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

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Gaier ED, Rodriguez RM, Zhou J, Ralle M, Wetsel WC, Eipper BA, Mains RE. In vivo and in vitro analyses of amygdalar function reveal a role for copper. J Neurophysiol 111: 1927–1939, 2014. First published February 19, 2014; doi:10.1152/jn.00631.2013.—Mice with a single copy of the peptide amidating monoxygenase (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 ED, Rodriguez RM, Ma XM, Sivaramakrishnan S, Bousquet-Moore D, Wetsel WC, Eipper BA, Mains RE. J Neurosci 30: 13656–13669, 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 wild-type slices with bathocuprine disulfonate, a highly selective, cell-impermeant Cu chelator. Interestingly, bath-applied CuSO4 had no effect on excitatory currents but reversibly potentiated the disynaptic inhibitory current. Bath-applied CuSO4 was highly selective, cell-impermeant Cu chelator. Interestingly, bath-applied CuSO4 had no effect on excitatory currents but reversibly potentiated the disynaptic inhibitory current. Bath-applied CuSO4 was sufficient to potentiate wild-type 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.

peptidylglycine alpha-amidating monoxygenase; fear; synaptic plasticity; learning and memory; GABA

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, respectively (La Fontaine and Mercer 2007; Lutsenko et al. 2007). Marginal dietary Cu deficiency in wild-type 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 monoxygenase (PAM), one of several enzymes essential for neuropeptide biosynthesis (Mains and Eipper 1999), and dopamine β-monoxygenase, the enzyme that converts dopamine into norepinephrine (Kliman 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 midgestation, but mice with a single functional copy of Pam (Pam+/−) grow and reproduce normally (Czyzyk et al. 2005). Although indistinguishable in appearance from wild-type mice, Pam+/− mice display several striking behavioral deficits: impaired thermoregulation, increased seizure susceptibility, enhanced anxiety-like behavior, deficient fear conditioning, and fear potentiation of the acoustic startle response (Bousquet-Moore et al. 2009, 2010; Gaier et al. 2010). Since several of these behavioral phenotypes are seen in marginally Cu-deficient wild-type 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, 2010). Notably, amidated neuropeptide levels were only slightly altered by Pam heterozygosity or Cu supplementation (Bousquet-Moore et al. 2010; Czyzyk et al. 2005; Yin et al. 2011), 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 (Kim et al. 2010; Lutsenko et al. 2007). 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, nonenzymatic 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 (LeDoux 2007; Sah et al. 2003) and provides one of the best systems for relating behav-
ioral to electrophysiological studies (Kim and Jung 2006; Ledoux 2000; Maren and Quirk 2004).

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 affect ion channel function directly (Gaier et al. 2013a; Kardos et al. 1989; Mathie et al. 2006; Tamano and Takeda 2011), 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 whether Cu levels differed between PAM$^{+/−}$ and wild-type 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 amygdala synaptic physiology. Finally, we analyzed the role of Cu in normal amygdalar synaptic function in wild-type 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. Wild-type and PAM$^{+/−}$ littermates ($≥$20 generations backcrossed onto a C57BL/6J background) were weaned by postnatal day 21 (P21) and group-housed. Animals were maintained on a 12:12-h light-dark cycle (lights on at 0700) with ad libitum food and water. Behavioral experiments were conducted at Duke University between 1100 and 1400, where adult male and female wild-type and PAM$^{+/−}$ mice (12–20 wk of age) were tested. Since no sex differences were observed, these data were pooled. Dietary Cu supplementation included 300 parts per million (1.2 mM) CuSO$_4$ in reverse-osmosis drinking water for 10–14 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 National Institutes of Health guidelines for animal care.

