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Pam heterozygous mice reveal essential role for Cu in amygdalar behavioral and synaptic function

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Abstract

Cu is an essential element with many biological roles, but its roles in the mammalian nervous system are poorly understood. Mice deficient in the cuproenzyme peptidylglycine α-amidating monooxygenase ($PAM^{+/-}$ mice) were initially generated to study neuropeptide amidation. $PAM^{+/-}$ mice exhibited profound deficits in a few behavioral tasks, including enhancements in innate fear along with deficits in acquired fear. Interestingly, several $PAM^{+/-}$ phenotypes were recapitulated in Cu restricted wildtype mice and rescued in Cu supplemented $PAM^{+/-}$ mice. These behaviors correspond to enhanced excitability and deficient synaptic plasticity in the amygdala of $PAM^{+/-}$ mice, which are also rescued by Cu supplementation. Cu and ATP7A are present at synapses, in key positions to respond to and influence synaptic activity. Further study demonstrated that extracellular Cu is necessary for wildtype synaptic plasticity and sufficient to rescue $PAM^{+/-}$ LTP. These experiments support roles for PAM in Cu homeostasis and for synaptic Cu in amygdalar function.

Keywords

peptidylglycine alpha-amidating monooxygenase; copper; amygdala; fear conditioning; synaptic plasticity; ATP7A

Cu and PAM

Biologically, Cu is best known as a cofactor in oxidation-reduction reactions[1]. Only a handful of enzymes that require Cu for their activity are encoded by the human genome, yet these “cuproenzymes” are diverse in their biological roles[2–5]. Cu deficiency can be caused by mutation in the gene encoding the intracellular Cu transporter ATP7A and results in Menkes disease[6–8]. Menkes disease patients have low levels of brain Cu, and typically present in infancy with failure to thrive and seizures. Symptoms of Menkes disease are thought to correspond to insufficient activity of specific cuproenzymes; however the pathophysiology leading to seizures is not well understood[9].

Peptidylglycine α-amidating monooxygenase (PAM) is a vesicular transmembrane protein with two luminal enzymatic domains, one of which is a cuproenzyme[10]. Peptidylglycine α-

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hydroxylating monooxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL) act sequentially to catalyze the cleavage of the C-terminal glycine of their peptidylglycine substrate to yield an amidated product peptide[11,12]. The PHM domain requires 2 Cu molecules for its activity[13], while PAL uses Zn[14]. PAM is the only enzyme in mammals that can accomplish this amidation reaction[15,16], which is essential to the biosynthesis of approximately half of all neuropeptides; α-amidation is the final step required to confer full biological activity to many neuropeptides[12,17].

PAM function is critical for development and nervous system function [16,18]. Aside from their role in multiple endocrine systems, peptides can also act as neurotransmitters and neuromodulators in the nervous system to regulate neuronal and synaptic physiology and function[19]. Amidation is impaired in animal models of Menkes disease[20]; levels of key neuropeptides are reduced with brain region-specificity[21].

PAM$^{+/-}$ mice and fear

To study the function of PAM in animals, our group developed a mouse genetically deficient in the gene encoding PAM. Mice homozygous for the deleted *Pam* gene die in utero with profound edema[16], a phenotype nearly identical to a knockout model of adrenomedullin, an amidated peptide[22]. By contrast, mice heterozygous for the gene deletion grow and reproduce normally, but exhibit a striking set of behavioral and physiological deficits when challenged (Table 1). PAM$^{+/-}$ mice and their wildtype littermates are bred from wildtype dams and PAM$^{+/-}$ sires for use in all experiments (Fig. 1A). PAM$^{+/-}$ mice cannot maintain their body temperature in the cold, secondary to impairments in vasoconstriction[23]. When injected with the $GABA_A$ receptor antagonist pentylenetetrazol, PAM$^{+/-}$ mice have more severe seizures at lower doses than their wildtype littermates[24]. When tested in fear-related tasks, PAM$^{+/-}$ mice display an interesting dichotomy of behaviors[24,25].

Innate fear in rodents is typically tested in the elevated zero maze (Fig. 1B). In this task, animals are placed onto an elevated circular platform with four segments, half of which have tall walls (closed) and the other very low walls (open)[26]. Rodents instinctually spend more time in the closed regions due to an innate fear of open spaces, a behavior that likely evolved to avoid predation. The amount of time spent in the open regions can be used to quantify the degree of innate fear for comparison between different genetic and/or pharmacologic conditions. PAM$^{+/-}$ mice placed in the elevated zero maze spend significantly less time in the open areas than their wildtype littermates[24,25]. Anxiolytic medications increase the proportion of time animals spend in the open areas, owing to a reduction in innate fear. Interestingly, PAM$^{+/-}$ mice respond to low doses of diazepam, a $GABA_A$ receptor allosteric modulator and anxiolytic, by increasing the amount of time spent in the open regions while wildtype mice are unaffected[25]. Thus PAM$^{+/-}$ mice show enhanced innate fear behavior and sensitivity to a GABAergic anxiolytic in the elevated zero maze.

