In vivo and in vitro analyses of amygdalar function reveal a role for copper

Abbreviated Title: Dietary Cu alters amygdalar function in vivo and in vitro

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ABSTRACT
Mice with a single copy of the peptide amidating monooxygenase (Pam) gene (PAM+/-) are impaired in contextual and cued fear conditioning. These abnormalities coincide with deficient long-term potentiation (LTP) at excitatory thalamic afferent synapses onto pyramidal neurons in the lateral amygdala. Slice recordings from PAM+/- mice identified an increase in GABAergic tone (Gaier et al. 2010). Biochemical data indicate a tissue-specific deficit in Cu content in the amygdala; amygdalar expression of Atox-1 and Atp7a, essential for transport of Cu into the secretory pathway, is reduced in PAM+/- mice. When PAM+/- mice were fed a diet supplemented with Cu, the impairments in fear conditioning were reversed and LTP was normalized in amygdala slice recordings. A role for endogenous Cu in amygdalar LTP was established by the inhibitory effect of a brief incubation of wildtype slices with bathocuproine disulfonate, a highly selective cell-impermeant Cu chelator. Interestingly, bath applied CuSO4 had no effect on excitatory currents, but reversibly potentiated the di-synaptic inhibitory current. Bath applied CuSO4 was sufficient to potentiate wildtype amygdala afferent synapses. The ability of dietary Cu to affect signaling in pathways that govern fear-based behaviors supports an essential physiological role for Cu in amygdalar function at both the synaptic and behavioral levels. This work is relevant to neurological and psychiatric disorders in which disturbed Cu homeostasis could contribute to altered synaptic transmission, including Wilson’s, Menkes, Alzheimer’s and prion-related diseases.

INTRODUCTION

The critical role played by Cu in neuronal function is illustrated by the neurological and neuropsychiatric deficits observed in Menkes’ and Wilson’s Diseases. Mutations in the P-type ATPases (ATP7A and ATP7B) that transport Cu from the cytosol into the secretory pathway result in brain Cu levels that are too low or too high (La Fontaine and Mercer 2007; Lutsenko et al. 2007). Marginal dietary Cu deficiency in wildtype mice causes impaired thermoregulation, increased seizure susceptibility, and increased anxiety-like behavior (Bousquet-Moore et al. 2010). These deficits could reflect impaired cuproenzyme function or a direct effect of diminished Cu levels. Peptidylglycine α-amidating monooxygenase (PAM), one of several enzymes essential for neuropeptide biosynthesis (Mains and Eipper 1999), and dopamine β-monooxygenase (DBM), the enzyme that converts dopamine into norepinephrine (Klinman 2006), both require Cu. In addition, release of Cu loaded into vesicles by Atp7a, which is...
present in dendrites (Gaier et al. 2013b), can yield synaptic Cu concentrations sufficient to alter ion channel function (Kardos et al. 1989; Mathie et al. 2006; Tamano and Takeda 2011).

Although Cu is not tightly bound to PAM, the enzyme is inactive without it (Mains et al. 1986; Prigge et al. 2000). Embryos lacking Pam die at mid-gestation, but mice with a single functional copy of Pam (PAM+) grow and reproduce normally (Czyzyk et al. 2005). While indistinguishable in appearance from wildtype mice, PAM+/- mice display several striking behavioral deficits: impaired thermoregulation, anxiety-like behavior, deficient fear conditioning, and fear potentiation of the acoustic startle response (Bousquet-Moore et al. 2009; Bousquet-Moore et al. 2010; Gaier et al. 2010). Since several of these behavioral phenotypes are seen in marginally Cu deficient wildtype mice (Bousquet-Moore et al. 2010), the ability of dietary Cu supplementation to reverse the deficits was assessed. Supplemental dietary Cu reversed the thermoregulatory deficit and normalized anxiety-like behavior in PAM+/- mice, but did not ameliorate seizure susceptibility (Bousquet-Moore et al. 2009; Bousquet-Moore et al. 2010). Notably, amidated neuropeptide levels were only slightly altered by Pam heterozygosity or Cu supplementation (Czyzyk et al. 2005; Yin et al. 2011; Bousquet-Moore et al. 2010), suggesting that neuropeptidergic fluctuations cannot account for the aberrant PAM+/- behaviors.

Whole body Cu homeostasis involves tissue-specific expression and regulation of many proteins dedicated to the transport, binding and delivery of Cu (Lutsenko et al. 2007; Kim et al. 2010). Liver levels of Cu are elevated in PAM+/- mice, and manipulation of Cu alters PAM secretion and trafficking (Bousquet-Moore et al. 2010; De et al. 2007). This bidirectional interaction suggests that PAM serves a tissue-specific, non-enzymatic role in Cu homeostasis. We recently found that expression of both Atp7a and Atox-1, the cytosolic chaperone that delivers Cu to Atp7a, were diminished in the amygdala, but not in the hippocampus, of PAM+/- mice (Gaier et al. 2013b). The amygdala plays a key role in the neural pathways involved in fear learning and memory (Sah et al. 2003; LeDoux 2007) and provides one of the best systems for relating behavioral to electrophysiological studies (Ledoux 2000; Maren and Quirk 2004; Kim and Jung 2006).

By recording from pyramidal neurons in the lateral nucleus of the amygdala, we previously demonstrated that synaptic plasticity at thalamic afferent synapses is impaired in PAM+/- mice, correlating with fear memory deficits (Gaier et al. 2010). We concluded that altered GABAergic transmission contributed to these changes; PAM and Atp7a are expressed at especially high levels in these neurons (Gaier et al. 2013b). Cu is secreted at synapses at concentrations sufficient to directly affect ion channel function (Kardos et al. 1989; Mathie et al. 2006; Tamano and Takeda 2011; Gaier et al. 2013a), but the role of endogenous Cu in synaptic function has never been studied. We hypothesize that altered Cu homeostasis caused by Pam heterozygosity underlies several of the electrophysiological and behavioral deficits. To study the role of Cu in this system, we first asked if Cu levels differed between PAM+/- and wildtype amygdala and whether dietary Cu supplementation could reverse the PAM+/- deficits in fear-based memory. We conducted in vitro slice electrophysiology studies to examine corresponding alterations in amygdalar synaptic physiology. Finally, we analyzed the role of Cu in normal amygdalar synaptic function in wildtype mice to assess how dysfunction of Cu signaling could contribute to the deficits seen in PAM+/- mice.

