ± 0.24</td>
<td>0.76 ± 0.26</td>
<td>.958</td>
<td><strong>.015</strong></td>
</tr>
<tr>
<td>Get up and Go (secs)</td>
<td>10.7 ± 4.8</td>
<td>11.2 ± 3.4</td>
<td>15.6 ± 7.2</td>
<td>.698</td>
<td><strong>.008</strong></td>
</tr>
<tr>
<td>PASE score</td>
<td>212.6 ± 103.5</td>
<td>182.2 ± 119.8</td>
<td>164.4 ± 150.6</td>
<td>.334</td>
<td>.654</td>
</tr>
<tr>
<td>SPPB score</td>
<td>9.8 ± 2.1</td>
<td>9.3 ± 2.0</td>
<td>7.8 ± 3.3</td>
<td>.305</td>
<td><strong>.076</strong></td>
</tr>
</tbody>
</table>

### Table 4-3b. Copper:Zinc grouping by frailty and physical performance

<table>
<thead>
<tr>
<th></th>
<th>Low &lt;0.93</th>
<th>Reference 1.12 - 1.27</th>
<th>High &gt;1.51</th>
<th>P values</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=24)</td>
<td>(n=27)</td>
<td>(n=23)</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Nonfrail % (n)</td>
<td>7 (2)</td>
<td>15 (4)</td>
<td>4 (1)</td>
<td>.413</td>
<td>.172</td>
</tr>
<tr>
<td>Prefrail % (n)</td>
<td>85 (22)</td>
<td>70 (19)</td>
<td>65 (17)</td>
<td>.215</td>
<td>.697</td>
</tr>
<tr>
<td>Frail % (n)</td>
<td>8 (2)</td>
<td>15 (4)</td>
<td>31 (8)</td>
<td>.413</td>
<td>.165</td>
</tr>
<tr>
<td>ASM (Kg)</td>
<td>22.8 ± 3.6</td>
<td>23.6 ± 2.9</td>
<td>21.0 ± 3.1</td>
<td>.406</td>
<td><strong>.003</strong></td>
</tr>
<tr>
<td>ASM/Ht</td>
<td>7.7 ± 1.0</td>
<td>7.9 ± 1.0</td>
<td>7.3 ± 0.9</td>
<td>.590</td>
<td><strong>.042</strong></td>
</tr>
<tr>
<td>Whole body fat mass (Kg)</td>
<td>29.3 ± 5.3</td>
<td>28.8 ± 6.8</td>
<td>26.2 ± 8.9</td>
<td>.765</td>
<td>.232</td>
</tr>
<tr>
<td>Whole body lean mass (Kg)</td>
<td>53.8 ± 7.7</td>
<td>55.5 ± 5.1</td>
<td>50.0 ± 5.8</td>
<td>.350</td>
<td><strong>.001</strong></td>
</tr>
<tr>
<td>Leg Press Strength (Newton)</td>
<td>713 ± 228</td>
<td>697 ± 190</td>
<td>564 ± 202</td>
<td>.779</td>
<td><strong>.021</strong></td>
</tr>
<tr>
<td>Leg Press Power (Watts)</td>
<td>309 ± 163</td>
<td>296 ± 137</td>
<td>195 ± 94</td>
<td>.754</td>
<td><strong>.005</strong></td>
</tr>
<tr>
<td>Handgrip (Kg)</td>
<td>24.2 ± 8.1</td>
<td>29.5 ± 8.7</td>
<td>23.0 ± 8.0</td>
<td>.029</td>
<td><strong>.009</strong></td>
</tr>
<tr>
<td>Chair rise time (secs)</td>
<td>12.9 ± 2.6</td>
<td>14.6 ± 5.2</td>
<td>17.5 ± 8.0</td>
<td>.150</td>
<td>.139</td>
</tr>
<tr>
<td>Walk best time (m/sec)</td>
<td>0.95 ± 0.16</td>
<td>0.95 ± 0.25</td>
<td>0.81 ± 0.26</td>
<td>.994</td>
<td>.064</td>
</tr>
<tr>
<td>Get up and Go (secs)</td>
<td>10.6 ± 2.0</td>
<td>11.4 ± 4.4</td>
<td>14.6 ± 7.0</td>
<td>.433</td>
<td>.056</td>
</tr>
<tr>
<td>PASE score</td>
<td>239.2 ± 110.6</td>
<td>184.1 ± 131.5</td>
<td>178.2 ± 170.0</td>
<td>.139</td>
<td>.860</td>
</tr>
<tr>
<td>SPPB score</td>
<td>10.4 ± 1.3</td>
<td>9.7 ± 2.2</td>
<td>7.8 ± 3.0</td>
<td>.178</td>
<td><strong>.014</strong></td>
</tr>
</tbody>
</table>

**Bold** represents p values < .05. P values were based on independent t-tests comparing mean values between copper level groups.
By contrast, subjects in the low serum Cu groups were statistically less likely to meet criteria for frailty and had stronger leg press performances than the reference group (Table 4-3A).

In the Cu/Zn ratio analysis, similarly impaired scores in most assessments were apparent in the high group (Table 4-3B). However, these deficits were focused on measures of body composition (lean body mass) and muscle strength (leg and handgrip strength and power), rather than speed (Fig. 4-6B). Moreover, impairments in rise time, walk time, and get-up-and-go did not reach statistical significance in the high Cu/Zn ratio group. Poorer performance in the SPPB for high Cu/Zn subjects was statistically significant (Fig. 4-7). Subjects in the high PAL group also had significantly diminished muscle strengths and lower SPPB scores near statistical significance (not shown). The only difference in physical performance measures for the low Cu/Zn ratio group was an isolated decrease in handgrip strength (Table 4-3B).

Figure 4-6. Speed and Muscle Strength. (A) Subjects were tested for speed with the get-up-and-go task. Individuals in the high serum Cu group took significantly more time to complete the get-up-and-go task than the reference group. (B) Subjects were also tested for muscle strength by measuring power generated by right leg press. Individuals in the high Cu/Zn ratio generated significantly less power than the reference group.
Overall Health. Measures of overall health included cognitive and emotional battery test scores, blood lipid profiles, hematocrit values, and need for assistance with IADLs and/or ADLs. Some similar trends between the high serum Cu and Cu/Zn ratio groups emerged with these measures (Table 4-4). No significant differences were observed between serum Cu or Cu/Zn ratio groups for cognitive performance (MMSE) or depression scale (CESD) scores. To verify the results of a previous study (Arnal et al., 2010), we made comparisons between Cu/Cp ratio groups (representing non-Cp-bound Cu) in the assessment of cognitive impairment in our cohort. Subjects in the high Cu/Cp ratio group performed more poorly on the MMSE than those in the reference group (Fig. 4-7B), confirming previous reports.

Interesting interactions between serum metals and PAL activity were found for lipid profile measures. No differences in lipid profiles were observed between serum Cu (Table 4-4A) or PHM activity groups. Subjects in the high Cu/Zn ratio group had significantly lower total cholesterol levels and triglyceride levels (Table 4-4B; Fig. 4-8A).
Since triglycerides contribute to total cholesterol levels and no differences were observed for LDL or HDL, the effect of Cu/Zn ratio grouping on lipid profile appeared to be specific to triglycerides. This relationship was carried by significantly lower triglyceride levels in the low Zn group (Fig. 4-8B). There was also a significant reduction in total cholesterol for subjects in the high PAL group (Fig. 4-8C); this effect was carried by a significant reduction in LDL levels rather than triglycerides (Fig. 4-8D).