Acquired fear, or fear learning and memory, can be assessed in many ways. One commonly used method is fear conditioning, which tests an animal's ability to associate a previously neutral stimulus (conditioned stimulus) with a footshock (unconditioned stimulus) (Fig.

1C)[27]. Two types of fear conditioning are typically tested. In contextual fear conditioning, the conditioned stimulus is the environment, consisting of the smell, shape and texture of the conditioning chamber. In cued fear conditioning, a single discrete auditory tone serves as the conditioned stimulus. Cued testing occurs in a different chamber where the exact same tone can be played. The conditioned and unconditioned stimuli are presented together during training, and the conditioned stimulus is presented alone during testing. Subsequent presentation of the conditioned stimulus causes the animal to outstretch the front paws and cease all motion aside from breathing, a readily quantifiable response referred to as "freezing". The portion of time the animal spends freezing can be used as a direct measure of fear learning and memory.

$PAM^{+/-}$ mice freeze for less time than their wildtype littermates during both contextual and cued testing, indicating a deficiency in both forms of fear conditioning[25]. $PAM^{+/-}$ mice are also deficient in another fear learning and memory task, fear-potentiated startled. These results stand in stark contrast to the enhanced innate fear we observe in $PAM^{+/-}$ mice. Other paradigms aimed at manipulating neuropeptidergic signaling, including specific peptide knockouts, generally have similar effects on both innate and learned fear[28–33]. This paradox between $PAM^{+/-}$ innate and learned fear behaviors intrigued us and led us to perform physiological and biochemical experiments aimed at understanding amygdalar function in $PAM^{+/-}$ mice.

$PAM^{+/-}$ amygdalar physiology

The amygdala is a part of the brain's limbic system situated in the medial temporal lobe in mammals and plays a role in both innate and learned fear behaviors (Fig. 2A)[34]. Manipulation of neuronal activity in the amygdala has direct effects on innate fear responses[35–37]. Amygdala function is required for both cued and contextual forms of fear conditioning; structural ablation or pharmacological blockade of synaptic transmission in the amygdala before or during, but not after, fear training abolishes learned fear responses[27].

With the goals of elucidating the neurobiological underpinnings of the $PAM^{+/-}$ paradoxical fear behaviors, we performed whole cell patch clamp recordings in the basolateral complex of the amygdala. Baseline excitatory synaptic transmission is enhanced in the $PAM^{+/-}$ amygdala, and $PAM^{+/-}$ amygdalar neurons are hyperexcitable when compared to wildtype amygdalar neurons[25]. Interestingly, baseline inhibitory synaptic transmission is also increased, which may relate to the observed heightened sensitivity to benzodiazepines. These findings may relate to the increased innate fear of $PAM^{+/-}$ mice.

Long-term potentiation (LTP) at thalamic afferent synapses in the lateral nucleus of the amygdala is a well-accepted and extensively studied correlate to cued fear conditioning in mice (Fig. 3)[38–40]. We induce LTP in acute brain slices prepared from wildtype and $PAM^{+/-}$ mice by pairing thalamic afferent stimulations with post-synaptic depolarization to trigger action potentials in single neurons in the presence of the $GABA_A$ receptor blocker picrotoxin[25,41] [as is necessary in these types of experiments[42]]. After induction, synaptic responses in $PAM^{+/-}$ neurons fail to increase above baseline, and are significantly lower than those recorded in wildtype neurons. This result corresponds to the deficit in cued fear

memory[25](Gaier et al, resubmitted). Notably, full blockade of GABAergic transmission with the addition of a $GABA_B$ receptor antagonist (CGP35348) restores $PAM^{+/-}$ LTP to wildtype levels (Fig. 3). It was initially hypothesized that these behavioral and physiological deficits arose from impairments in amidation of specific neuropeptides; however, for the few peptides examined, only modest reductions in amidation have been observed in $PAM^{+/-}$ mice[16,43].