**Fear Conditioning**

Mice were tested in a Med Associates chamber (St. Albans, VT) in either context or cued fear conditioning 24 h after training (Porton et al. 2010; Taylor et al. 2008) and were conditioned with a single 30-s, 72-dB tone (CS) that 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 heated to 32°C and perfused with aerated aCSF. Electrode placement in the internal capsule recruited thalamic afferent axons (Weiskopf and Ledoux 1999). For Cu wash-in experiments, neurons were clamped at $V_{holding} = −35$ mV to record excitatory and feedforward inhibitory transmission. Evoked inhibitory postsynaptic currents (eIPSCs) 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 CuSO$_4$. Paired pulses were applied at a 50-ms interval when indicated. Paired-pulse ratios (PPRs) were calculated as the excitatory postsynaptic potential ratio of EPSP$_2$ to EPSP$_1$ (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; Weiskopf et al. 1999) and 1 μM CGP-35348 (CGP; Tocris Biosciences, Ellisville, MO) where indicated (Gaier et al. 2010; Shaban et al. 2006). The holding current was adjusted to maintain $V_m = −70$ mV throughout the course of experiments. Stimulation strength was adjusted to produce a 3- to 6-mV EPSP. The 10–90% rise slope of 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 1-type voltage-gated Ca$^{2+}$ 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 at least +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 postinduction; 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. CGP (1 μM) was used to block slow GABA$_A$ receptor-mediated transmission. AMPA receptors were

**Electrophysiology**

**Slice preparation.** Male wild-type and PAM$^{+/−}$–P49 littermates were decapitated, and their brains were quickly removed, placed into ice-cold artificial cerebrospinal fluid (aCSF) containing, in mM, 125 NaCl, 26 NaHCO$_3$, 10 glucose, 2.3 KCl, 2 CaCl$_2$, 2 MgSO$_4$, 1.26 KH$_2$PO$_4$ (310 mosmol/kgH$_2$O, pH 7.3), and aerated with 95% O$_2$–5% CO$_2$ (Zhou et al. 2008). Coronal slices, 300 μm thick, were incubated at room temperature for ≥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- to 5-MΩ tips. The internal pipette solution contained, in mM, 135 K-gluconate, 10 HEPES, 10 phosphocreatine (Na salt), 3 Na$_2$ATP, 2 MgCl$_2$, 0.3 Na$_2$GTP, pH 7.3, 285 mosmol/kgH$_2$O (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 on achieving whole cell configuration (25 × 50-pA steps, −300 to +900 pA; 500-ms steps), and the firing pattern was used to verify pyramidal cell type. Input resistance ($R_i$) was continuously monitored using either a 5-mV voltage or 25-pA current step. Neurons were eliminated from analysis if $R_i$ changed by >20%. Bath application of 10 μM CuSO$_4$ did not affect $R_i$ (aCSF, 151.1 ± 6.2 MΩ; CuSO$_4$, 140.9 ± 9.5 MΩ; n = 7 wild-type 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 (Weiskopf and Ledoux 1999). For Cu wash-in experiments, neurons were clamped at $V_{holding} = −35$ mV to record excitatory and feedforward inhibitory transmission. Evoked inhibitory postsynaptic currents (IPSCs) 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 CuSO$_4$. Paired pulses were applied at a 50-ms interval when indicated. Paired-pulse ratios (PPRs) were calculated as the excitatory postsynaptic potential ratio of EPSP$_2$ to EPSP$_1$ (Gaier et al. 2010).

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Cu Measurements

Amygdalae and dorsal hippocampi were isolated from individual adult male mice (9 wild-type; 8 PAM**/−**); 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 of 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 20,000 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 1,550 W, an argon plasma gas flow rate of 15 l/min, and an argon 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 of 50% HNO3 (TraceMetal Grade; Fisher Scientific) solution. Following overnight, room temperature digestion of the samples, 850 µl of 1% HNO3 (TraceMetal Grade; Fisher Scientific) was added to the sample. CuSO4 was dissolved in H2O to yield a stock solution, which was then diluted in aCSF. Cu at 10 µM has been used in previous studies demonstrating synaptic effects (Doreuеe et al. 1997; Leiva et al. 2000; Peters et al. 2011).