Table 4-4a. Copper groupings for overall health

<table>
<thead>
<tr>
<th></th>
<th>Low &lt;836.69 ppb (n=26)</th>
<th>Reference 906.44 - 1013.91 ppb (n=27)</th>
<th>High &gt;1193.06 ppb (n=20)</th>
<th>P values Low</th>
<th>P values High</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMSE score</td>
<td>28.4 ± 1.7</td>
<td>27.9 ± 3.1</td>
<td>28.0 ± 1.28</td>
<td>.480</td>
<td>.869</td>
</tr>
<tr>
<td>CESD score</td>
<td>6.1 ± 5.1</td>
<td>7.3 ± 6.0</td>
<td>5.5 ± 4.4</td>
<td>.435</td>
<td>.267</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>192 ± 34</td>
<td>183 ± 47</td>
<td>175 ± 43</td>
<td>.443</td>
<td>.552</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>42 ± 11</td>
<td>44 ± 11</td>
<td>45 ± 10</td>
<td>.604</td>
<td>.666</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>124 ± 30</td>
<td>114 ± 42</td>
<td>111 ± 36</td>
<td>.335</td>
<td>.811</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>152 ± 116</td>
<td>117 ± 55</td>
<td>101 ± 46</td>
<td>.154</td>
<td>.294</td>
</tr>
<tr>
<td>IADL needs assistance % (n)</td>
<td>8 (2)</td>
<td>18 (5)</td>
<td>26 (5)</td>
<td>.245</td>
<td>.528</td>
</tr>
<tr>
<td>ADL needs assistance % (n)</td>
<td>8 (2)</td>
<td>4 (1)</td>
<td>15 (3)</td>
<td>.507</td>
<td>.170</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>43.5 ± 3.2</td>
<td>41.5 ± 5.0</td>
<td>38.5 ± 4.2</td>
<td>.079</td>
<td>.030</td>
</tr>
</tbody>
</table>

Table 4-4b. Copper:Zinc groupings for overall health

<table>
<thead>
<tr>
<th></th>
<th>Low &lt;0.93 &lt;n=24&gt;</th>
<th>Reference 1.12 - 1.27 &lt;n=27&gt;</th>
<th>High &gt;1.51 &lt;n=23&gt;</th>
<th>P values Low</th>
<th>P values High</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMSE score</td>
<td>28.4 ± 1.2</td>
<td>28.1 ± 1.5</td>
<td>28.0 ± 3.4</td>
<td>.510</td>
<td>.879</td>
</tr>
<tr>
<td>CESD score</td>
<td>5.9 ± 5.5</td>
<td>8.8 ± 8.9</td>
<td>6.9 ± 3.9</td>
<td>.176</td>
<td>.348</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>194 ± 40</td>
<td>193 ± 29</td>
<td>173 ± 40</td>
<td>.333</td>
<td>.041</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>44 ± 12</td>
<td>45 ± 14</td>
<td>46 ± 12</td>
<td>.794</td>
<td>.739</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>120 ± 41</td>
<td>120 ± 27</td>
<td>111 ± 31</td>
<td>.981</td>
<td>.318</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>131 ± 115</td>
<td>138 ± 64</td>
<td>84 ± 49</td>
<td>.785</td>
<td>.001</td>
</tr>
<tr>
<td>IADL needs assistance % (n)</td>
<td>4 (1)</td>
<td>11 (3)</td>
<td>32 (7)</td>
<td>.357</td>
<td>.074</td>
</tr>
<tr>
<td>ADL needs assistance % (n)</td>
<td>4 (2)</td>
<td>0</td>
<td>22 (5)</td>
<td>.284</td>
<td>.011</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>43.0 ± 2.9</td>
<td>43.0 ± 3.8</td>
<td>39.0 ± 5.1</td>
<td>.990</td>
<td>.002</td>
</tr>
</tbody>
</table>

**Bold** represents p values < .05. P values were based on independent t-tests comparing mean values between copper level groups.
Hematocrit values were significantly lower in both the high serum Cu and Cu/Zn ratio groups compared to their respective reference groups (Table 4-4; Fig. 4-9A,B). The same significant reduction was evident for the high serum PHM and PAL activity groups (not shown). Homeostatic pathways and Fe mobilization require the activity of Cu-dependent ferroxidases. Thus alterations in Cu levels could affect ferroxidase activity and Fe transport. To determine whether Fe transport was affected in these high metal and PAM activity subjects, we compared serum Fe levels between those groups with...
their respective reference groups. There was a significant reduction in serum Fe for subjects in the high Cu/Zn ratio group (Fig. 4-9C). However, in the high serum Cu, PHM activity and PAL activity groups, no significant decreases in serum Fe were observed. Subjects in these high metal or enzyme activity groups did not have daily dietary Fe intakes that differed from their respective reference groups (not shown), suggesting that the deficit in hematocrit and/or serum Fe does not result from reduced Fe intake. No significant differences in hematocrit were found for the low Cu or low Cu/Zn ratio groups (Table 4-4).

Function and independence were assessed in our cohort by determining whether each subject required assistance with IADLs or both ADLs and IADLs (Table 4-4). A greater proportion of individuals in the high Cu/Zn ratio group required ADL assistance (Table 4-4B); a similar trend was observed for the high serum Cu group, but this increase was not statistically significant (Table 4-4A). Serum PHM and PAL groups were not associated with changes in IADL or ADL assistance (not shown). These data suggest a reduction in independent function in individuals with high serum Cu/Zn ratios that likely stems from impairments in physical function and/or overall health.
**Figure 4-9. Hematocrit.** Individuals in the high serum Cu (A) and the high Cu/Zn ratio (B) groups all had significantly lower hematocrit values than subjects in their respective reference groups. (C) The high Cu/Zn ratio subjects also had significantly lower serum Fe levels than their reference group cohorts.
DISCUSSION

In the present study we found significant relationships between serum metals and PAM enzymatic activity with clinically relevant measures of health and independence in a cohort of frail elderly men. Several recent studies have demonstrated a clear link between increased serum Cu levels and several progressive, degenerative diseases (Arnal et al., 2010; Viktorinova et al., 2009) and increased risk for all-cause mortality in the elderly (Leone et al., 2006; Malavolta et al., 2010). In many of these cases, the Cu/Zn ratio proved to be a better predictor for disease and mortality than levels of either trace element alone. This phenomenon speaks to the tight relationships between homeostatic pathways for various trace metals as well as their respective essential yet potentially dangerous roles in biology. Here, we have provided evidence for deficits in bone health and physical performance associated with high serum Cu/Zn ratios, which may contribute to disease progression and mortality in elderly populations.

Our measures of serum Cu and Zn by inductively coupled plasma mass spectrometry were consistent with previously reported values using similar methods (Easter et al., 2010; Leibovitz et al., 2009; Malavolta et al., 2010). Serum metal values were not related to dietary intake, consistent with the presence of an intact regulatory system in our cohort (Kim et al., 2010b). Even within normal ranges, high serum Cu and Cu/Zn ratio values predicted impairments in bone density and turnover, muscle mass and strength, hematocrit, and activities of daily living. While these relationships cannot implicate any causative effects of high serum Cu or the Cu/Zn ratio, they highlight the importance of serum Cu and the Cu/Zn ratio as well as their potential clinical utility as markers for overall health and independence.
Serum Metals and Enzyme Activities

Consistent with previous reports, serum Cu/Zn ratios appeared to provide more useful information than serum Cu levels alone. Our results support the potential role of the Cu/Zn ratio as a functional and predictive biomarker (Malavolta et al., 2010) and provide evidence that a secreted enzyme may contribute to the pathologies commonly associated with aging.

Since PHM and PAL are derived from the same gene product, with PHM requiring Cu and PAL using Zn, assessment of their serum levels and their associations provided important functional insight regarding these serum trace metals. Levels of both PHM and PAL were correlated with serum Cu, not with serum Zn or the Cu/Zn ratio, suggesting that Cu is a key determinant of serum PAM levels. While studies of PAM processing and secretion in a pituitary tumor cell line identified a regulatory role for copper, it is not yet possible to extend these in vitro studies of one cell type to the in vivo situation (De et al., 2007). Current studies in the lab are aimed at elucidating this interaction using a PAM deficient mouse model (Gaier et al., in preparation).