Cu rescue of $PAM^{+/-}$ phenotypes

Knowing that PAM requires Cu for its catalytic activity, we manipulated dietary Cu in wildtype and $PAM^{+/-}$ mice and performed the same behavioral and physiological experiments. Mild dietary Cu restriction (Cu depleted food for 4–6 weeks) in wildtype mice impairs thermoregulation, lowers seizure threshold and promotes anxiety-like behaviors very similar to $PAM^{+/-}$ mice at baseline (Table 1)[24]. Cu restriction has little to no effect on the $PAM^{+/-}$ phenotype. On the other hand, dietary Cu supplementation (300 ppm in the drinking water) in $PAM^{+/-}$ mice restores thermoregulation, ameliorates the anxiety-like behaviors, and rescues cued fear conditioning and fear potentiated startle [24](Gaier et al, resubmitted). Contextual fear conditioning and seizure susceptibility are the only behaviors examined that are not affected by dietary Cu supplementation in $PAM^{+/-}$ mice. Importantly, contextual fear conditioning tests an animal's ability to integrate discrete sensory modalities, a task that requires the hippocampus, in addition to forming simple associations as in cued conditioning, which requires the amygdala, but not the hippocampus[44]. The difference in the ability of Cu to rescue only select fear memory tasks could reflect differences in the nature of the aberration in the $PAM^{+/-}$ amygdala and hippocampus. The hippocampus is also a very epileptogenic brain region and is likely involved in the seizure susceptibility of $PAM^{+/-}$ mice. That Cu supplementation distinguishes abnormal behaviors involving the hippocampus and amygdala strong suggests distinct aberrations in these brain regions and that $PAM^{+/-}$ amygdalar dysfunction is directly related to Cu.

We also tested the ability of Cu to rescue amygdalar LTP. Wildtype and $PAM^{+/-}$ mice were supplemented with dietary Cu, exactly as in the behavioral experiments, and recordings were performed on slices prepared from these mice. Dietary Cu supplementation restores $PAM^{+/-}$ LTP to normal levels and has no effect on wildtype LTP (Fig. 3; Table 1)(Gaier et al, resubmitted). These results draw a clear parallel between behavior and physiology in which the $PAM^{+/-}$ phenotype is rescued by dietary Cu. Interestingly, dietary Cu supplementation has no effect on peptide amidation, strongly arguing against our initial hypothesis that deficiencies of various amidated neuropeptides accounts for the $PAM^{+/-}$ phenotype[24,43]. Rather, these results suggest a role for Cu itself in mediating the effects of *Pam* heterozygosity and dietary rescue.

ATP7A and Cu at synapses

At this point we had gathered evidence that a deficiency in Cu in the $PAM^{+/-}$ amygdala could contribute to the synaptic impairments likely underlying the fear learning and memory deficits of these mice. Inherent to this hypothesis is the presumption that Cu and its handling machinery are present at synapses. We employed a well-established subcellular fractionation

technique to directly study Cu biochemistry down to the synaptic level[45]. This technique requires amounts of tissue that exceed the volume of the mouse amygdala, so we used cortical tissue samples from individual animals. We focused on wildtype mice, knowing that changes in Cu and its chaperones/transporters are region specific in PAM$^{+/-}$ mice[46].

We found that ATP7A is enriched in fractions containing synaptic vesicles and synaptosomal membranes (Fig. 4)[46]. The synaptic membrane fraction was further separated on a sucrose gradient and then solubilized using Triton X-100, allowing separation of insoluble proteins; neither cytoskeletal proteins nor the proteins concentrated in the post-synaptic density (PSD) are solubilized by Triton x-100. The gigadalton PSD complex forms the electron dense structure located at the tips of dendritic spines receiving excitatory inputs[45]. The majority of the ATP7A in the synaptosomal membrane fraction is Triton-soluble, but there is a clear and consistent portion of ATP7A that segregates with the Triton-insoluble PSD[46]. The C-terminal PDZ-binding motif of ATP7A may contribute to its localization in the PSD fraction[47,48]. The majority of Cu is in the cytosolic fraction, where Cu bound to chaperones and metallothioneins would be found[46,49]. Cu is also present in the synaptosomal membrane and cytosol fractions, totaling approximately 40% of the total Cu content. These data compellingly support the presence of ATP7A and Cu at synapses, but how ATP7A is distributed among synapses remained to be seen.

To address this issue, we simultaneously visualized ATP7A, VGlut1 (a presynaptic marker) and PSD95 (a post-synaptic marker) in cultured cortical neurons prepared from wildtype mice[46]. We found almost no pre-synaptic localization of ATP7A; ATP7A is primarily post-synaptic, partially overlapping with PSD95 (Fig. 4). ATP7A staining near the dendritic membrane typically either co-localizes or is juxtaposed with PSD95. It is possible that co-staining puncta represent ATP7A within the PSD that we observed in the subcellular fractionation studies. Thus, ATP7A is present only at select synapses, begging the question – what is the functional difference between ATP7A-positive and ATP7A-negative synapses? The developmental expression of ATP7A suggests a potential role in synaptogenesis[50,51], so the presence of Cu in the adult animal may reflect an ongoing role in synaptic maintenance and plasticity. Nevertheless, these data provide compelling evidence for the presence of Cu and Cu handling machinery at synapses.