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 (Stats 20 program; IBM SPSS Statistics, Chicago, IL). The fear conditioning data were analyzed by repeated-measures ANOVA (RMANOVA) and Bonferroni-corrected pairwise comparisons as the post hoc test. Synaptic potentiation above baseline was assessed using the nonparametric Wilcoxon signed-rank test; comparisons between conditions were made using Student’s t-test (unpaired, 2-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, 2010), we reasoned that the amygdalar dysfunction exhibited by PAM**+/−** mice (Gaier et al. 2010) may result from deficiency in Cu in this brain region. We tested this hypothesis directly by measuring total tissue Cu in punches of amygdala and hippocampus taken from wild-type and PAM**+/−** mice (Fig. 1). Two-way ANOVA found a significant main effect of genotype with lower concentrations of Cu in PAM**+/−** mice compared with wild-type (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 wild-type 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 wild-type 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 wild-type 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. Wild-type and PAM**+/−** mice were fed either a copper-replete diet (3 ppm Cu) or a Cu-deficient diet (0.01 ppm Cu) from weaning. After 4 weeks of dietary treatment, the mice were rechallenged with the CS+/− and CS− to test baseline fear memory. There were no differences in fear memory between wild-type mice fed the Cu-replete or Cu-deficient diets (Fig. 2A). In contrast, the Cu-deficient diet in PAM**+/−** mice reduced fear memory (P < 0.05) compared with wild-type mice (Fig. 2A). These data are consistent with the hypothesis that Cu is necessary for normal fear memory consolidation in PAM**+/−** mice.

Fig. 1. Reduced Cu in PAM**+/−** amygdala. Bilateral amygdalae (Amyg) and dorsal hippocampi (Hipp) were isolated from individual wild-type (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 with Wt mice within brain region; †P < 0.05, compared with amygdala within genotype; n = 7–9 mice/genotype/brain region.
PAM\textsuperscript{+/-} mice received reverse-osmosis drinking water (control) or drinking water supplemented with CuSO\textsubscript{4} 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).

Wild-type and PAM\textsuperscript{+/-} mice given control water were indistinguishable throughout conditioning, and both genotypes showed similar small increases in freezing immediately following the CS-US pairing (data not shown). Twenty-four hours later, mice were examined in a test for contextual fear conditioning (Fig. 2A). RM ANOVA revealed 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\textsuperscript{+/-} mice consistently had lower levels of freezing for all time points compared with wild-type controls \((P < 0.05)\). 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 before CS presentation. RM ANOVA revealed that, relative to the pretone interval, both genotypes responded to the CS by freezing over the 3-min exposure interval \((P < 0.05)\). Significant time by genotype and time by genotype by Cu condition interactions \((P < 0.05)\) were observed. A posteriori comparisons under the control water condition demonstrated that PAM\textsuperscript{+/-} mice froze significantly less to the CS than wild-type mice \((P < 0.05)\). Freezing by wild-type mice was unaffected by Cu supplementation \((P > 0.05)\). Notably, Cu supplementation increased freezing of the PAM\textsuperscript{+/-} mice \((P < 0.05)\) to the level of the Cu-supplemented wild-type 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\textsuperscript{+/-} 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\textsuperscript{+/-} 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 wild-type or PAM\textsuperscript{+/-} mice affected LTP at thalamic afferent synapses within the lateral nucleus of the amygdala (Fig. 3). Thalamic afferent fiber bundles were stimulated and EPSPs were recorded from pyramidal neurons using whole cell patch-clamp methods. LTP was induced using a paired LTP induction protocol (Bauer et al. 2002; Gaier et al. 2010; Fig. 3A). In acute slices of wild-type amygdala, reliable induction of LTP requires limitation of GABAergic inhibition, typically using the GABA\textsubscript{A} receptor antagonist PTX \((100 \mu M\) in the perfusate; Bauer et al. 2002; Gaier et al. 2010; Tully et al. 2007). EPSPs recorded from pyramidal neurons of wild-type mice given control water were potentiated, whereas EPSPs recorded from PAM\textsuperscript{+/-} 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\textsuperscript{+/-} compared with wild-type 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 PPR (EPSP\textsubscript{2}/EPSP\textsubscript{1}) was monitored throughout the LTP time course (Fig. 3C). As expected for glutamatergic synapses, the PPR was >1.0 at thalamic afferent synapses, reflecting the contribution of accumulated Ca\textsuperscript{2+} at presynaptic terminals after the first stimulation. There were no differences in PPRs between genotypes at baseline (Fig. 3C, inset). Wild-type LTP coincided with a significant reduction in the PPR, indicating an enhancement in initial neurotransmitter release. By contrast, PAM\textsuperscript{+/-} 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\textsuperscript{+/-} amygdala and indicate a presynaptic component in the mechanism of potentiation.