Specific Measures

The importance of Cu in bone health has been studied in the context of Cu deficiency and osteoporosis (Palacios, 2006; Chaudhri et al., 2009; Lowe et al., 2002). The bone mineral densities of our cohort fit criteria for osteopenia (t-scores between -1.0 and -2.5) for measurements in the femur (neck and trochanter) and radius, but not for total body density. Chaudhri et al. (Chaudhri et al., 2009) found a linear relationship between serum Cu values and bone density in post-menopausal women. We found that our subjects with low serum Cu levels had increased values of bone turnover markers...
(specifically BAP), suggesting ongoing bone loss; however, their bone mineral densities were similar to those of the reference group. Our results disagree, however, with respect to men with high serum Cu levels; high serum Cu and a high Cu/Zn ratio were associated with lower bone mineral densities. Parathyroid hormone elevation and concomitant vitamin D reduction may contribute to progressive bone loss in these groups. Importantly, measures of vitamin D alone were not significantly associated with high serum Cu levels or high Cu/Zn ratios. Thus, serum Cu and the Cu/Zn ratio could serve as a useful biomarker for determining individuals at risk for bone loss in elderly populations. These findings have significant clinical relevance since the head and neck of the femur (sites for several associations in this study) are common sites for bone loss and clinically important fracture in the elderly.

The serum Cu/Zn ratio has been studied in the context of several chronic progressive diseases. A high ratio was found to predict mortality in cardiovascular and cancer patients in several prospective studies with 3.5 to 18 year follow-ups (Leone et al., 2006; Malavolta et al., 2010; Reunanen et al., 1996). In the present study we found a similar trend in physical tasks, but with a clear distinction between deficits associated with high serum Cu levels and Cu/Zn ratios. Elderly men in the high serum Cu group demonstrated deficits in tasks with a stronger cardiovascular component (slower walk and get-up-and-go times), whereas men in the high serum Cu/Zn ratio group displayed more impaired performances in discrete tasks of muscle strength (weaker leg press and hand grip). Men with high serum Cu/Zn ratios also had significantly lower muscle mass (ASM and whole body lean mass) than their cohorts. This curious divergence likely speaks to distinct interactions between Cu and Zn in cardiovascular versus musculoskeletal systems, since quintile comparisons for serum Zn alone revealed no clear trends in physical performance tasks.
Not all of our data were consistent with previous reports. In a cross-sectional study, Mezzeti et al. (Mezzetti et al., 1998) found an association between high serum Cu and LDL and triglycerides that purportedly contributed to the association with cardiovascular disease. We found that a high Cu/Zn ratio predicted lower triglyceride and total cholesterol levels. These conflicting results may reflect a survivor effect in the frail elderly and/or differences in participant selection criteria between studies.

A significantly decreased hematocrit was evident in groups with elevated serum Cu, Cu/Zn ratio, PHM andPAL; thus reduced hematocrit was the most dominant finding of this study. A well-documented symptom of chronic Cu deficiency is anemia that is refractory to Fe supplementation (Uauy et al., 1998;Halfdanarson et al., 2008) and does occur in elderly populations (Carmel, 2008). In contrast, we observed a reduction in hematocrit in the high serum Cu and Cu/Zn ratio groups and not in any of the low metal groups. This reduction in hematocrit was accompanied by a reduction in serum Fe levels in subjects in the high Cu/Zn ratio group. This unexpected result likely reflects the complex interconnected pathways of Fe and Cu homeostasis that extend far beyond Cu-dependent ferroxidases such as ceruloplasmin (Uriu-Adams and Keen, 2005;Uauy et al., 1998;Collins et al., 2010). Subclinical anemia could also contribute to a heightened risk of cardiovascular mortality through long-term increased demand on the heart. Additionally, the poor physical performance of elderly men with high serum Cu and Cu/Zn ratios could arise through inadequate delivery of oxygen to muscle.

Independent of the cause, poor physical performance of men with high Cu/Zn ratios has high clinical and functional relevance. Strength, speed and stability contribute to an individual’s level of independence through his ability to care for himself. Indeed, we found a significantly increased proportion of the men in the high Cu/Zn group required
ADL assistance. This novel finding suggests an increased demand for daily care in individuals with high serum Cu/Zn ratios in addition to risks for disease and mortality. In fact, this parameter alone could contribute to the association between high serum Cu and Cu/Zn ratio with mortality risk through psychological mechanisms. Assuming that serum Cu and the Cu/Zn ratio are important causative factors, intervention to normalize serum Cu and the Cu/Zn ratio may reduce frailty and increase independence and life expectancy.

**Potential Roles for Cu and Zn**

The oxidative properties of Cu and the antioxidant properties of Zn are hypothesized to be influential factors in chronic progressive conditions such as cardiovascular disease (Leone et al., 2006; Reunanen et al., 1996), neurodegenerative disease (Mezzetti et al., 1998; Arnal et al., 2010), and secondary complications of diabetes mellitus (Viktorinova et al., 2009). Many studies consistently report an age-dependent increase in serum Cu, but a concomitant reduction in serum Zn predicts disease in an age-independent manner (Malavolta et al., 2010; Mezzetti et al., 1998). Imbalance of Cu and Zn is thought to result in oxidation of lipids, which in turn compromises the integrity of vascular and neuronal membranes. In combination with other factors, oxidative stress mediated through trace metal imbalances could contribute to the increase in susceptibility to and progression of degenerative diseases with age.

A regulatory system for Cu transport to the heart was recently identified (Kim et al., 2010b). Kim and colleagues generated a cardiac-specific knockout of the major plasma membrane Cu transporter CTR1. In addition to impairments in cardiac function, these transgenic mice had reduced levels of Cu in the liver and increased expression of the Cu transporter ATP7A in the liver and gut. The changes in ATP7A expression were shown
to be mediated through a serological factor. These data also highlight the importance of adequate Cu levels in cardiac function. While the details of this system are still unclear, we can draw the hypothesis that the elderly men in the high serum Cu and Cu/Zn ratio groups may suffer from perturbations in the very same system that results in cardiovascular impairments. Alternatively, cardiovascular impairment may be primary, and high Cu levels represent a reactive engagement of a functioning Cu regulatory system. This regulatory system is surely not exclusive to cardiac function; other organ systems that are heavily reliant on Cu and participate in serum Cu regulation could contribute to the alterations associated with age. Clearly this topic requires further investigation to elucidate the interaction between serum Cu and multiple organ systems, particularly as it relates to elderly populations.

In conclusion, we have characterized a set of significant relationships between serum Cu and Zn, and clinically relevant measures of physical function and independence in frail elderly men. Our findings highlight the importance and potential clinical utility of trace elements as serological markers in identifying at-risk elderly and raise a major question for future study: Can manipulation of Cu and the Cu/Zn ratio alone improve physical performance and independence? Prospective studies will be needed to test whether serum Cu and the Cu/Zn ratio change over time with and without treatment within individuals and to determine the predictive power of serum Cu and Cu/Zn ratios in long-term health.
REFERENCE LIST


CHAPTER 5: Kalirin7 is required for synaptic structure and function

CONTRIBUTIONS

In addition to PAM, our lab studies the physiological roles of Kalirin, a guanine nucleotide exchange factor (GEF). One isoform of Kalirin, Kalirin7, is the predominant form of Kalirin in the brain and localizes to the post-synaptic density of dendritic spines. The role of GEFs in signal transduction combined with the localization of Kalirin7 highly suggested a role for Kalirin7 in synaptic function. Our lab employed a variety of techniques to test this hypothesis. The results of these experiments were published in the Journal of Neuroscience in November, 2008. I conducted electrophysiological recordings in hippocampi of wildtype and Kalirin7 knockout mice, collected and analyzed results, and wrote a section of the paper based on my findings. This section comprised one full figure and one supplemental figure.