Cu is sufficient and necessary for PAM$^{+/-}$ LTP

Knowing that Cu and its handling machinery are present at synapses, we manipulated Cu in our *in vitro* slice recordings to study the functional role of Cu at amygdalar synapses (Gaier et al, resubmitted). If Cu is the key to rescuing PAM$^{+/-}$ amygdalar dysfunction, then acute application of Cu would be sufficient for LTP. Indeed Cu (10 μM) supplied in the bath solution is sufficient to potentiate amygdalar afferent synapse (Fig. 3). Previous studies in the hippocampus have found that 10 μM Cu inhibits LTP, but we found that the concentration of Cu has no effect on wildtype LTP in the amygdala. Again, this likely reflects differences in synaptic properties in these respective brain regions.

Logically, we next asked whether Cu is necessary for LTP. We used the membrane impermeant, Cu-selective chelator bathocuproine disulfonate (BCS; 50 μM). BCS is highly

selective for Cu at this concentration, and application just prior to LTP induction minimized concerns about intracellular Cu depletion[52–54]. Inclusion of BCS in the perfusate eliminates LTP in wildtype mice and has no effect in $PAM^{+/-}$ mice (Table 1; Fig. 3)(Gaier et al, resubmitted). Addition of CGP35348 to block $GABA_B$ receptors in addition to picrotoxin to block $GABA_A$ receptors, permits LTP in both genotypes, but addition of BCS under these conditions still abolishes LTP in both genotypes. Together this suggests that while altered GABAergic signaling is involved in the $PAM^{+/-}$ LTP deficit, Cu is necessary for LTP in both genotypes. To our knowledge, this was the first set of synaptic plasticity experiments employing a Cu chelator. Previous studies have found that Cu chelation enhances NMDA receptor activity[55], which is necessary for some forms of amygdalar LTP[41]. However, our induction protocol has been previously shown to be dependent on voltage-gated calcium channels rather than NMDA receptors[41], possibly accounting for this discrepancy. Moreover, previous physiological studies employing Cu chelators were performed in hippocampal neurons, not amygdalar neurons[55].

PAM and Cu: reciprocal influences

All of our data up to this point suggest that deficits in PAM reversibly affect Cu homeostasis, with behavioral and physiological consequences. How PAM affects Cu homeostasis is not clear. *In vitro* experiments using a well-studied corticotropic tumor cell line (AtT-20) have been conducted to study the bidirectional influences between PAM and Cu. Low Cu levels (achieved by BCS application) promote proteolytic cleavage of PAM and PHM secretion, whereas high Cu conditions promote retention of intact PAM on the membrane surface[54]. Along the same lines, dietary Cu deficiency promotes serum amidating activity[24]. Dietary Cu deficiency also affects proteolytic processing of membrane PAM. However, dietary Cu supplementation has no effect on serum amidating activity, likely due to the mild amount of Cu provided through this supplementation protocol. In the other direction, overexpression of PAM in the same cell line results in up-regulation of Atox-1, a chaperone that delivers Cu to the secretory pathway[56]. The cytosolic domain of PAM (sfCD) could be cleaved and signal to the nucleus to affect gene transcription (Fig. 4)[57]. In mice, *Pam* heterozygosity alters markers of Cu homeostasis in the liver and heart[24]. These interactions between PAM and Cu on the cellular lever reflect a role for PAM in the regulation of Cu homeostasis[58].

Our behavioral and physiological data strongly suggest a deficiency of Cu at synapses in the $PAM^{+/-}$ amygdala. However, total brain Cu levels are unchanged in $PAM^{+/-}$ mice, and dietary Cu supplementation does not affect brain Cu levels[24]. Immunohistochemical staining of brain slices containing the amygdala reveal normal amygdala structure with similar staining patterns of both PAM and ATP7A[25,46]. Staining for both PAM and ATP7A was high in the lateral and basolateral nuclei of the amygdala. Levels of ATP7A protein and mRNA are reduced in the $PAM^{+/-}$ amygdala and normal in the hippocampus. The same pattern of expression was seen when we analyzed transcript levels of Atox-1, the cytosolic chaperone that delivers Cu to ATP7A in the secretory pathway. Levels of Cu itself are reduced in homogenized brain punches of the amygdala isolated from $PAM^{+/-}$ mice compared to wildtype(Gaier et al, resubmitted). This effect is also region-specific as there is no genotypic difference in Cu levels seen in the hippocampus. The deficit in amygdalar Cu

has effects on other cuproenzyme functions, namely dopamine β-monooxygenase, that is also region-specific[46]. This finding reflects not only a consequence of Cu deficiency, but also implies that Cu functioning in the secretory pathway in particular is deficient in the $PAM^{+/-}$ amygdala. Thus, *Pam* heterozygosity affects Cu homeostasis specifically in the amygdala, perhaps explaining the Cu-sensitive behavioral and physiological dysfunction we observed in studying the $PAM^{+/-}$ amygdala.