METHODS

Animals

Mice were generated from PAM+/- matings in the University of Connecticut Health Center (UCHC) animal facility. Wildtype and PAM+/- littermates (>20 generations backcrossed onto a C57BL/6J background) were weaned by P21 and group-housed. Animals were maintained on a 12 h light/dark cycle (lights on at 0700) with ad lib food and water. 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, these data were pooled. Dietary Cu supplementation included 300 ppm (1.2 mM) CuSO4 in reverse-osmosis drinking water for 14 days. All experiments were conducted with approved protocols from the UCHC and the Duke University Institutional Animal Care and...
Use Committees and in accordance with NIH guidelines for animal care.

**Fear conditioning**

Mice were tested in a MedAssociates chamber (St. Albans, VT) in either context or cued fear conditioning 24 h after training (Taylor et al. 2008; Porton et al. 2010), and were conditioned with a single 30 s 72 dB tone (CS) which terminated with a 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 it had been conditioned in the absence of the CS and US. For cued testing, mice were placed into a novel chamber whose color, texture, shape, dimensions, and level of illumination were different from that of the conditioning chamber. All tests were filmed and behaviors were scored by a trained observer blinded to the genotype and Cu status of the mice using Noldus Observer software (Leesburg, VA). Freezing was defined as the lack of all non-respiratory movement by the animal for >1 s (Anagnostaras et al. 2000; Porton et al. 2010). 214

**Electrophysiology**

**Slice preparation.** Male wildtype and PAM+/- littermates were decapitated and their brains quickly removed into ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 26 NaHCO3, 1.26 KH2PO4 (310 mOsm/kg, pH 7.3), and aerated with 95% O2/5% CO2. Recording pipettes contained (in mM) 135 K-gluconate, 10 HEPES, 2.3 KCl, 2 Na2ATP, 3 Na2GTP, pH 7.3, and aerated with 95% O2/5% CO2 (Zhou et al. 2008). Patching was guided by 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 was used to verify pyramidal cell type. Input resistance was continuously monitored using either a 5 mV voltage or 25 pA current step. Neurons were eliminated from analysis if input resistance changed by more than 20%. Bath-application of 10 µM CuSO4 did not affect input resistance (aCSF 151.1 ± 6.2 MΩ, CuSO4 140.9 ± 9.5 MΩ; N=7 Wt neurons).

**Evoked synaptic transmission.** Synaptic activity was evoked using 200 µs current pulses 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 recruited thalamic afferent axons (Weisskopf and Ledoux 1999). For Cu wash-in experiments, neurons were clamped at VHolding = -35 mV to record excitatory and feed-forward inhibitory transmission. Evoked inhibitory post-synaptic currents were elicited through placement of the stimulating electrode in the lateral amygdala nucleus; stimulation strength was adjusted to yield the half-maximal response. A 5 mV, 25 ms hyperpolarizing step was applied 800 ms after each synaptic response to monitor passive membrane properties. The perfusate was changed during continuous recordings to aCSF containing 10 µM CuSO4. Paired pulses were applied at a 50 ms interval when indicated. Paired pulse ratios were calculated as the ratio EPSP2/EPSP1 (Gaier et al. 2010).

**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) and 1 µM CGP35348 where indicated (Shaban et al. 2006; Gaier et al. 2010). The holding current was adjusted to maintain Vm = -70 mV throughout the course of experiments.

**Stimulation strength was adjusted to produce a 3-6 mV EPSP.** The 10-90% rise slope of excitatory post-synaptic potentials (EPSPs) was used as the measure of synaptic efficacy. After establishment of a steady baseline, long-term potentiation (LTP) was induced using an action potential pairing induction protocol reported to be L-type voltage-gated Ca2+ channel-dependent and N-methyl-D-aspartate (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 in +50 pA increments 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.** Picrotoxin (PTX; 100 µM; Sigma-Aldrich) was used to block fast GABA<sub>A</sub> receptor-mediated transmission. CGP35348 (1 µM; Tocris Biosciences, Ellisville, MO) was used to block slow GABA<sub>B</sub> receptor-mediated transmission. AMPA receptors were blocked with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 µM; Tocris Biosciences) and NMDA receptors with 3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP; 3 µM; Tocris Biosciences). CNQX stock was prepared in DMSO; the final concentration of DMSO in the perfusate was 0.01% (Gaier et al. 2010). Bath-application of bathocuproine disulfonate (BCS; 50 µM; Sigma-Aldrich) was used to selectively chelate extracellular Cu<sup>2+</sup> (De et al. 2007). The open voltage-gated Na<sup>+</sup> channel blocker QX-314 (3 µM; Sigma-Aldrich) was added to the internal pipette solution where indicated to block action potential activity in the patched neuron (Duvarci et al. 2011). CuSO<sub>4</sub> was dissolved in H<sub>2</sub>O to yield a stock solution, which was then diluted in aCSF. Cu at 10 µM has been used in previous studies demonstrating synaptic effects (Doreulee et al. 1997; Leiva et al. 2000; Peters et al. 2011).

### Cu measurements

Amygdalae and dorsal hippocampi were isolated from individual adult male mice (9 wildtype; 8 PAM<sup>−/−</sup>); brains were placed into ice-cold phosphate-buffered saline solution (pH 7.4). Bilateral 2 mm diameter, 1 mm thick tissue punches were taken from both sides of the appropriate coronal slice. Excess saline was removed, and tissue punches were transferred into microfuge tubes containing 100 µl 20 mM Na TES, 10 mM mannitol, pH 7.4 and frozen. Samples were later thawed and tissue fragments were homogenized with a handheld pestle; following two cycles of freezing and thawing, particulate material was removed by centrifugation for 1 min at 20 x g and the protein content of the supernatant was quantified using the bicinchoninic acid assay (Pierce) with bovine serum albumin as the standard. Inductively coupled plasma mass spectrometry (ICPMS) 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 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, 50 µl homogenates were transferred into nitric acid washed 15 ml conical centrifuge tubes and digested with 100 µl 50% HNO<sub>3</sub> (trace metal grade, Fisher Scientific) solution. Following overnight, room temperature digestion of the samples, 850 µl of 1% HNO<sub>3</sub> (trace metal grade, Fisher Scientific) was added, the samples were spun at 4,700 x g for 10 min to remove insoluble material, and the supernatants were transferred into clean, acid treated 15 ml conical tubes. Data were quantified using a 9-point calibration curve [0, 0.5, 1, 2, 5, 10, 50, 100, 1000 ppb (ng/g)] with external standards for Fe, Cu, and Zn (Common Elements Mix 2, Multi-Element Aqueous Standard, VHG Labs) in 1% HNO<sub>3</sub>. For each sample, data were acquired in triplicate and averaged. An internal standard (Internal Standard Multi-Element Mix 3, VHG Labs) introduced with the sample was used to correct for plasma instabilities, and frequent measurements of a 10 ppb Cu solution as well as a blank (containing 1% HNO<sub>3</sub> only) were used for quality control and to determine the coefficient of variance. To access recovery rates of elements and probe background contamination from buffers and containers, the following controls were treated, prepared, and analyzed using the same method as the samples: certified NIST standard reference material (serum; 1598a), homogenization buffer, acid control (containing 50% HNO<sub>3</sub> only), and certified elemental standard for ICPMS (Common Elements Mix 2, Multi-Element Aqueous Standard, VHG Labs).