After that paper was published, I performed similar recordings from nucleus accumbens medium spiny neurons from wildtype and Kalirin7 knockout mice. Those data have been added to the results section.
ABSTRACT

Rho GTPases activated by GDP/GTP exchange factors (GEFs) play key roles in the developing and adult nervous system. Kalirin-7 (Kal7), the predominant adult splice form of the multifunctional Kalirin RhoGEF, includes a PDZ binding domain and localizes to the postsynaptic side of excitatory synapses. In vitro studies demonstrated that overexpression of Kal7 increased dendritic spine density whereas reduced expression of endogenous Kal7 decreased spine density. To evaluate the role of Kal7 in vivo, mice lacking the terminal exon unique to Kal7 were created. Mice lacking both copies of the Kal7 exon (Kal7\textsuperscript{KO}) grew and reproduced normally. Golgi impregnation and electron microscopy revealed decreased hippocampal spine density in Kal7\textsuperscript{KO} mice. Behaviorally, Kal7\textsuperscript{KO} mice showed decreased anxiety-like behavior in the elevated zero maze and impaired acquisition of a passive avoidance task, but normal behavior in open field, object recognition and radial arm maze tasks. Kal7\textsuperscript{KO} mice were deficient in hippocampal long-term potentiation. Western blot analysis confirmed the absence of Kal7 and revealed compensatory increases in larger Kalirin isoforms. PSDs purified from the cortices of Kal7\textsuperscript{KO} mice showed a deficit in Cdk5, a kinase known to phosphorylate Kal7 and play an essential role in synaptic function. The early stages of excitatory synaptic development proceeded normally in cortical neurons prepared from Kal7\textsuperscript{KO} mice, with decreased excitatory synapses apparent only after 21 days in vitro. Expression of exogenous Kal7 in Kal7\textsuperscript{KO} neurons rescued this deficit. Kal7 plays an essential role in synaptic structure and function, affecting a subset of cognitive processes.
METHODS

Slice Preparation. Mice of 4-5 weeks of age were sacrificed by decapitation and their brains quickly removed in ice-cold artificial cerebral spinal fluid solution (ACSF; in mM): 125 NaCl, 26 NaHCO₃, 10 glucose, 2.3 KCl, 2 CaCl₂, 2 MgSO₄, 1.26 KH₂PO₄ (aerated with 95% O₂ and 5%CO₂; pH=7.3; 310 mOsm/kg) (Zhou et al., 2008b). Coronal slices, 300 µm thick, were allowed to incubate at room temperature for at least 1 hour before recordings.

Whole Cell Recordings. Slices were transferred to a recording chamber heated to 32°C and perfused with aerated ACSF with the addition of 50 µM Picrotoxin (PTX; Sigma-Aldrich) to isolate excitatory neurotransmission. Recording pipettes with 3-5 MΩ tips were filled with internal solution containing (in mM): 135 KGluconate, 10 HEPES, 10 PCreatine, 3 Na₂ATP, 2 MgCl₂, 0.3 Na₂GTP (pH=7.3; 285 mOsm/kg) (Zhou et al., 2008b). Cells were patched under visual guidance using infrared differential interference contrast optics. Data were collected using a Multiclamp 700B amplifier (Axon Instruments/Molecular Devices Sunnyvale, CA) and recorded/analyzed using pClamp 9.2 software (Axon Instruments).

Intrinsic Membrane Properties. Integrity of each patch was assessed through a series of negative and positive steps in current clamp mode (20 x 100 pA steps, -600 pA to +1300 pA; 100 msec steps). The three initial negative steps were used to calculate membrane resistance; single exponential curve fits yielded tau (τ) and capacitance. Action potential threshold was defined as the point of voltage inflection for the first spike fired in the lowest current step. Inter-spike interval (ISI) was assessed in steps eliciting 3 or more spikes and defined as time between peaks of the last two spikes within a step.
Spontaneous Glutamatergic Activity. We recorded spontaneous Excitatory Post Synaptic Currents (EPSCs) while voltage clamped at -70 mV for 1 minute for each cell. Events observed in the full minute were analyzed using MiniAnalysis (Synaptosoft Inc, Chapel Hill, NC). Two outlier cells from each group were excluded from statistical analysis; their event frequency values were far greater than 2 standard deviations above their group mean.

Long Term Potentiation. Bipolar tungsten electrodes (World Precision Instruments, Sarasota, FL) were placed in the stratum radiatum to stimulate Schaffer collateral axons. EPSCs were recorded in voltage clamp mode at -70 mV, with stimulation strength adjusted to obtain 20-50 pA EPSCs at baseline. Test pulses were applied at 0.1 Hz to establish averaged baseline (10-25 pulses) and post-LTP induction EPSC values (25-50 pulses, recorded within 15 min after LTP induction). EPSC amplitudes were measured as the absolute difference between baseline and peak inward current within 50 ms after the stimulus artifact. A small subset of cells was recorded in the absence of PTX, and included in the analysis because their electrophysiological measures were consistent. LTP induction was initiated using Theta Burst Pairing in voltage clamp mode and always within 15 minutes after achieving whole cell configuration. LTP induction consisted of 15 4-pulse bursts at 100 Hz separated by 200 ms intervals, and each burst was paired with a 50 msec depolarizing voltage step to 0 mV. Cells that required greater than 200 µA of stimulation or showed LTP smaller than 120% were excluded from analyses (Kauer and Malenka, 2007). Statistical comparisons were made using Student’s T-test.
RESULTS

Kalirin7KO electrophysiology in hippocampus

To assess the functional consequences of Kal7 knock-out, we performed electrophysiological recordings from acute slice preparations. First, we evaluated passive membrane properties. Capacitance is a measure of cell surface area and dendritic spines contribute 40–70% of the total surface area of hippocampal CA1 pyramidal cells (Harris and Stevens, 1989; Mainen et al., 1996; Inoue et al., 2001). Given the reduction in spine density observed in the CA1 pyramidal neurons of Kal7KO mice, we expected to see slightly reduced capacitance values for Kal7KO versus Wt neurons. Hyperpolarizing and depolarizing current steps were applied to patched CA1 hippocampal neurons to provide measures of membrane resistance and 𝜏 so that capacitance could be calculated (Fig. 5-1A). On average, Kal7KO neurons had higher input resistance (Wt, 122 ± 7 MΩ; Kal7KO, 147 ± 5 MΩ; 𝑝 = 0.008; 32 Wt neurons and 29 Kal7KO neurons) and lower membrane capacitance (Wt, 66 ± 3 pF; Kal7KO, 57 ± 2 pF; 𝑝 = 0.028); time constants did not differ (Wt, 7.6 ± 0.3 ms; Kal7KO, 8.1 ± 0.3 ms; 𝑝 = 0.19).