Future directions and application

The $PAM^{+/-}$ mouse is a model connecting brain and behavior ideal for studying the role of Cu in synaptic function. Combined, the data we have summarized here support the suggestion that Cu is released with neuronal activation and signals to the pre-synaptic terminal to promote vesicle release. Future studies should determine the cells in which ATP7A and Cu are essential for synaptic plasticity and fear conditioning, namely the prevalent glutamatergic neurons or the interneurons with high PAM and ATP7A expression. Conditional knockout models for ATP7A are the key to conducting such studies, especially when combined with induction via viral vectors using cell-type specific promoters to target ATP7A knockout. Cultured neuronal systems are more easily manipulated and will serve as ideal models for studying the role of Cu in neuronal and synaptic function. Moreover, culture models can be combined with promising new methods to visualize Cu at subcellular levels in real time[59,60]. We anticipate a time when these techniques will greatly expand our ability to visualize the dynamics of Cu homeostasis at synapses.

We have shown that secreted Cu is necessary for amygdalar LTP, but exactly how Cu modulates synaptic transmission and plasticity remains unclear. Cu interacts with several different receptors, binding proteins and enzymes at synapses and could therefore affect synaptic function in a multitude of ways [61]. We have studied how manipulation of Cu affects behavioral and physiology, but no experiments we have performed to date have gone in the other direction. It would be interesting to test whether neuronal ATP7A and Cu localization can be manipulated by neuronal stimulation *in vivo*, perhaps through seizure induction or fear conditioning training. We hypothesize that the ATP7A distribution between the Triton-soluble and PSD fractions will change with stimulation, but even more interesting will be to test whether and how those responses may be altered in $PAM^{+/-}$ mice. Investigating these dynamics of Cu homeostasis at synapses is critical to understanding the mechanism by which Cu regulates synaptic plasticity.

All together these findings suggest an essential role for Cu in amygdalar synaptic functions with behavioral relevance. The $PAM^{+/-}$ model therefore may be particularly relevant to studies of post-traumatic stress disorder (PTSD), in which the amygdala is known to play a critical role [62]. Progressive strengthening of traumatic memories, which drives baseline anxiety and memory-related fear, is at the heart of PTSD[63]. Thus, identification of Cu signaling as a critical player in amygdalar synaptic plasticity and fear memory formation may have therapeutic potential as a pharmacological target in the treatment of PSTD. Expanding the study of $PAM^{+/-}$ mice and Cu signaling in amygdala function to the other half of fear conditioning, extinction (not discussed here), would have even higher relevance to novel treatments for PTSD[64]. Aside from anxiety disorders, disruptions in Cu

homeostasis have been implicated in several neurodegenerative diseases[65,66] including Alzheimer's disease[67,68], prion-related diseases[69] and multiple sclerosis[70].

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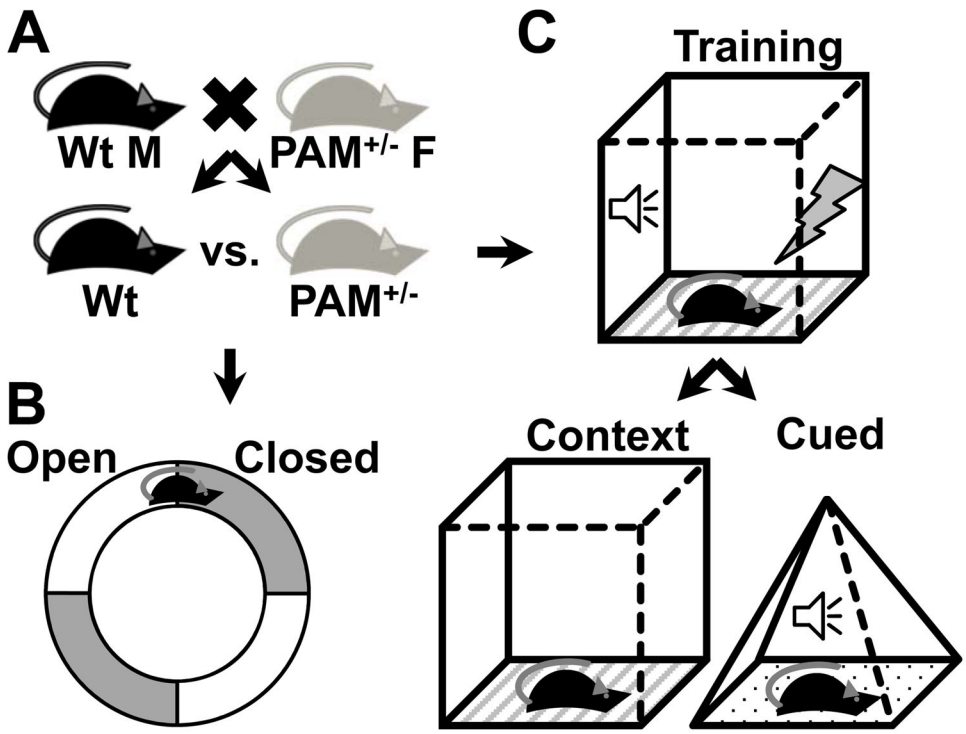