Since intrinsic membrane properties can influence synaptic plasticity through postsynaptic mechanisms, we compared measures of passive (resting membrane potential, RMP; \(R_i\); membrane capacitance, \(C_m\)) and active (action potential threshold, APT; action potential rheobase, APR) membrane proper-

![Fig. 2. Cu supplementation does not affect contextual fear conditioning while rescuing PAM\textsuperscript{+/-} responses in cued testing. Two cohorts of Wt and PAM\textsuperscript{+/-} littermates were given either control water or Cu-supplemented water (Cu Supp) for 14 days before conditioning. A: for 1 cohort, percentage of time freezing during the 5-min contextual test was assessed 24 h after conditioning (each minute averaged). B: for the other cohort, percentage of time freezing in the 2 min before (PreTone) and the 3 min during presentation (Tone) of the single 30-s, 72-dB tone (CS) in cued testing was assessed 24 h after conditioning. *\(P < 0.05\), compared with Wt mice within the Cu condition; #\(P < 0.05\), compared with Cu condition within genotype; \(n = 9–11\) mice/genotype/Cu condition.](http://jn.physiology.org/)

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Fig. 3. Cu supplementation rescues the PAM\(^{+/−}\) amygdalar synaptic plasticity deficit. A, left: schematic of a coronal brain slice containing the amygdala; recording electrode (rec) 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), and basolateral and central nuclei of the amygdala (BL and C). Right: schematic illustrating recording from a lateral amygdala pyramidal neuron (PN), stimulation of thalamic afferents, and feedforward inhibition, which is mediated by GABAergic interneurons (IN) and was blocked in these experiments by inclusion of 100 \(\mu\)M picrotoxin (PTX). B: Wt and PAM\(^{+/−}\) littermates were fed control water before amygdalar slice preparation for long-term potentiation (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 excitatory postsynaptic potentials (NL EPSP slope) were used to measure synaptic efficacy. Baseline values correspond to responses during minutes \(-5\) to 0, and postinduction LTP values correspond to responses during minutes \(30–40\). Traces above plots depict averaged EPSPs corresponding to the time periods outlined by solid (baseline) and dashed (postinduction) lines. C: paired-pulse ratios (PPRs; rise slope ratio EPSP\(_2\) to EPSP\(_1\)) were recorded throughout the LTP time course in B and are plotted in 5-min bins by genotype. Absolute PPR values averaged over the baseline (BL; \(-5\) to 0 min) and postinduction (LTP; \(30–40\) min) are plotted by genotype in the inset. D and 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 vs. 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 with baseline within genotype and Cu condition; NS, not significant compared with baseline (Wilcoxon signed-rank test); \(*P < 0.05\), compared with Wt mice within Cu condition; \#P < 0.05, compared with control water within genotype (unpaired t-test); \(n = 7–8\) mice/genotype/Cu condition.
there were no significant differences in the current-voltage relationship between wild-type and PAM\textsuperscript{+/-} amygdalar neurons (Fig. 3D). APT was depolarized by <3 mV in PAM\textsuperscript{+/-} neurons (wild-type: -32.5 ± 0.8 mV; PAM\textsuperscript{+/-}: -29.7 ± 0.7 mV; n = 13 wild-type, 13 PAM\textsuperscript{+/-}; P < 0.05); however, a difference of this magnitude is unlikely to be the sole postsynaptic factor driving the difference in synaptic plasticity. APR was not different between genotypes (wild-type: 169.2 ± 16.5 pA; PAM\textsuperscript{+/-}: 146.2 ± 16.5 pA; n = 13 wild-type, 13 PAM\textsuperscript{+/-}; P > 0.05). Therefore, intrinsic membrane properties are unlikely to contribute to the deficit in synaptic plasticity in PAM\textsuperscript{+/-} amygdalar neurons under these conditions.