The resting potential and action potential threshold for knock-out and Wt pyramidal neurons were identical (Fig. 5-2A). In knock-out neurons, the number of action potentials fired during weak depolarizing current steps was slightly increased (Fig. 5-2B), whereas latency to fire the first action potential after step initiation was decreased (Fig. 5-2C); both results are consistent with the decreased membrane capacitance (decreased surface area) observed in Kal7KO neurons. There was no significant difference in interspike interval between the two groups. Because the CA1 pyramidal neurons of Kal7KO mice have fewer dendritic spines, we assessed spontaneous EPSCs (Fig. 5-1B); as predicted, spontaneous EPSC frequency was significantly decreased in Kal7KO neurons. No difference was observed in amplitude (Fig. 5-2). We went on to
Figure 5-1. Electrophysiological deficits. (A) Representative traces from Wt and Kal7\textsuperscript{KO} CA1 pyramidal neurons of membrane responses to positive and negative 200 pA somatic current injections. (B) Composite spontaneous EPSC frequency (\(N = 24\) Wt, 18 KO neurons; \(p = 0.023\); left) and LTP (\(N = 7\) Wt, 13 KO neurons; \(p = 0.0079\); right) data. Error bars indicate SEM. (C) Averaged traces of EPSCs recorded from single CA1 pyramidal neurons from Wt and Kal7\textsuperscript{KO} mice before and within 15 min after theta burst pairing.

compare plastic properties of Wt and Kal7\textsuperscript{KO} synapses. EPSCs, evoked via Schaffer
collateral stimulation, were of similar amplitude and required similar stimulation strengths in Wt and Kal7KO neurons (Fig. 5-2E,F). Taken with the lack of difference in spontaneous EPSC amplitude, these data suggest normal strength of basal synaptic transmission in Kal7KO neurons. To measure LTP, EPSCs were examined before and after applying a TBP paradigm. A single TBP trial resulted in significant LTP that was maintained for at least 1 h in both Wt and Kal7KO slices (Fig. 5-1C). LTP was quantified by comparing EPSC amplitudes within 15 min after the TBP trial to averaged baseline EPSC amplitudes. Compared with Wt neurons, Kal7KO neurons showed markedly blunted LTP (Fig. 5-1B,C). This deficiency in LTP may correlate with the decreased ability to acquire behavioral training, because LTP is typically considered to be one cellular correlate of learning.
Figure 5-2. **Other electrophysiological deficits.** (A) Resting membrane potential and action potential threshold were similar in Wt and KO cells. (B-C) For the 100 pA, 200 pA and 300 pA injections, average values for the number of action potentials in the 100 msec step (B) and latency to fire the first spike (C) are plotted. KO cells only fired significantly more action potentials in the 100 pA step ($p=0.0499$). KO cells fired the first spike significantly sooner in the 200 pA and 300 pA steps ($p=0.0021$ and $p=0.0045$, respectively). (D) There was no significant difference in spontaneous EPSC amplitude. (E) There was no difference in the amplitude of evoked EPSCs recorded for baseline values in LTP experiments. (F) There was no difference in the stimulation strengths used to elicit those responses.
**Kalirin7KO electrophysiology in nucleus accumbens**

To determine whether the synaptic aberrations present in the Kal7KO hippocampus also extend to the nucleus accumbens, a specialized forebrain structure involved in the dopaminergic reward pathway. Involvement of this pathway has been implicated in addiction for several drugs of abuse including cocaine. Kal7KO mice show abnormal responses to cocaine and other members of the lab were investigating this phenotype along with molecular changes in the nucleus accumbens in Kal7KO mice. To gain insight into physiological changes that may contribute to baseline differences in nucleus accumbens function of Kal7KO mice, I recorded membrane and synaptic responses in nucleus accumbens medium spine neurons. Methods for this project were identical to those in the previous section.

Membrane responses to hyperpolarizing and depolarizing current steps elicited changes in membrane potential that yielded quantifiable information regarding passive and active membrane properties of the patched neuron. There were no differences between wildtype and Kal7KO in membrane potential (Fig. 5-3A) or parameters of single action potential kinetics (not shown). Membrane resistances were substantially higher for medium spiny neurons compared to hippocampal pyramidal neurons. There was a significant reduction in the membrane resistance of Kal7KO neurons compared to wildtype (Fig. 5-3B). In contrast to the hippocampus, Kal7KO nucleus accumbens neurons had capacitance values similar to their wildtype counterparts (Fig. 5-3C). Depolarizing current steps elicited action potentials in both medium spiny neurons of both genotypes. The interval between the first two action potentials in the train was significantly longer in Kal7KO neurons compared to wildtype (Fig. 5-4). Notably, this effect was present in both hippocampal CA1 neurons and medium spiny neurons of the nucleus accumbens. Therefore, differences in passive membrane properties cannot
Spontaneous glutamatergic synaptic activity was also recorded in Figure 5-3. Kal7KO nucleus accumbens medium spiny neurons have decreased input resistances. Hyperpolarizing current steps elicited changes in membrane potential and parameters relating to passive membrane properties were quantified. Plots depicting resting membrane potential (RMP) (A), membrane resistance (B), and capacitance are plotted (D). The change in voltage caused by 3 hyperpolarizing steps (-50 to -150 pA) are depicted for each genotype. N = 15 Wt; 15 KO. * indicates p<0.05 between genotypes.
nucleus accumbens medium spiny neurons (Fig. 5-5). Unlike the hippocampus, there was no difference in sEPSC frequency in the nucleus accumbens of wildtype and Kal7 KO neurons (Fig. 5-5A). There was also no difference in the amplitude of sEPSCs (Fig. 5-5B). However, the average decay time (to 37% of the maximum) of sEPSCs recorded from Kal7 KO neurons was prolonged compared to Wt (Fig. 5-5C). This difference also enhanced the charge of Kal7 KO events as assessed by sEPSC area (Fig. 5-5D). This trend in prolonged decay and enhanced charge was also present in sEPSCs recorded from hippocampal neurons, but those changes did not reach statistical significance.

Figure 5-4. Prolonged inter-spike-interval in Kal7 KO neurons is not region-specific. Depolarizing current steps elicited action potentials in neurons of both genotypes. The time between the peaks of the first 2 action potentials within train elicited by 200 and 300 pA 500 ms current steps was measured and compared between genotypes in CA1 of the hippocampus (A) and the nucleus accumbens (B). N = 15 Wt; 14 KO. * indicates p<0.05 between genotypes.
Figure 5-5. Prolonged decay and enhanced charge of spontaneous glutamatergic events recorded from Kal7KO medium spiny neurons. sEPSCs were recorded at $V_{\text{holding}} = -70$ mV in medium spiny neurons in the wildtype and Kal7KO nucleus accumbens. Plots depicting the average frequency (A), amplitude (B), decay time (C) and charge (D) of these events are shown. * indicates $p<0.05$ between genotypes.
DISCUSSION

Kal7 plays an essential role in neuronal function *in vivo*

A series of *in vitro* studies demonstrated that Kal7 is necessary for the formation and maintenance of dendritic spines (Ma et al., 2003; Ma et al., 2008b). We show here that mice lacking Kal7 have deficits severe enough to affect synaptic transmission and specific behaviors. Shank1 and Kal7 are essential for dendritic spine formation *in vivo* and in culture, but Kal7KO produces a greater drop in hippocampal spine density than Shank1 (15 vs 6%) (Hung et al., 2008), making Kal7 an important model for human disease. Mental retardation syndromes are often associated with dysregulation of dendritic spines (Benarroch, 2007; Laumonnier et al., 2007). For example, Patau syndrome (trisomy 13), Down syndrome (trisomy 21), and fragile X all have alterations in the number and shape of dendritic spines (Kaufmann and Moser, 2000; Govek et al., 2004; Grossman et al., 2006). Understanding why a subset of behaviors is affected by loss of Kal7 whereas other learning tasks are not affected should provide new insights into signaling.

Comparing *in vitro* and *in vivo* phenotypes

As observed for other PSD protein knock-out mice (Migaud et al., 1998; Elias et al., 2006; Varoqueaux et al., 2006), the phenotype of the Kal7KO mouse is less severe than would have been predicted from *in vitro* studies. Several factors may contribute to this difference. Most importantly, alternative splicing allows the larger isoforms of Kalirin to accumulate in Kal7KO neurons. Lacking the Kal7 exon, the splicing machinery associated with Kalirin transcripts would be expected to splice exon 33 to exon 34, creating the increased levels of Kal8, Kal9, and Kal12 in total homogenates. The other targeting strategy considered, insertion of a stop codon within the Kal7 exon preceding the PDZ
binding motif, would have generated a truncated product, complicating analysis in a different manner. Because the Sec14p and spectrin-like repeats target Kalirin to membranes, the nine copies of Kal7 present at the “typical” synapse in wild-type mice may be replaced by six copies of these larger Kalirin isoforms, which could help preserve excitatory synapses and alter synaptic function in a region- and context-specific manner.