Fig 1. Innate and learned fear behavioral testing

(**A**) PAM$^{+/-}$ mice are generated from wildtype (Wt) dams and PAM$^{+/-}$ sires, yielding 50% Wt and 50% PAM$^{+/-}$ offspring. Wt and PAM$^{+/-}$ littermates are used in all experiments. (**B,C**) Innate fear is tested in the elevated zero maze, and fear learning and memory is tested in fear conditioning. (**B**) The elevated zero maze is comprised of four quadrants, two of which are “open” (have low walls) and two of which are “closed” (have high walls). Mice are placed into the maze for a fixed length of time, and the amount of time spent in the open versus the closed arms of the maze is recorded. The percentage of time spent in the closed arm of the maze corresponds directly to the amount of anxiety-like behavior of the animal in this test. (**C**) Fear conditioning tests begin with training, during which a tone is paired with an aversive footshock. Subsequently, mice are either tested in contextual (left) or cued (right) fear conditioning. In contextual fear conditioning, mice are placed in the same chamber in which training occurred. In cued testing, mice are placed in a novel chamber and the same tone that was played during training is played. The amount of time the animal spends freezing (immobile except for breathing) while in the training context or during the tone is recorded. The percentage of time spent freezing is a direct measure of how well the animal learned and remembers the association between the conditioned stimulus (context or tone) and the unconditioned stimulus (footshock).