Next, we examined neuronal and synaptic function of amygdalar neurons from wild-type and PAM\textsuperscript{+/-} 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 (wild-type: -69.6 ± 3.2 mV; PAM\textsuperscript{+/-}: -68.5 ± 2.7 mV; n = 7 wild-type, 8 PAM\textsuperscript{+/-}; P > 0.05). R\textsubscript{i} (wild-type: 125.3 ± 10.6 MΩ; PAM\textsuperscript{+/-}: 163.1 ± 16.9 MΩ; n = 7 wild-type, 8 PAM\textsuperscript{+/-}; P > 0.05), or C\textsubscript{m} (wild-type: 100.1 ± 7.5 pF; PAM\textsuperscript{+/-}: 96.5 ± 4.1 pF; n = 7 wild-type, 8 PAM\textsuperscript{+/-}; P > 0.05). Moreover, there were no significant differences in the current-voltage plot relationship between amygdalar neurons from wild-type and PAM\textsuperscript{+/-} mice that were supplemented with dietary Cu (Fig. 3E). Likewise, there were no genotypic differences in active membrane properties with respect to APT (wild-type: -35.0 ± 3.4 mV; PAM\textsuperscript{+/-}: -34.4 ± 2.4 mV; n = 7 wild-type, 8 PAM\textsuperscript{+/-}; P > 0.05) or APR (wild-type: 150.0 ± 10.9 pA; PAM\textsuperscript{+/-}: 131.3 ± 9.1 pA; n = 7 wild-type, 8 PAM\textsuperscript{+/-}; P > 0.05). Two-way ANOVA found no main effects of or interactions between genotype and dietary condition with respect to parameters of passive and active membrane properties (P values >0.05). Cu supplementation exerted no gross effects on the voltage responses to LTP induction in wild-type mice (Fig. 3F). Together, these results suggest no influence of dietary Cu supplementation on intrinsic membrane properties of wild-type or PAM\textsuperscript{+/-} 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 wild-type mice exhibited LTP that was indistinguishable from LTP in wild-type mice receiving control water (Fig. 3, G vs. B). By contrast, Cu supplementation increased PAM\textsuperscript{+/-} LTP to levels significantly above PAM\textsuperscript{+/-} 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\textsuperscript{+/-} amygdala.

The fact that providing PAM\textsuperscript{+/-} and wild-type 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\textsuperscript{+/-} 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 wild-type and PAM\textsuperscript{+/-} 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\textsuperscript{+/-} mice are the result of deficient amygdalar Cu, one would predict an effect of exogenous Cu on synaptic plasticity at amygdalar afferent synapses. We first assessed the effects of Cu on baseline synaptic transmission in the wild-type amygdala. We clamped pyramidal neurons of the lateral nucleus in wild-type mice at V\textsubscript{holding} = -35 mV; this allowed us to monitor simultaneously excitatory and inhibitory responses to thalamic afferent stimulation when 10 μM CuSO\textsubscript{4} was perfused (Doreullee et al. 1997; Leiva et al. 2003). PTX was not present for these experiments (Fig. 4A). As expected, stimulation of thalamic glutamatergic afferents produced a monosynaptic excitatory inward current that was closely followed by a disynaptic inhibitory outward current (Fig. 4A). Notably, the stimulation current necessary to elicit biphasic responses was substantially greater than currents used in plasticity experiments. Disynaptic outward currents represent fast feedforward inhibition of thalamic afferent synapses mediated by GABA\textsubscript{A} receptors (Duarvari 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\textsubscript{A} receptor blockade with 100 μM PTX (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 slope of the inward current and the efficacy of feedforward inhibition using the peak-to-peak slope. Inward and outward events were monitored before, during, and after the addition of 10 μM CuSO\textsubscript{4} to the perfusate (Fig. 4B). Estimates of synaptic Cu levels vary, but 10 μM is well within the range thought to be physiologically relevant (Doreullee et al. 1997; Goldschmith et al. 2005; Leiva et al. 2003; McGee et al. 2013; Peters et al. 2011). 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-min Cu washout period. These data support a net GABAergic inhibitory role for bath-applied Cu in the wild-type amygdala.