Recent studies assigning an essential role for Kal7 in activity-dependent activation of the Rac and N-cadherin/afadin pathways used an shRNA directed against the spectrin repeat region (nucleotides 1229–1250; spectrins 2–3) (Xie et al., 2007; Xie et al., 2008), reducing expression of all major forms of Kalirin, not just Kal7. Although this approach led to the conclusion that Kal7 regulates the GluR1 content of pyramidal neuron dendritic spines and AMPA receptor-mediated synaptic transmission (Xie et al., 2007), the GluR1 content of PSDs purified from Kal7KO neurons is indistinguishable from wild-type. Selective elimination of Kal7 using antisense or shRNA targeted to its unique 3′-untranslated region (Ma et al., 2008b) produced a more profound decrease in spine density (approximately two-fold) than observed in cultures prepared from Kal7KO mice. A Kal7-specific shRNA would not be expected to increase levels of the larger kalirin isoforms; in addition, breakdown products generated from Kal7 transcripts targeted by the shRNA could contribute to the effects observed (Kim and Rossi, 2008).

**Spine and plasticity analysis**

Ultrastructural analyses demonstrated a substantial decrease in the number of synapses (PSD with presynaptic terminal) in CA1 hippocampal neurons from Kal7KO mice. Our cell culture data showed a similar decline in synapses assessed as Vglut1/PSD-95 clusters. Golgi staining revealed a less dramatic decrease in linear spine density. Although
Vglut1-positive terminals in wild-type neurons usually align with PSD-95 clusters, this strict association is lost in Kal7KO neurons. Neither unpaired spine-like structures nor unpaired presynaptic endings would be counted by EM analysis. The PSDs in Kal7KO mice were narrower and thinner than in wild-type mice. These changes in PSD dimensions were consistent and comparable with changes seen in Shank1KO mice (Hung et al., 2008).

Unlike pyramidal neurons in the hippocampus, medium spiny neurons in the nucleus accumbens have normal dendritic spine densities. The electrophysiological data presented here, including normal capacitance and sEPSC frequency, are consistent with this in distinguishing nucleus accumbens recordings from those in the hippocampus. Thus these electrophysiological studies in Kal7KO mice substantiate the biochemical analyses in these respective brain regions.

Tracking the development of synapses in cortical cultures prepared from Kal7KO mice was revealing. Both in vivo and in culture, synapses begin to form before Kal7 is expressed. Synaptic development proceeded normally for 21 d in the absence of Kal7; Vglut1-positive presynaptic endings contacted dendritic shafts aligned with PSD-95 clusters. Although spine density increased between DIV21 and DIV28 in wild-type neurons, no increase was observed in Kal7KO neurons; it is not clear what distinguishes the spines formed between P21 and P28. Kal7 may play an essential role in the maturation and/or maintenance of dendritic spines, as proposed for the neuroligins (Varoqueaux et al., 2006). Consistent with the conclusion that Kal7 has a role late in synaptic development, no differences in LTP were apparent when slices from P21 and younger mice were examined. When synaptic plasticity was tested in Kal7KO mice older than P28, deficits were apparent.
Although PSD length and width were reduced in Kal7<sup>KO</sup> mice, the effect was small, consistent with our inability to see dramatic changes in PSD content of a number of proteins. Indeed, similar results have been seen with other PSD-protein knock-outs. Ablation of PSD-95 caused no change in spine volume, but made spines longer and thinner and decreased the number of AMPA receptors (Beique et al., 2006; Elias et al., 2006). Knock-out of Shank1, which caused a decrease in spine number and PSD size, decreased levels of GKAP (guanylate kinase-associated protein) and Homer and weakened basal synaptic transmission, leaving synaptic plasticity unaltered (Hung et al., 2008).

**Potential mechanisms**

Determining how the absence of Kal7 causes these changes in spine number and synaptic function will require detailed analysis of the deficits. Kal7<sup>KO</sup> PSDs contained less Cdk5 and less NR2B than wild-type PSDs. Cdk5 plays a complex and important role in postsynaptic signaling and architecture (Benavides and Bibb, 2004; Cheung et al., 2006; Cheung and Ip, 2007) and the decrease in Cdk5 may contribute to many of the changes observed. For example, the GEF activity of Kal7 is increased after Cdk5-catalyzed phosphorylation of Thr<sup>1590</sup> (Xin et al., 2008). Although Kal7 with either a T<sup>1590A</sup> or T<sup>1590D</sup> mutation caused spine formation when expressed in rat cortical neurons, spine morphologies differed (Xin et al., 2008). Based on the fact that dominant-negative Cdk5 blocked the ability of Kal7 to affect PC12 cell morphology, this interaction appears to play a critical role in Kalirin function.

Like Kal7, the Cdk5/p35 complex affects Rac/Pak signaling (Nikolic et al., 1998), which alters cytoskeletal dynamics in dendritic spines (Tashiro et al., 2000). Through its effects on ephrins, the decreased Cdk5 in Kal7<sup>KO</sup> PSDs may contribute to the dearth of dendritic
spines. Cdk5 plays a role in EphA-dependent spine retraction (Fu et al., 2007), whereas Kal7 plays a role in EphB-dependent spine maturation (Penzes et al., 2003). Cdk5 phosphorylates PSD-95, regulating its clustering (Morabito et al., 2004), which might contribute to the changes in PSD size and shape seen in Kal7KO mice. Importantly, Cdk5 plays a role in the phosphorylation of NR2B, resulting in its stabilization in the membrane (Zhang et al., 2008). The decreased levels of Cdk5 in Kal7KO PSDs may cause the decreased levels of NR2B. Additional studies are underway to determine how Kal7 and Cdk5 interact to contribute to the changes observed in Kal7KO mice.

**Kal7 is essential for specific memory processes**

Given the spine changes seen in mental retardation and the role of dendritic spines in memory formation (Benarroch, 2007), it is not surprising that Kal7KO animals exhibit learning deficits. Although numerous knock-out mice have demonstrated deficits in hippocampal learning processes, few have revealed disparities in appetitive spatial and aversive contextual hippocampal-dependent learning paradigms (Bach et al., 1995; Hung et al., 2008). It has been posited that different mechanisms underlie single-trial versus more gradual repetitively learned tasks (Bach et al., 1995; Hung et al., 2008). The Kal7KO mice were normal in nonaversive tests of learning and memory (object recognition, radial arm maze), but abnormal in tests of anxiety and fear learning (elevated zero maze, passive avoidance). Perhaps specific proteins or signaling pathways are essential for the formation/function of synapses necessary for particular types of learning (e.g., fear learning) but not others (e.g., spatial learning). The hypothesis that specific synapses or molecular pathways underlie different forms of hippocampal memory was put forth previously (Bach et al., 1995), but has received little additional investigation. Future behavioral and neurochemical mapping studies of Kal7KO and Kal7CKO mice will help clarify the role of different spine proteins in different types of learning.
**Kal7 plays a key role in synaptic plasticity and in human psychiatric conditions**

Kal7 is the only RhoGEF specifically trafficked to the PSD (Sheng and Hoogenraad, 2007) and the only RhoGEF identified in the complex of NR2B-associated proteins (Collins et al., 2006). Decreased levels of Kal7 were observed in postmortem cortices from schizophrenics and Alzheimer disease patients (Tataki et al., 2005; Hill et al., 2006; Youn et al., 2007). In addition to the present findings, preliminary studies with Kal7\(^{\text{KO}}\) mice revealed aberrant responses to drugs of abuse and additional deficits in synaptic plasticity and cognitive function. Kal7\(^{\text{KO}}\) mice have now emerged as a valuable model for understanding synaptic malfunctions and human psychiatric disorders.
REFERENCE LIST