### Statistics

Cu measurements were assessed by Two-way ANOVA for genotype and brain region. The behavioral data are presented as means and standard errors of the mean (SPSS-20 program; IBM SPSS Statistics, Chicago, IL). The fear conditioning data were analyzed by repeated-measures ANOVA (RMANOVA) and Bonferroni corrected pair-wise comparisons as the post-hoc test. Synaptic potentiation above baseline was...
assessed using the non-parametric Wilcoxon signed rank test; comparisons between conditions were made using Students’ t-test (unpaired, two-tailed, unequal variance assumed). In all cases, $p<0.05$ was considered significant.

**RESULTS**

**Cu is reduced in the PAM+/- amygdala**

Since several physiological and behavioral deficits observed in PAM+/- mice were ameliorated by Cu supplementation (Bousquet-Moore et al. 2009; Bousquet-Moore et al. 2010), we reasoned that the amygdalar dysfunction exhibited by PAM+/- mice (Gaier et al. 2010) may result from deficient in Cu in this brain region. We tested this hypothesis directly by measuring total tissue Cu in punches of amygdala and hippocampus taken from wildtype and PAM+/- mice (Fig. 1). Two-way ANOVA found a significant main effect of genotype, with lower concentrations of Cu in PAM+/- mice compared to wildtype ($p<0.05$). There was also a significant main effect of brain region, with a lower concentration of Cu in the amygdala than in the hippocampus from wildtype mice ($p<0.05$), consistent with previous results in rats (Jackson et al. 2006). Bonferroni pairwise comparisons revealed a significantly lower concentration of Cu in the PAM+/- amygdala than in the wildtype amygdala ($p<0.05$) (Fig. 1). By contrast, there was no significant effect of genotype on Cu concentration in the hippocampus. There was a significantly lower Cu concentration in the PAM+/- amygdala than in the hippocampus ($p<0.05$). There was no effect of brain region on Cu concentration in wildtype mice. These data demonstrate a region-specific deficit in Cu in the PAM+/- amygdala that may contribute to the deficits in short- and long-term fear memory and fear potentiated-startle observed previously in these mice (Gaier et al. 2010).

**Dietary Cu rescues fear conditioning deficits in PAM+/- mice**

Next, we asked whether the fear memory deficits were responsive to dietary Cu supplementation. Wildtype and PAM+/- mice received reverse-osmosis drinking water (control) or drinking water supplemented with CuSO4 for 10-14 days. Mice were trained with a single fear conditioning trial (CS-US pairing) and tested either in contextual or cued fear conditioning 24 h later (Fig. 2). Wildtype and PAM+/- mice given control water were indistinguishable throughout conditioning and both genotypes showed similar small increases in freezing immediately following the CS-US pairing (not shown). Twenty-four h later, mice were examined in a test for contextual fear conditioning (Fig. 2A). RMANOVA found that both genotypes increased freezing over the 5 min exposure ($p<0.05$) with no significant within-subject interactions. Between subjects, there was a significant effect of genotype ($p<0.05$) with no significant interactions. Regardless of Cu condition, PAM+/- mice consistently had lower levels of freezing for all time points compared to wildtype controls ($p$ values<0.05). Hence, Cu supplementation exerted comparable effects in contextual fear conditioning, increasing freezing responses for both genotypes while maintaining the difference between genotypes.

A separate set of mice was evaluated for cued fear conditioning (Fig. 2B). Both genotypes, regardless of Cu condition, displayed similarly low levels of freezing during the 2 min prior to CS presentation. RMANOVA revealed that, relative to the pre-tone interval, both genotypes responded to the CS by freezing over the 3 min exposure interval ($p$ values<0.05). Significant time by genotype and time by genotype by Cu condition interactions ($p$ values<0.05) were observed. A posteriori comparisons under the control water condition demonstrated that PAM+/- mice froze significantly less to the CS than wildtype mice ($p<0.05$). Freezing by wildtype mice was unaffected by Cu supplementation ($p<0.05$). Notably, Cu supplementation increased freezing of the PAM+/- mice ($p<0.05$) to the level of the Cu supplemented wildtype mice. Together these results show that Cu supplementation eliminated the effect of PAM+/- heterozygosity on cued but not on contextual fear conditioning.

**Dietary Cu rescues synaptic plasticity in PAM+/- mouse amygdala**

Impaired synaptic plasticity at thalamic afferent synapses in the amygdala is thought to reflect neurophysiological impairments responsible for the fear memory deficits observed in PAM+/- mice (Gaier et al. 2010). Since some aspects of the fear memory deficiencies were ameliorated with Cu supplementation, we next asked whether in vivo dietary Cu supplementation of wildtype or PAM+/- mice affected LTP at thalamic afferent synapses within the lateral nucleus of
the amygdala (Fig. 3). Thalamic afferent fiber bundles were stimulated and excitatory post-synaptic potentials (EPSPs) were recorded from pyramidal neurons using whole-cell patch clamp methods. LTP was induced using a paired pulse induction protocol (Bauer et al. 2002; Gaier et al. 2007; Gaier et al. 2010). EPSPs recorded from pyramidal neurons of wildtype mice given control water were potentiated, while EPSPs recorded from PAM+/- mice under the same condition failed to become potentiated, confirming our previous results (Gaier et al. 2010) (Fig. 3B). In addition, normalized EPSP rise slopes were reduced in PAM+/- compared to wildtype neurons 30-40 min after LTP induction, indicating a significant difference in LTP (p<0.05).