To examine the effect of Cu on GABA\textsubscript{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\textsuperscript{+} channels were blocked exclusively in the clamped cell by the inclusion of membrane impermeant QX-314 in the intracellular pipette solution. The slower GABA\textsubscript{B} receptor-mediated component of inhibition was blocked by QX-314 (Nathan et al. 1990). To elicit 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 of the maximal eIPSC. Perfusion of CuSO\textsubscript{4} and subsequent wash had no effect on eIPSCs (Fig. 4C). Therefore, Cu is unlikely to enhance feedforward 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 postsynaptic neuron in the absence of stimulation (Fig. 4D). This effect did not correspond to any significant changes in $R_E$ (see METHODS). Inclusion of PTX 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. To relate these experiments to the synaptic plasticity data, we performed these experiments in current-clamp in the presence of PTX. Amygdalar neurons from wild-type 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 with wild-type neurons (wild-type: $−70.6 ± 0.6$ mV; PAM$^{+/−}$: $−67.6 ± 1.0$ mV; $n = 15$ wild-type, 16 PAM$^{+/−}$; $P < 0.05$); however, this small difference is unlikely to influence plasticity experiments. There were no significant genotypic differences in $R_E$ (wild-type: 135.4 ± 9.8 $MΩ$; PAM$^{+/−}$: 132.7 ± 7.3 $MΩ$; $n = 15$ wild-type, 16 PAM$^{+/−}$; $P > 0.05$) or $C_m$ (wild-type: 89.6 ± 4.9 pF; PAM$^{+/−}$: 91.4 ± 3.8 pF; $n = 15$ wild-type, 16 PAM$^{+/−}$; $P > 0.05$). Moreover, there were no significant differences in the current-voltage plot relationship between amygdalar neurons from wild-type 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 (wild-type: $−30.9 ± 1.0$ mV; PAM$^{+/−}$: $−29.4 ± 0.7$ mV; $n = 15$ wild-type, 16 PAM$^{+/−}$; $P > 0.05$) or APR (wild-type: 173.3 ± 14.5 pA; PAM$^{+/−}$: 159.4 ± 9.4 pA; $n = 15$ wild-type, 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 ~2-mV depolarization in APT. Together, these results suggest no influence of CuSO$_4$ on intrinsic membrane properties of wild-type 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 wild-type mice given control water (Fig. 4F). EPSPs recorded from wild-type neurons potentiated with Cu perfusion compared with 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 PTX has a long-lasting, potentiating effect on presynaptic glutamate release at thalamic afferent amygdalar synapses.