CHAPTER 6: Future directions
Demonstrating causality of Cu mishandling in PAM+/− emotional dysregulation

The data presented in Chapter 3 strongly suggest that alterations in Cu homeostasis underlie the PAM+/− deficits in emotionality, learning and memory. Through the pursuit of these mechanisms, we have uncovered several novel and interesting aspects of Cu usage in the brain. Moreover, we have shown that region-specificity and subcellular distribution of molecules that bind and transport Cu across membranes are likely to be important to normal neuronal/synaptic function with alterations resulting in behavioral consequences. While PAM+/− mice exhibit abnormal central and peripheral Cu handling (Chapter 3)(Bousquet-Moore et al., 2010b), a direct link between these abnormalities and the physiological and behavioral deficits observed in these mice has yet to be shown. Possible alternative explanations include abnormal neuropeptide signaling, developmental abnormalities, and disruptions of catecholaminergic signaling. One important future direction is to test the hypothesis that there is a causal relationship between disrupted Cu handling and the neurophysiological and behavioral dysfunction. To do this, PAM and other molecules essential to Cu handling must be selectively targeted.

One effective contemporary approach is conditional knockout of PAM and ATP7A using the Cre/lox system. By flanking an early and essential exon of a gene with loxP sites, expression of that gene can be selectively targeted for deletion with great experimental control. Crossing heterozygote or homozygote floxed animals with transgenic mice expressing Cre under cell- or tissue-specific promoters will selectively target the gene of interest. Alternatively, injection of a virus encoding Cre recombinase into specific brain regions can selectively target floxed genes. Behavioral and physiological comparisons
between targeting conditions within floxed mice will demonstrate specific causal relationships.

One interesting avenue for this approach involves conditionally knocking out ATP7A expression in bilateral basolateral amygdalae via viral-mediated Cre expression driven by the CamKII or GAD65 promoter. This should selectively impair the ability of pyramidal neurons or interneurons of the basolateral complex to secrete Cu through an ATP7A-dependent process. Based on the data presented in Chapter 3, we expect that impairing Cu secretion will abolish LTP at thalamic amygdalar synapses and impair cue-dependent fear conditioning. It will be interesting to see whether this effect arises from targeting the ATP7A knockout to pyramidal neurons or interneurons.

Another advantage of using the viral-mediated knockout system is that it provides the ability to make within subject comparisons. Animals could be run through two different discrete fear learning tasks (e.g. one involving a light and the other a tone). Mice could receive an infusion of virus encoding Cre recombinase into the amygdala before the second test and performance compared to mice that had completed the task before virus injection. This type of analysis provides substantial power in determining causality, assuming the proper controls are performed. A pharmacological approach to these studies could involve acute injection of the Cu-specific chelator BCS into the amygdala prior to fear conditioning. Based on our slice recordings, we predict that ATP7A knockout or BCS injection in the amygdala will impair the expression of fear associations learned after treatment and spare those learned before.

Furthering our understanding of Cu signaling in the amygdala is highly pertinent to several neurological and psychiatric disorders in humans including epilepsy, anxiety, post-traumatic stress disorder (PTSD), Alzheimer’s disease, and prion-related diseases.
In the case of PTSD, the role of Cu in amygdala memory formation could be taken to a clinical level. It has been proposed by others that pharmacological agents could be used during therapy sessions to target synapses representing the traumatic memory. During recall, these synapses are temporarily rendered labile. Cu chelators, such as trientine or disulfiram which are already FDA approved for use in Wilson’s disease and alcohol dependence, respectively, could be candidates for one of these therapeutic agents for PTSD if the connection between amygdalar Cu and fear learning and memory can be further validated (see above).

**Does amygdalar Cu go beyond fear?**

We have shown that amygdalar Cu is important for fear-related behaviors and anxiety-like behavior (Chapter 3), but this is only one face of amygdalar function. The amygdala also plays essential roles in mediating appetitive behaviors, reward and social interactions. Autism spectrum disorders comprise a heterogeneous class of CNS illnesses with neurological and psychiatric manifestations that are often associated with deficits in learning/memory, emotionality and social interactions. While many studies have asked whether alterations in amygdalar function are associated with autism, few have explored a connection with Cu homeostasis. A connection between autism and oxidative stress has been proposed (Chauhan and Chauhan, 2006), and the ratio of serum Cu to Zn is elevated in autistic patients (Faber et al., 2009). One study suggested that Cu levels are elevated in the hair of autistic patients, but a more recent meta-analysis disproved this assertion (De Palma et al., 2011). Probing for changes in the periphery or at the level of the whole brain will not necessarily yield relevant information. For example, PAM^+/- mice do not show altered levels of total brain Cu but clearly display region-specific alterations in Cu homeostasis with profound behavioral correlates
(Chapters 2 and 3). In addition, given the broad spectrum and multifactorial pathogenesis of autism spectrum disorders, it is difficult to rule out a possible connection with amygdalar Cu that may be relevant to a subset of autistic cases.

Social interaction behavioral testing is simple to perform, and properly done analyses provide objective measures. Evaluation of social interaction in PAM\(^{+/−}\) mice and/or in a model of amygdala-specific knockout of ATP7A would yield valuable information regarding the role of amygdalar Cu in mediating social interactions. Again, the pharmacological approach of using BCS injection directly into the amygdala just prior to testing could substantiate findings made in transgenic models. The hypothesis is that mice with deficient Cu signaling in the amygdala will show reductions in social interaction behaviors.

**Altering Cu homeostasis with neuronal excitation**

Studies in cultured neurons demonstrated that calcium influx in response to neuronal activity induced up-regulation of ATP7A and shifted ATP7A into dendrites (Schlief et al., 2005; Dodani et al., 2011). This phenomenon has only been demonstrated in cultured neurons, never in vivo. Seizure activity induced by electroconvulsive shock should produce extensive, broad and coordinated neuronal activation. Mice sacrificed 1, 3, 6 and 12 hours after shock could be analyzed in order to determine whether a similar response occurs in vivo and its time-course. Based on in vitro data, we would expect ATP7A mRNA levels and the ATP7A protein levels to increase with shock. It may also be possible to observe insertion of ATP7A into the PSD of synapses at earlier time-points, perhaps allowing synaptic release of Cu.
This same experimental design could be extrapolated to a behaviorally-relevant model. Specifically, shifts in ATP7A expression and localization may be detectable in the amygdala following fear conditioning. It may also be interesting to determine whether PAM heterozygosity affects the response of ATP7A expression and localization to shock or fear training. We would expect fear conditioning to affect ATP7A expression and localization in the same way as ECS. Whether PAM\(^{+/−}\) ATP7A is affected in the same way will also provide valuable insight into how PAM\(^{+/−}\) amygdala synapses are altered. Data from PAM\(^{+/−}\) cortex suggests that ATP7A is abnormally distributed at baseline. A lack of ATP7A response in the amygdala to fear conditioning could indicate abnormalities in mechanisms upstream that trigger changes in ATP7A. The number of neurons incorporated into a fear memory trace has been estimated to be at or below 20% (Reijmers et al., 2007; Zhou et al., 2009). Admittedly, detection of these changes in a small subset of amygdala neurons may not be possible using these methods. Stronger fear conditioning training protocols may be needed to elicit detectable changes at the level of the whole amygdala.