In an effort to localize the site of synaptic potentiation using this induction protocol, this experiment was conducted using paired pulses (50 ms interval) and the paired pulse ratio (PPR; EPSP2/EPSP1) was monitored throughout the LTP time course (Fig. 3C). As expected for glutamatergic synapses, the PPR was greater than 1.0 at thalamic afferent synapses, reflecting the contribution of accumulated Ca2+ at presynaptic terminals after the first stimulation. There were no differences in PPRs between genotypes at baseline (Fig. 3C inset). Wildtype LTP coincided with a significant reduction in the PPR, indicating an enhancement in initial neurotransmitter release. By contrast PAM+/- PPRs remained unchanged on average following induction, coinciding with unchanged synaptic efficacy. These data confirm the previously reported deficit in synaptic plasticity in the PAM+/- amygdala and indicate a presynaptic component in the mechanism of potentiation.

Since intrinsic membrane properties can influence synaptic plasticity through post-synaptic mechanisms, we compared measures of passive (resting membrane potential, Rm; input resistance, R; membrane capacitance, C) and active (action potential threshold, APT; action potential rheobase, APR) membrane properties between genotypes under these same conditions. Consistent with previous results (Gaier et al. 2010), we found no significant genotypic differences in Rm (Wt: -69.6 ± 1.2 mV; PAM+/-: -67.5 ± 0.5 mV; N= 13 Wt, 13 PAM+/-; p>0.05), R (Wt: 126.5 ± 11.6 MΩ; PAM+/-: 147.1 ± 12.2 MΩ; N= 13 Wt, 13 PAM+/-; p>0.05), or Cm (Wt: 84.1 ± 4.7 pF; PAM+/-: 85.1 ± 3.9 pF; N= 13 Wt, 13 PAM+/-; p>0.05). Moreover, there were no significant differences in the current-voltage relationship between wildtype and PAM+/- amygdalar neurons (Fig. 3D). APT was depolarized by less than 3 mV in PAM+/- neurons (Wt: -32.5 ± 0.8 mV; PAM+/-: -29.7 ± 0.7 mV; N= 13 Wt, 13 PAM+/-; p<0.05), however, a difference of this magnitude is unlikely to be the sole post-synaptic factor driving the difference in synaptic plasticity. APR was not different between genotypes (Wt: 169.2 ± 16.5 pA; PAM+/-: 146.2 ± 16.5 pA; N= 13 Wt, 13 PAM+/-; p>0.05). Therefore, intrinsic membrane properties are unlikely to contribute to the deficit in synaptic plasticity in PAM+/- amygdalar neurons under these conditions.

Next we examined neuronal and synaptic function of amygdalar neurons from wildtype and PAM+/- mice supplemented with Cu through the diet. Intrinsic membrane properties were assessed under the same recording conditions as the previous experiment. There were no genotypic differences in passive membrane properties with respect to RMP (Wt: -69.6 ± 3.2 mV; PAM+/-: -68.5 ± 2.7 mV; N= 7 Wt, 8 PAM+/-; p>0.05). Cu supplementation exerted no gross effects on the voltage responses to LTP induction in wildtype mice (Fig. 3F). Together, these results suggest no influence of dietary Cu supplementation on intrinsic membrane properties of wildtype or PAM+/- amygdalar neurons relevant to synaptic plasticity.
Using the same LTP induction protocol, we then tested whether dietary Cu supplementation had an effect on amygdalar LTP. Neurons from Cu supplemented wildtype mice exhibited LTP that was indistinguishable from LTP in wildtype mice receiving control water (Fig. 3G versus Fig. 3B). By contrast, Cu supplementation increased PAM$^+$ LTP to levels significantly above PAM$^+$ mice given control water ($p<0.05$). Moreover, Cu supplementation abolished the genotypic differences in LTP that were observed in mice maintained on control water (Fig. 3E). Thus, Cu supplementation successfully eliminated the synaptic plasticity deficit observed in the PAM$^+$ amygdala.

The fact that providing PAM$^+$ and wildtype mice with supplementary Cu rescues their behaviors in cued fear conditioning, abrogating the genotype differences in LTP, removes concerns that the differences observed in PAM$^+$ mice arise from permanent alterations that occur during development. Changes in the biosynthesis of amidated neuropeptides are also unlikely to be a contributing factor because amidating activity was unaffected by Cu supplementation (Bousquet-Moore et al. 2010). Therefore, our data support the hypothesis that Cu supplementation suppresses or abrogates behavioral and physiological differences between wildtype and PAM$^+$ mice through its effects on Cu homeostasis.

**Bath applied Cu modulates inhibitory synaptic transmission and is sufficient for LTP**

If the alterations in synaptic plasticity observed in PAM$^+$ mice are the result of deficient amygdalar Cu, one would predict an effect of exogenous Cu on synaptic plasticity at amygdala afferent synapses. We first assessed the effects of Cu on baseline synaptic transmission in the wildtype amygdala. We clamped pyramidal neurons of the lateral nucleus in wildtype mice at $V_{holding} = -35\text{ mV}$; this allowed us to simultaneously monitor excitatory and inhibitory responses to thalamic afferent stimulation when 10 $\mu\text{M CuSO}_4$ was perfused (Doreulee et al. 1997; Leiva et al. 2003). Picrotoxin was not present for these experiments (Fig. 4A). As expected, stimulation of thalamic glutamatergic afferents produced a mono-synaptic excitatory inward current which was closely followed by a di-synaptic inhibitory outward current (Fig. 4A). Notably, the stimulation current necessary to elicit bi-phasic responses was substantially greater than currents used in plasticity experiments. Di-synaptic outward currents represent fast feed-forward inhibition of thalamic afferent synapses mediated by GABA$_A$ receptors (Duvarci and Pare 2007). As expected, both excitatory and inhibitory currents were sensitive to AMPA receptor antagonism with CNQX and only the inhibitory currents were sensitive to GABA$_A$ receptor blockade with 100 $\mu\text{M}$ picrotoxin (Fig. 4A inset).

Since the excitatory and inhibitory events partially overlapped in time, we assessed the efficacy of excitatory transmission using the 0-70% rise of the inward current, and the efficacy of feed-forward inhibition using the peak-to-peak slope. Inward and outward events were monitored before, during and after the addition of 10 $\mu\text{M CuSO}_4$ to the perfusate (Fig. 4B). Estimates of synaptic Cu levels vary, but 10 $\mu\text{M}$ is well within the range thought to be physiologically relevant (Doreulee et al. 1997; Goldsclimith et al. 2005; Leiva et al. 2003; Peters et al. 2011; McGee et al. 2013). Bath application of Cu had no effect on excitation, but exerted a profound potentiating effect on inhibition ($p<0.05$). Importantly, the effect of Cu on the inhibitory current was completely reversible; outward currents returned to baseline during the 30 minute Cu washout period. These data support a net GABAergic inhibitory role for bath applied Cu in the wildtype amygdala.