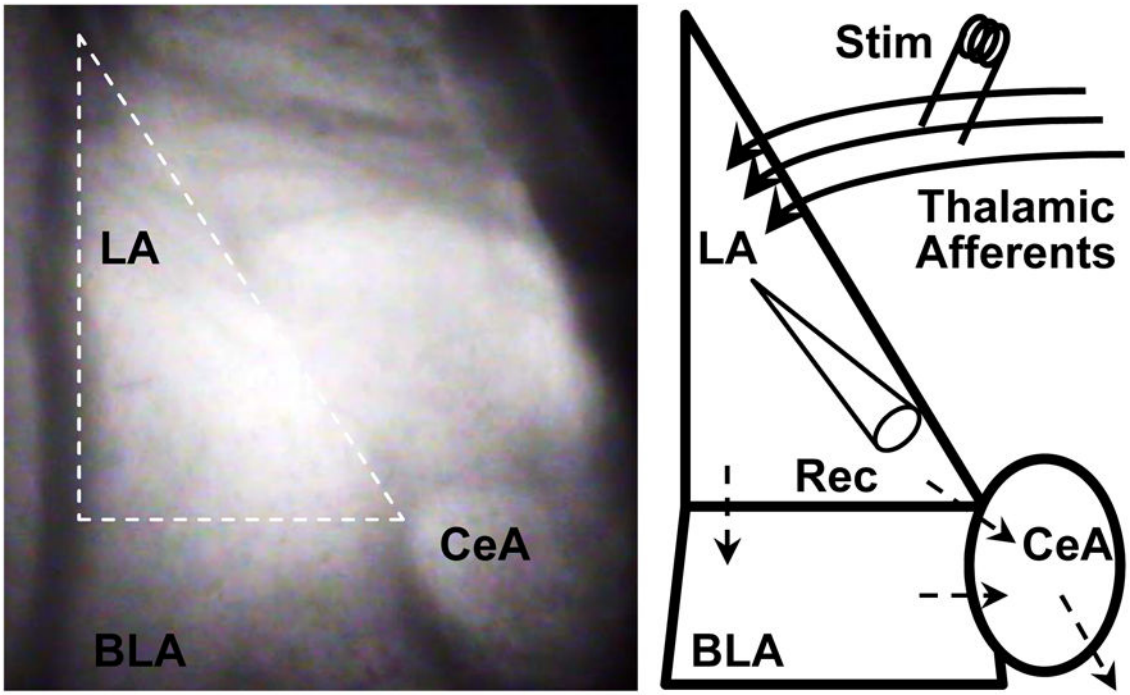

Fig 2. Amygdala structure, circuitry and recording methods

(Left) Infrared differential interference contrast image taken at 40× of a coronal mouse brain slice containing the amygdala. The amygdala is comprised of lateral (LA), basolateral (BLA) and central (CeA) nuclei. We performed our experiments in the LA (outlined in white dashes). (Right) Schematic depicting amygdala nuclei. Thalamic afferent axons enter the LA and synapse onto LA neurons. LA neurons send their projections to the BLA and CeA; the BLA sends its projections to the CeA as well. CeA neurons send their projections to midbrain and brainstem nuclei responsible for evoking the fight or flight response in mammals, which contributes to anxiety-like behaviors and freezing in fear conditioning. We stimulated (Stim) thalamic afferent synapses and recorded post-synaptic responses (Rec; whole cell patch clamp) in LA pyramidal neurons.

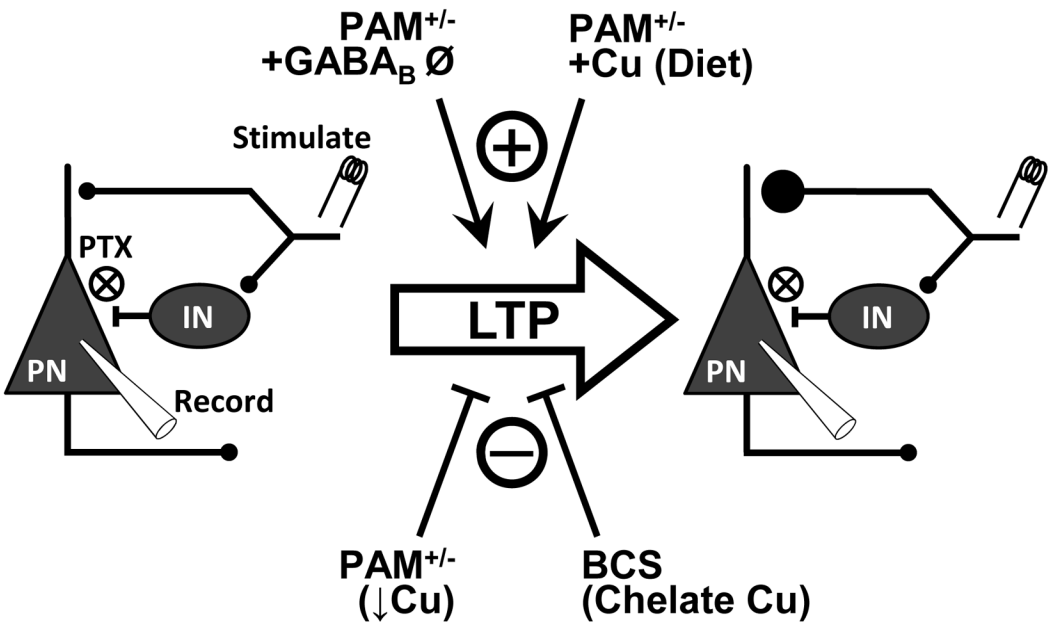

Fig 3. Amygdalar synaptic plasticity data summarized

(Left) Schematic of neuronal/synaptic circuitry in the LA depicting methods for stimulating afferents to and recording from LA pyramidal neurons (PN). We stimulated thalamic afferents to the LA and recorded from PNs that receive those excitatory inputs. GABAergic interneurons (INs) also receive collateral thalamic afferent inputs and mediate feed-forward inhibition of PNs. Picrotoxin (PTX) is used in all experiments to block $GABA_A$ receptors to eliminate the fast component of feed-forward inhibition and permit LTP. Pairing of pre-synaptic stimulation with post-synaptic depolarization induces LTP (large arrow), resulting in potentiation of synaptic efficacy (right). LTP in this system under these conditions includes increased pre-synaptic neurotransmitter release. Several conditions are permissive to (+) or abolish (gray X) LTP. *Pam* heterozygosity (a low Cu condition) abolishes LTP, but not in the presence of $GABA_B$ receptor blockade (using CGP35348). Dietary Cu supplementation prior to slice preparation or inclusion of Cu in the perfusate (10 μM $CuSO_4$) rescues PAM+/- LTP. On the other hand, inclusion of the membrane impermeant, Cu-specific chelator BCS abolishes LTP in both Wt and $PAM^{+/-}$ mice even when $GABA_B$ receptors are blocked. Combined, these results indicate that Cu is necessary for LTP and sufficient to rescue LTP in $PAM^{+/-}$ mice.