**Elucidating the essential role of Cu at amygdala afferent synapses**

Inclusion of BCS in the perfusate completely eliminated LTP induced by AP pairing in the amygdala independent of GABAergic transmission (Chapter 3). The AP pairing induction protocol used in these studies was previously shown to be VGCC-dependent and NMDA receptor-independent (Bauer et al., 2002). It would be interesting to determine whether BCS also abolishes NMDA receptor-dependent LTP. This could be tested using a theta burst stimulation paradigm. PAM\(^{+/−}\) mice are deficient in theta burst-induced LTP at thalamic afferent synapses of the amygdala (Chapter 2). Consistent with our hypothesis that the PAM\(^{+/−}\) phenotype results from impaired Cu signaling, we would
expect that NMDA receptor-dependent LTP is impaired bath application of BCS. On the other hand, NMDA receptor function is inhibited by Cu (Schlief et al., 2006), so removal of endogenous extracellular Cu may enhance NMDA receptor-dependent LTP.

Shin and colleagues (Shin et al., 2010) recently characterized two induction protocols that selectively induce pre- and post-synaptic forms of LTP. With different mechanisms of LTP expression, these two induction protocols should be extremely helpful in narrowing down the LTP signaling pathway for which Cu is required.

Several signaling pathways have been characterized at thalamic afferent synapses important for LTP induction and maintenance. These include cAMP-PKA signaling (Huang and Kandel, 1998) and NO-cGMP-PKG signaling (Ota et al., 2008) pathways. Cell-permeable stable analogs of cAMP and cGMP, 8-Br-c(A/G)MP, can lower the threshold for LTP at amygdala afferent synapses when present in the perfusate. It would be interesting to test whether these analogs have the same effect in the presence of BCS. If the efficacy of one is impaired with BCS, it would suggest that Cu signaling is involved in that signaling pathway and not the other. If the efficacy of both analogs is impaired with BCS, it would suggest that secreted Cu is involved in the initiating signals and/or induction of LTP upstream of cyclase activation.

MMP9 and BDNF may also be involved in LTP induction through a mechanism involving Cu (Chapter 1). Previous studies connecting Cu to MMP9 and BDNF were performed in the hippocampus, but BDNF signaling is also known to play a role in amygdala-dependent learning and memory (Rattiner et al., 2005). MMP9 and BDNF are sufficient to induce LTP in the hippocampus. If extracellular Cu is required for MMP9- and/or BDNF-induced LTP, neither compound will be active in the presence of BCS. Assuming the mechanism of MMP9/BDNF-dependent LTP in the amygdala is similar to the
hippocampus, we would expect MMP9-LTP to be sensitive to BCS and BDNF-LTP not to be sensitive. This result would confirm an essential role for Cu in facilitating MMP9 activity, thus enhancing BDNF cleavage, release and receptor binding (see Chapter 1).

Assessment of hippocampal function in PAM^{+/−} mice

Neurophysiological and behavioral testing of PAM^{+/−} mice has focused on the amygdala up to this point. No behavioral tests have been conducted to directly assess the status of hippocampal function in this mouse model. PAM^{+/−} mice are deficient in contextual fear conditioning, a behavioral learning task that requires the hippocampus. The majority of neurophysiological studies concerning Cu have been performed in hippocampal neurons. Thus information regarding the status of PAM^{+/−} hippocampal function could be interpreted in the context of a broad foundation of knowledge regarding potential neurophysiological mechanisms.

Tests of hippocampal function typically involve components testing spatial learning and memory. Tests such as the Barnes and Morris water mazes use negative reinforcement to motivate the animal to a “safe” area. In the Barnes maze, mice are motivated to escape an open area through holes surrounding the open area. The animals must use spatial memory to recognize which holes lead to a drop box to escape more efficiently. In the Morris water maze, mice are placed in murky water and forced to swim until they reach a hidden platform. The mice must rely on spatial cues to find the platform faster on subsequent days. Importantly, the anxiety-like phenotype of PAM^{+/−} mice (Chapter 2) may confound the animal’s performance in these tasks through a stronger motivation to escape the open area or find the platform in the Barnes and Morris mazes, respectively.
Thus hippocampal tests that do not use negative reinforcement are better suited to testing hippocampal function in PAM^{+/-} mice.

The radial arm maze should allow evaluation of hippocampal function in PAM^{+/-} mice. In this task, mice must find food at the end of discrete arms of the maze labeled with different symbols. Mice must use these symbols to locate the food more efficiently. Food provides positive reinforcement to motivate animals to learn. However, it is important to show that hunger is an equally motivating factor for PAM^{+/-} and wildtype mice. In an unrelated experiment not mentioned in this body of work, I tested feeding and satiety in PAM^{+/-} mice. Since many neuropeptides, including vasoactive intestinal peptide and cholecystokinin, are involved in signaling hunger and satiety in mammals, I hypothesized that hunger and satiety signaling would be altered in PAM^{+/-} mice. I removed wildtype and PAM^{+/-} littermate females from their normal ad libitum feeding schedule and gave them free access to food for only 1 h per day. I monitored the weight of each animal and the food consumed over the 5 days on the diet. All mice lost weight and consumed more food each day as the experiment progressed. PAM^{+/-} weight loss and food consumption over the 5 days were indistinguishable from that of wildtype mice, suggesting normal hunger and satiety signaling in PAM^{+/-} mice. These results serve as an important control for testing hippocampal function of PAM^{+/-} mice in the radial arm maze.

If radial arm maze performance is impaired in PAM^{+/-} mice, it would suggest that the synaptic plasticity deficits in the amygdala that correlate with fear learning and memory deficits can be extrapolated to hippocampal synapses. If not, it suggests that normal PAM levels are not required for normal hippocampal function. In either case, synaptic plasticity should be assessed at Schaffer-CA1 synapses using NMDA receptor-
dependent and -independent LTP induction protocols. The role of endogenous secreted Cu in hippocampal LTP should also be tested using bath-applied BCS (Chapter 3).

**Uncovering the mechanism underlying the anxiolytic effects of TRH**

During the summer of 2009, I hosted 3 students from Farmington High School in our lab. We were to complete a small project for them to present at the end of two weeks. I chose a simple project aimed at investigating the effect of an amidated peptide, thyrotropin releasing hormone (TRH; pyro-Glu-His-Pro-NH$_2$) on GABAergic transmission in the amygdala. Behaviorally, TRH has a potent anxiolytic effect in mammals (Yarbrough et al., 2007; Gutierrez-Mariscal et al., 2008) and TRH receptor 1 deficient mice exhibit increased anxiety-like behavior (Zeng et al., 2007). However, the neurophysiological effects of TRH in the amygdala, a region of the brain involved in anxiety-like emotional behaviors, have never been investigated and published. Only one study investigated the effects of TRH on limbic GABAergic signaling; TRH enhanced GABAergic signaling in the hippocampus (Deng et al., 2006).

In the summer project, we recorded spontaneous and evoked IPSCs in BLA pyramidal neurons as 1 or 10 µM TRH was applied through the perfusate. We found a robust enhancement of evoked IPSC responses when TRH was applied. These experiments were performed in a small number of neurons and were never replicated. A proper study of the effects of TRH on synaptic transmission and membrane excitability would be simple to complete and would provide valuable insight into the potential neural mechanisms underlying TRH-mediated anxiolysis.

Whole-cell patch clamp recordings should focus on pyramidal neurons of the BLA. Membrane properties can be tested through episodic current step application to initiate
action potential firing. A sensitive way to monitor synaptic function is by recording at $V_{\text{holding}} = -35$ mV while stimulating afferent fibers. This allows simultaneous monitoring of excitatory and inhibitory signals. Lidocaine should be added to the pipette solution to avoid synaptically evoked voltage-gated Na currents. The stimulating electrode should be placed in the LA to record intra-amygdaloid signaling. In separate experiments monitoring membrane or synaptic transmission, TRH should be bath applied as it was previously. These two experiments should be very sensitive to any effects of TRH. Additional experiments would be needed to pursue the effects of TRH on particular currents in the case of membrane excitability or particular synapses in the case of synaptic function.