In order to examine the effect of Cu on GABA$_A$ receptor-mediated inhibition, we performed the same experiment with CNQX and CPP included in the perfusate to block AMPA and NMDA receptors, respectively. Voltage-gated Na$^+$ channels were blocked exclusively in the clamped cell by the inclusion of membrane impermeant QX-314 in the intracellular pipette solution. The slower GABA$_B$ receptor mediated component of inhibition was blocked by QX-314 (Nathan et al. 1990). To elicit inhibitory post-synaptic currents (eIPSCs), we directly stimulated the interneuronal network by placing the stimulating electrode in the lateral amygdala nucleus (Gaier et al. 2010). Stimulation current was adjusted to elicit half the maximal eIPSC. Perfusion of CuSO$_4$ and subsequent wash had no effect on eIPSCs (Fig. 4C). Therefore, Cu is unlikely to enhance feed-forward inhibition by directly activating interneurons.
Bath application of Cu increased the holding current ($p<0.05$), representing a rapidly reversible hyperpolarizing effect of Cu on the post-synaptic neuron in the absence of stimulation (Fig. 4D). This effect did not correspond to any significant changes in input resistance (see Methods). Inclusion of picrotoxin in the perfusate along with QX-314 in the pipette solution abolished this effect, suggesting that Cu affects the holding current through GABA-mediated signaling.

Next we tested the effect of CuSO$_4$ perfusion on isolated excitatory synaptic transmission. In order to relate these experiments to the synaptic plasticity data, we performed these experiments in current clamp in the presence of picrotoxin. Amygdalar neurons from wildtype and PAM$^{+/-}$ mice were patched in the continuous presence of 10 $\mu$M CuSO$_4$ and measures of intrinsic membrane properties were assessed. There was a small but significant depolarization of RMP in PAM$^{+/-}$ compared to wildtype neurons ($Wt: -70.6 \pm 0.6$ mV; PAM$^{+/-}: -67.6 \pm 1.0$ mV; $N=15$ Wt, 16 PAM$^{+/-}$; $p<0.05$). Moreover, there were no significant differences in the current-voltage plot relationship between amygdalar neurons from wildtype and PAM$^{+/-}$ mice in the presence of CuSO$_4$ (Fig. 4E).

Likewise, there were no genotypic differences in active membrane properties with respect to APT ($Wt: -30.9 \pm 1.0$ mV; PAM$^{+/-}: -29.4 \pm 0.7$ mV; $N=15$ Wt, 16 PAM$^{+/-}$; $p>0.05$) or APR ($Wt: 173.3 \pm 14.5$ pA; PAM$^{+/-}: 159.4 \pm 9.4$ pA; $N=15$ Wt, 16 PAM$^{+/-}$; $p>0.05$). Two-way ANOVA revealed a main effect of genotype on RMP ($p<0.05$), corresponding to the depolarization of RMP in the CuSO$_4$ condition. Two-way ANOVA also revealed a main effect of genotype on APT ($p<0.05$), wherein PAM$^{+/-}$ neurons had an approximately 2 mV depolarization in APT. Together, these results suggest no influence of CuSO$_4$ on intrinsic membrane properties of wildtype or PAM$^{+/-}$ amygdalar neurons relevant to synaptic plasticity.

We measured EPSP responses to a 10 min pulse of 10 $\mu$M CuSO$_4$ perfusion in neurons from wildtype mice given control water (Fig. 4F).

EPSPs recorded from wildtype neurons potentiated with Cu perfusion compared to baseline. This effect remained after 30 min of Cu washout, suggesting Cu-mediated potentiation of excitatory transmission is long-lasting.

To relate this finding to our LTP experiments, we simultaneously assessed the EPSP PPR with CuSO$_4$ perfusion and subsequent washout (Fig. 4G). There was a decrease in the PPR that corresponded with EPSP potentiation as was seen with LTP induction (Fig. 3C). Together these data indicate Cu perfusion in the presence of picrotoxin has a long-lasting, potentiating effect on pre-synaptic glutamate release at thalamic afferent amygdalar synapses.

Secreted endogenous Cu is essential for LTP

Since supplementation of Cu, both in vivo through the diet and in vitro through the perfusate, enhanced synaptic efficacy in the lateral amygdala, we next asked whether manipulation of endogenous Cu affected synaptic plasticity at these same synapses. Bathocuproine disulfonate (BCS), a cell-impermeant, highly specific Cu(I) chelator (De et al. 2007; Hawkins and Perrin 1963) was used in the presence of picrotoxin to chelate extracellular Cu. With BCS in the bath, there were no genotypic differences in passive membrane properties including RMP ($Wt: -71.7 \pm 1.1$ mV; PAM$^{+/-}: -68.8 \pm 1.6$ mV; $N=11$ Wt, 10 PAM$^{+/-}$; $p>0.05$), Ri ($Wt: 137.7 \pm 10.3$ M$\Omega$; PAM$^{+/-}: 139.3 \pm 9.1$ M$\Omega$; $N=11$ Wt, 10 PAM$^{+/-}$; $p>0.05$), or Cm ($Wt: 85.7 \pm 5.5$ pF; PAM$^{+/-}: 89.6 \pm 6.5$ pF; $N=11$ Wt, 10 PAM$^{+/-}$; $p>0.05$). Moreover, there were no significant differences in the current-voltage plot relationship between amygdalar neurons from wildtype and PAM$^{+/-}$ mice recorded in BCS (Fig. 5A). Likewise, there were no genotypic differences in active membrane properties with respect to APT ($Wt: -29.9 \pm 0.6$ mV; PAM$^{+/-}: -30.5 \pm 0.9$ mV; $N=11$ Wt, 10 PAM$^{+/-}$; $p>0.05$) or APR ($Wt: 181.8 \pm 16.9$ pA; PAM$^{+/-}: 165.0 \pm 10.7$ pA; $N=11$ Wt, 10 PAM$^{+/-}$; $p>0.05$). Two-way ANOVA revealed a main effect of genotype on RMP ($p<0.05$), representing a depolarization of RMP in PAM$^{+/-}$ neurons of less than 3 mV. Two-way ANOVA also revealed a genotype by pharmacological condition (absence versus presence of BCS) on APT ($p<0.05$), wherein wildtype APT was depolarized less than 3 mV by addition of BCS and PAM$^{+/-}$ neuronal APT remained unaffected. Again, these small differences are unlikely to correspond to any significant changes in input resistance. The Cu$^{2+}$ concentration in water was not significantly different from the Cu$^{2+}$ concentration in control water. Therefore, any effects seen in the presence of Cu$^{2+}$ were due to addition of Cu$^{2+}$ and not due to a change in Cu$^{2+}$ concentration. To test this hypothesis, we measured the Cu$^{2+}$ concentration in water before and after perfusion with Cu$^{2+}$ using a PicoSorb 4G ICP-MS sensor (Sartorius, Goettingen, Germany). We found that the Cu$^{2+}$ concentration in water was not significantly different from the Cu$^{2+}$ concentration in control water. Therefore, any effects seen in the presence of Cu$^{2+}$ were due to addition of Cu$^{2+}$ and not due to a change in Cu$^{2+}$ concentration.
have any influence on plasticity experiments.