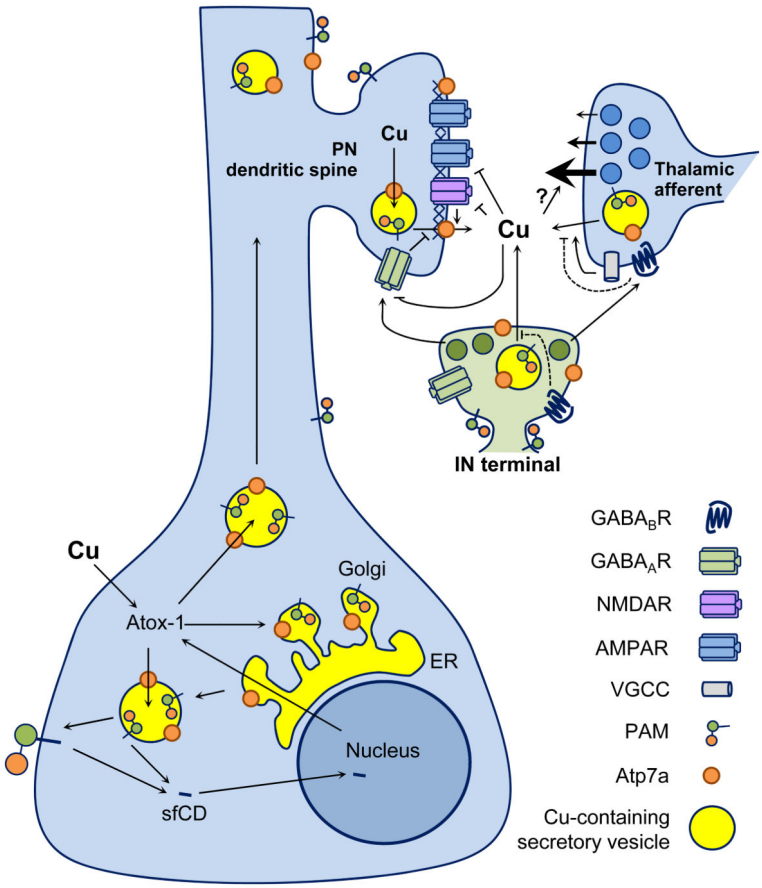

Fig 4. Cu and ATP7A are present at synapses

Schematic summarizing biochemical and synaptic plasticity studies with respect to Cu and ATP7A. (Soma) Cu enters neurons and binds the chaperone Atox-1 in the cytosol, which transports Cu to ATP7A for delivery into the secretory pathway. PAM is also present in the secretory pathway and co-localizes with ATP7A in neurons. A cytosolic portion of PAM is cleaved and enters the nucleus, affecting transcription and up-regulation of Atox-1. Vesicles containing PAM, ATP7A and Cu are transported into the dendrite. (Synapse) Thalamic afferent glutamatergic terminals innervate pyramidal neuron (PN) dendritic spines. Interneuron (IN) terminals release GABA which inhibits excitatory transmission and excitation through activation of $GABA_A$ and $GABA_B$ receptors. Notably, INs express the highest levels of PAM and ATP7A. Extracellular Cu could come from any number of sources including the post-synaptic pyramidal neurons, pre-synaptic afferents, or INs. Inhibitory effects of Cu on synaptic receptors and ion channels including $GABA_A$ receptors, NMDA receptors and L-type voltage-gated Ca channels have been described (see text), but how Cu influences synaptic plasticity remains unknown. The role of Cu in modulating synaptic transmission and plasticity is complex and requires further study.

Table 1

PAM$^{+/-}$ behavioral and physiological phenotypes and their responses to dietary Cu condition.

Phenotype	Cu restriction mimic	Cu supp rescue	Reference
Thermoregulation deficit	yes	yes	[23,24]
Vasoconstriction deficit		yes	[23]
Seizure hypersensitivity	yes	no	[24]
Anxiety-like behaviors	yes	yes	[24]
Fear conditioning – contextual		no	[25]Gaier et al, resubmitted
Fear conditioning – cued		yes	[25]Gaier et al, resubmitted
Fear-potentiate startle		yes	[25]Gaier et al, resubmitted
Amygdalar LTP		yes	[25]Gaier et al, resubmitted