Importantly, Cu chelation exerted no gross effects on the voltage responses to LTP induction in wildtype mice (Fig. 5B), eliminating the possibility of non-specific effects of BCS on membrane responses to the induction protocol that might account for its effects on LTP. Finally, baseline EPSP slopes were no different in the absence and presence of BCS in neurons from wildtype and PAM+/- mice. Two-way ANOVA revealed no main effects of or interactions between genotype and pharmacological condition on baseline EPSP slope (p values>0.05). Together these data argue strongly against any non-specific effects of BCS.

Establishment of LTP in wildtype slices (Fig. 5C). EPSPs recorded from PAM+/- amygdalar neurons, which do not exhibit LTP under these conditions, were unaffected by the addition of BCS to the perfusate. Washout of BCS was not feasible because of the additional time required. These experiments suggest that endogenous Cu normally plays a role in the establishment of LTP at thalamic afferent synapses.

We showed previously that LTP can be induced at thalamic afferents in slices from PAM+/- mice after a GABA_A receptor antagonist (CGP35348; 1 µM) is added to the perfusate along with picrotoxin (Gaier et al. 2010). GABA_A receptors are found both on the afferent presynaptic glutamatergic terminals and on the dendrites of the pyramidal neurons themselves (McDonald et al. 2004; Pan et al. 2009). Therefore, we next tested the effect of BCS on LTP in slices prepared from both wildtype and PAM+/- mice during blockade of both GABA_A and GABA_B receptors (Fig. 5D). The presence of BCS completely eliminated LTP in both wildtype and PAM+/- slices. These data suggest an essential role for endogenous, secreted Cu in amygdalar excitatory synaptic plasticity even in the absence of GABAergic transmission. Furthermore, the fact that chelation of extracellular Cu eliminated the genotypic difference in LTP in both pharmacological conditions supports the hypothesis that altered Cu secretion underlies the deficit in PAM+/- LTP.

**DISCUSSION**

In the current study, we showed that the fear memory deficit observed in PAM+/- mice was sensitive to dietary Cu supplementation. This response to dietary Cu was mimicked in vitro through correction of the PAM+/- synaptic plasticity deficit. Cu levels in the PAM+/- amygdala were decreased, suggesting that these fear memory and synaptic plasticity impairments resulted from a deficiency of Cu.

Extracellular Cu is necessary and sufficient for LTP at thalamic afferent amygdalar synapses. Together these data identify Pam heterozygosity as a cause of amygdalar Cu deficiency and reveal a role for Cu in amygdalar synaptic function that is critical to fear-based learning and memory (Fig. 6).

**Behavioral effects of dietary Cu:**

Supplemental Cu reversed the deficiency of the PAM+/- mice in cued fear conditioning to wildtype control levels (Fig. 2B); Cu-supplementation provoked increased freezing behavior among PAM+/- animals without affecting wildtype freezing. For cued fear conditioning, the behavioral responses are supported by thalamic and cortical inputs converging on the lateral amygdala, which activates the central amygdala (Davis 1990). Outputs from the central amygdala regulate emotional reactions that mediate freezing behaviors as well as autonomic and endocrine responses comprising the defensive fear response. Strikingly, genotypic differences in contextual fear conditioning, which is dependent on both hippocampal and amygdalar function, remained following dietary Cu supplementation (Fig. 2A). This may reflect Cu-insensitive hippocampal dysfunction in PAM+/- mice. In support of this hypothesis, Cu content was selectively reduced in the PAM+/- amygdala, and not different from wildtype in the whole forebrain (Bousquet-Moore et al. 2010) or hippocampus (Fig. 1). Moreover, dietary Cu supplementation does not affect forebrain Cu levels. Region-specific Cu handling in neurons is complex and deserves dedicated investigation in a separated set of experiments (Gaier et al. 2013b).

Cu deficient rodents display increased baseline anxiety-like behaviors (Fujiwara et al. 2006; Railey et al. 2010; Bousquet-Moore et al. 2010)
and altered gene expression (Levenson 2005; Gonzalez et al. 2008). Cu deficiency enhances the baseline acoustic startle response while Cu supplementation reduces this response (Fujiwara et al. 2006). Zn supplementation causes dietary Cu deficiency and prolonged immobility in contextual, but not cued, fear conditioning (Maret and Sandstead 2006; Chrosniak et al. 2006; Railey et al. 2010). Another study found no effect of dietary Cu on fear memory (Fujiwara et al. 2006). Global Cu deficiency from dietary manipulation differs greatly from the region-specific Cu deficiency observed in the PAM+/− amygdala, and may affect other brain regions important in the assessment of fear behaviors. Overall, our results are in agreement with previous fear conditioning studies supporting a role for Cu in amygdala function.

**Synaptic effects of dietary Cu:** We investigated the effect of Cu supplementation on LTP at the synapses of thalamic afferents onto pyramidal neurons in the lateral amygdala (Ledoux et al. 1990). Dietary Cu supplementation abrogated the effect of Pam heterozygosity on LTP, with no effect on LTP in wildtype slices. Importantly, Cu supplementation of PAM+/− mice eliminated the necessity for added GABA<sub>B</sub> receptor blockade for LTP induction (Fig. 3E). Most notably, Cu deficiency can activate adenosine monophosphate-activated protein kinase (AMPK) in brain tissue without changing AMP levels (Gyblina and Prohaska2008; Gaier et al. 2013a). AMPK phosphorylates the GABA<sub>B</sub> receptor at Ser<sup>783</sup> and promotes downstream signaling (Kuramoto et al. 2007). GABA<sub>B</sub> receptors are found both pre- and post-synaptically in the amygdala (McDonald et al. 2004; Pan et al. 2009; Terunuma et al. 2010), where pre-synaptic GABA<sub>B</sub> receptors modulate afferent neuronal membrane that is GABA-mediated hyperpolarizing effect of Cu on the patched neuronal membrane that is GABA-mediated electrophysiological study and the increased sensitivity of PAM<sup>−/−</sup> mice to the anxiolytic effects of benzodiazepines (Gaier et al. 2010), this result was particular interesting. The synaptic plasticity experiments were performed in the presence of picrotoxin, whereas the Cu bath-application experiment was performed without any pharmacologic blockers, revealing a hyperpolarizing effect of Cu on the patched neuronal membrane that is GABA-mediated (Fig. 4D).

Despite these concerns, we found that bath application of 10 µM CuSO<sub>4</sub> to amygdala slices was sufficient to potentiate wildtype synapses without LTP induction (Fig. 4F). In addition, the potentiating effect of Cu is mediated through an increase in pre-synaptic release probability as indicated by a corresponding reduction in the PPR, just as we observed during LTP induction in our model. This result supports our hypothesis that Cu release during LTP induction plays a role in potentiation. Future experiments will focus on PAM<sup>−/−</sup> responses to Cu bath application, how Cu deficiency affects GABAergic systems and how this relates to Cu signaling at synapses.

Our observations in the amygdala contrast with the hippocampus (Gaier et al. 2013a), and could result from differences in GABAergic tone. Bath-applied Cu inhibits LTP in the hippocampus at concentrations close to those used in our study (Doreulee et al. 1997; Leiva et al. 2009; Leiva et al. 2003; Salazar-Weber and Smith 2011). GABAergic tone is much higher in the amygdala than in the hippocampus (Gaier et al. 2010), as evidenced by the necessity to block GABA<sub>A</sub> receptors to observe amygdalar but not hippocampal LTP (Bauer et al. 2002; Tully et al. 2007; Gaier et al. 2010; Ma et al. 2008). This
Physiological levels of Cu have inhibitory effects on multiple synaptic receptor/ion channels, many of which are important in gating and/or regulating LTP (Gaier et al. 2013). Synaptic Cu levels rise as high as 100-250 µM (Kardos et al. 1996), while low µM levels of Cu bind to and modulate GABA<sub>A</sub> receptors (Fisher and Frei 1995; McGee et al. 2013). We used BCS at 50 µM; other studies have used higher concentrations (200-360 µM) still at least 10-fold greater than its affinity for Cu(I). BCS has been used to chelate Cu (McGee et al. 2013), and Cu(I) specific cell impermeant chelator (Hawkins and Perrin 1963). Biochemically isolated synaptic fractions from the amygdala and hippocampus (Jackson et al. 2006; Akatsu et al. 2012). ATP7A and Cu are present in mouse brain analysis. Cu-binding proteins and transporters are redistributed to more peripheral cellular sites with increased Cu exposure and neuronal activity (Lutsenko et al. 2007; Schlief et al. 2006). PAM<sup>−/−</sup> animals have increased susceptibility to dietary Cu deficiency. It is our hypothesis that Cu depletion, suggesting disrupted total body Cu homeostasis. Manipulation of Cu availability in vitro and in vivo influences the expression and proteolytic processing of PAM (De et al. 2007; Bousquet-Moore et al. 2010). Dietary Cu supplementation has dramatic effects on PAM<sup>−/−</sup> synaptic function and behavior without changing total brain Cu levels in mice of either genotype (Bousquet-Moore et al. 2010). Total brain levels of Cu are normal in PAM<sup>−/−</sup> mice (Bousquet-Moore et al. 2010), but a clear deficit was found in the amygdala (Fig. 1), while Cu supplementation could go undetected in whole brain analysis. Cu-binding proteins and transporters are redistributed to more peripheral cellular sites with increased Cu exposure and neuronal activity (Lutsenko et al. 2007; Schlief et al. 2006). PAM may play a role in this regulatory system, which is disrupted with genetic PAM deficiency. It is our hypothesis that PAM plays an essential part in regulating Cu handling in the nervous system, likely with region-specificity, modulating gene expression and altering cytoskeletal organization.

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**Potential mechanism:** GABA<sub>A</sub>ergic inhibition presumably acts upstream to suppress Cu release, which is essential for amygdalar LTP.
University and Megan Duffy for performing the ICPMS measurement at the elemental analysis core facility at OHSU. We also thank Drs. Srdjan Antic and Eric Levine for many helpful discussions and solutions to electrophysiological challenges. This work was supported by grants from the National Institutes of Health: DK32949 (BAE, REM); NS41224; MH60451 (WCW).


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FIGURE LEGENDS

Fig. 1. Reduced Cu in PAM⁺⁻ amygdala.

Bilateral amygdalar and dorsal hippocampi were
isolated from individual Wt and PAM⁺⁻ mice by
tissue punch. Tissue samples were analyzed for
Cu concentration using Inductively Coupled
Plasma Mass Spectrometry; Cu levels were
normalized to protein concentration for
comparisons by genotype and brain region. Cu
concentrations according to protein are plotted
by genotype for amygdala and hippocampus.

⁎p<0.05, compared to Wt mice within brain
region; ⁎p<0.05, compared to amygdala within
genotype; n = 7-9 mice/genotype/brain region.

Fig. 2. Cu supplementation does not affect
contextual fear conditioning while rescuing
PAM⁺⁻ responses in cued testing. Two cohorts
of Wt and PAM⁺⁻ littermates were given either
control water or Cu-supplemented water for 14
days prior to conditioning. (A) For one cohort,
percent time freezing during the 5 min
contextual test was assessed 24 h after
conditioning (each minute averaged). (B) For the
other cohort, percent time freezing in the 2 min
prior to (PreTone) and the 3 min during
presentation (Tone) of the CS in cued testing
was assessed 24 h after conditioning. *p<0.05,
compared to Wt mice within the Cu condition;
⁎p<0.05, compared to Cu condition within
genotype; n = 9-11 mice/genotype/Cu condition.

Fig. 3. Cu supplementation rescues the
PAM⁺⁻ amygdalar synaptic plasticity deficit.

(A) Left, schematic of a coronal brain slice
containing the amygdala; recording electrode
(re) placement in the lateral nucleus of the
amygdala (L) and stimulating electrode (stim)
placement in thalamic afferent fibers are
illustrated. Also depicted for reference: cerebral
cortex (Cort), hippocampus (H), thalamus (Th),
hypothalamus (Hy), basolateral and central
nuclei of the amygdala (BL, C). Right, synaptic
schematic illustrating recording from a lateral
amygdala pyramidal neuron (PN), stimulation of
thalamic afferents and feed-forward inhibition,
which is mediated by GABAergic interneurons
and was blocked in these experiments by
inclusion of 100 µM picrotoxin (PTX). (B) Wt and
PAM⁺⁻ littermates were fed control water before
amygdalar slice preparation for LTP experiments
using an action potential pairing induction
paradigm starting at minute 0 (arrow). The time-
course (1 min bins) of averaged LTP
experiments for Wt (Black) and PAM⁺⁻ (gray)
neurons are shown. Normalized rise slopes of
the EPSPs (NL EPSP slope) were used to
measure synaptic efficacy. Baseline values
correspond to responses during min -5-0 and
post-induction LTP values correspond to
responses during min 30-40. Traces above plots
depict averaged EPSPs corresponding to the

time periods outlined by solid (baseline) and
dashed (post-induction) lines. (C) Paired pulse
ratios (PPR; rise slope ratio EPSP₂/EPSP₁) were
recorded throughout the LTP time course in B
and are plotted in 5 min bins for by genotype. .

Absolute PPR values averaged over the
baseline (BL; -5-0 min) and post-induction (LTP;
30-40 min) are plotted by genotype in the inset.

(D,E) Current-Voltage plots generated from
amygdalar neurons from Wt and PAM⁺⁻
littermates fed normal (C) and Cu-supplemented
(D) water. (F) Representative voltage responses
to the paired LTP induction protocol recorded in
amygdalar neurons from Wt mice fed control
water versus Cu supplemented water. (G) Wt
and PAM⁺⁻ littermates were fed Cu
supplemented water before amygdalar slice
preparation for LTP experiments, which were
conducted as in B. *p<0.05, compared to
baseline within genotype and Cu condition;
NSNot significant compared to baseline
(Wilcoxon Signed Rank Test); *p< 0.05,
compared to Wt mice within Cu condition;
⁎p<0.05, compared to control water within
genotype (unpaired t-test); n = 7-8

Fig. 4. Bath-applied CuSO₄ transiently
enhances inhibitory synaptic transmission
and restores PAM⁺⁻ LTP. (A) Pyramidal
neurons in the lateral amygdala of Wt mice were
patched with pipettes filled with ICS containing
QX-314 and voltage clamped at Vholding = -35
mV. Left, synaptic schematic depicting the
recording paradigm. Current responses to
thalamic afferent stimulation were recorded for
45 min while CuSO₄ (10 µM) was applied from
min 0-10 and subsequently washed out. Right,
averaged traces depicting a representative
response to Cu²⁺ application. Current responses
were biphasic: fast inward (downward) current
represents excitatory glutamatergic transmission
and delayed outward (upward) current
represents feed-forward GABAergic inhibitory
transmission. Traces correspond to the following
time bins: Baseline, -5-0 min; Cu, 5-10 min;
Wash, 35-40 min. (B) Averaged time-course
data depicting the responses of normalized
inward rise slope and peak-to-peak slope
representing excitatory and inhibitory
components of the response, respectively; n = 7
Wt neurons. (C) Time-course of normalized
inhibitory post-synaptic current (IPSC) slopes
recorded from a representative pyramidal
neuron at \( V_{\text{holding}} = -35 \text{ mV} \) in the presence of
CNQX to block AMPA glutamate receptors (from \( N=3 \)). The lateral amygdala parenchyma was
stimulated to directly activate the interneuronal
network to evoke IPSCs (inset). CuSO₄ was
applied as in B. Open diamonds represent
individual responses; Filled diamonds represent
averaged responses in 5 min bins. (D) Time-
course of normalized holding current (IHolding)
responses to CuSO₄ in the absence (aCSF) and
presence of PTX. + \( p < 0.05 \), compared to baseline
(Wilcoxon Signed Rank Test); n = 7 Wt neurons.

**Fig. 5. Extracellular Cu is essential for LTP at
thalamic afferent amygdalar synapses.** (A)
Current-Voltage plots generated from amygdalar
neurons from Wt and PAM+/- littermates in the
presence of 100 µM PTX and 50 µM BCS. (B)
Representative voltage responses to the paired
LTP induction protocol recorded from Wt
amygdalar neurons in the presence of PTX and
BCS. (C,D) LTP was induced at thalamic
afferent synapses of lateral amygdala pyramidal
neurons as in Fig. 4 using slices prepared from
Wt and PAM+/- littermates fed control water. The
experiment in (C) was conducted in the
presence of 100 µM PTX and 50 µM BCS; the
experiment in (D) also included 1 µM
CGP34358. Time-course of averaged LTP
experiments with representative traces depicted
above. Wt and PAM+/- LTP in the presence of
PTX alone and both PTX and CGP without BCS
was reported previously (Gaier et al. 2010);
levels are indicated for reference (dashed lines).
NSNot significant compared to baseline
(Wilcoxon Signed Rank Test); n = 11-12 Wt; 6-7
PAM+/-.

**Fig. 6. Synaptic model of Cu homeostasis.**
(A) Schematic diagram of an afferent synapse in
the lateral amygdala and LTP. The legend
identifies molecules important to Cu
homeostasis and LTP. The thalamic afferents
terminating on lateral amygdalar pyramidal
neurons in PAM+/- mice do not exhibit LTP under
the usual recording conditions, but *in vivo* Cu
supplementation restores LTP to the level seen
in Wt mice. Moreover, *in vitro* perfusion of
CuSO₄ (green) is sufficient to poteniate
amygdalar synapses in wildtype mice. Acute
removal of extracellular Cu using the chelator
BCS (red) eliminated LTP at Wt synapses and at
PAM+/- synapses in which LTP was uncovered
using a GABAB antagonist [as in (Gaier et al.
2010)]. Bath application of Cu to Wt slices,
which enhances inhibitory currents without
having a major effect on excitatory currents,
does not mimic the actions of endogenous Cu.
Ablation of LTP by Cu chelation, despite full
GABAergic blockade, indicates that Cu acts
downstream of GABAergic inhibition. Cu could
be contributed by presynaptic terminals,
postsynaptic spines or interneurons. Release of
Cu is facilitated by Ca²⁺ influx through voltage
gated Ca²⁺ channels or NMDA receptors, and
inhibited by GABA receptor activation. While the
evidence that Cu is important to LTP at this
synapse is compelling, the underlying
mechanism remains to be uncovered.

QX-314 also blocks the slow, GABAA-mediated
IPSP (Duvarci and Pare 2007).