**Secreted Endogenous Cu is Essential for LTP**

Since supplementation of the perfusate, enhanced synaptic efficacy in the lateral amygdala, we next asked whether manipulation of endogenous Cu affected synaptic plasticity at these same synapses. BCS, a cell-impermeant, highly specific Cu(I) chelator (De et al. 2007; Hawkins and Perrin 1963), was used in the presence of PTX to chelate extracellular Cu. With BCS in the bath, there were no genotypic differences in passive membrane properties including RMP (wild-type: $−71.7 ± 1.1$ mV; PAM$^{+/−}$: $−68.8 ± 1.6$ mV; $n = 11$ wild-type, 10 PAM$^{+/−}$; $P > 0.05$), $R_E$ (wild-type: 137.7 ± 10.3 $MΩ$; PAM$^{+/−}$: 139.3 ± 9.1 $MΩ$; $n = 11$ wild-type, 10 PAM$^{+/−}$; $P > 0.05$), or $C_m$ (wild-type: 85.7 ± 5.5 pF; PAM$^{+/−}$: 89.6 ± 5.5 pF; $n = 11$ wild-type, 10 PAM$^{+/−}$; $P > 0.05$). Moreover, there were no significant differences in the current-voltage plot relationship between amygdalar neurons from wild-type and PAM$^{+/−}$ mice recorded in BCS (Fig. 5A). Likewise, there were no genotypic differences in active membrane properties with respect to APT (wild-type: $−29.9 ± 0.6$ mV; PAM$^{+/−}$: $−30.5 ± 0.9$ mV; $n = 11$ wild-type, 10 PAM$^{+/−}$; $P > 0.05$) or APR (wild-type: 181.8 ± 16.9 pA; PAM$^{+/−}$: 165.0 ± 10.7 pA; $n = 11$ wild-type, 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 <3 mV. Two-way ANOVA also revealed a genotype by pharmacological condition (absence vs. presence of BCS) on APT ($P < 0.05$) wherein wild-type APT was depolarized $<3$ mV by addition of BCS and PAM$^{+/−}$ neuronal APT remained unaffected. Again, these small differences are unlikely to have any influence on plasticity experiments. Importantly, Cu chelation exerted no gross effects on the voltage responses to LTP induction in wild-type mice (Fig. 5B), eliminating the possibility of nonspecific effects of BCS on membrane responses to the induction protocol that might account for its effects on LTP. Finally, baseline EPSP slopes were not different in the absence and presence of BCS in neurons from wild-type and PAM$^{+/−}$ mice. Two-way ANOVA revealed no main effects of or interactions between genotype and pharmacological condition on baseline EPSP slope ($P > 0.05$). Together, these data argue strongly against any nonspecific effects of BCS on baseline neuronal or synaptic function in the wild-type or PAM$^{+/−}$ amygdala.

To assess the effects of extracellular Cu chelation on LTP in amygdalar neurons from wild-type and PAM$^{+/−}$ mice, BCS was added to the perfusate no more than 20 min before LTP induction to minimize the extraction of Cu from cells. The presence of BCS blocked the establishment of LTP in wild-type 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$_B$ receptor antagonist (CGP; 1 $\mu$M) is added to the perfusate along with PTX (Gaier et al. 2010). GABA$_B$ 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 wild-type and PAM$^{+/−}$ mice during blockade of both GABA$_A$ and GABA$_B$ receptors (Fig. 5D). The presence of BCS completely eliminated LTP in both wild-type 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 wild-type control levels (Fig. 2B); Cu supplementation provoked increased freezing behavior among PAM+/− animals without affecting wild-type 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 wild-type 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 (Bousquet-Moore et al. 2010; Fujiwara et al. 2006; Railey et al. 2010) and altered gene expression (Gonzalez et al. 2008; Levenson 2005). Cu deficiency enhances the baseline acoustic startle response, whereas 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 (Chrosniak et al. 2006; Maret and Sandstead 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 (Ledaux et al. 1990). Dietary Cu supplementation abrogated the effect of Pam heterozygosity on LTP with no effect on LTP in wild-type 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 (Gaier et al. 2013a; Gynbin and Prohaska 2008). AMPK phosphorylates the GAB<sub>A</sub>B<sub>2</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 postsynaptically in the amygdala (McDonald et al. 2004; Pan et al. 2009; Terrunna et al. 2010) where presynaptic GABA<sub>B</sub> receptors mediate afferent terminal release probability (Pan et al. 2009), important for our LTP paradigm (Fig. 2C). Additionally, GABA<sub>B</sub> receptor-mediated inhibition is an important regulator of NMDA receptor-mediated Ca<sup>2+</sup> signaling, triggering neuronal release of Cu (Schrief et al. 2005), and critical to many forms of LTP (Morrisett et al. 1991; Pan et al. 2009).

Synaptic Effects of Acute (Bath) Cu

In contrast to more physiological local synaptic Cu release, acute bath application of Cu to slice preparations makes Cu available simultaneously to all of its many potential targets. By evaluating the effects of bath-applied Cu in cells clamped at −35 mV, we gained information about simultaneous changes in both glutamatergic and GABAergic neurotransmission without PTX or CGP. Bath application of Cu (10 μM) was within the physiological range and limited to 10 min. Cu had no effect on the monosynaptic excitatory current but reversibly potentiated the disynaptic inhibitory current. This likely reflects disinhibition of interneurons, which express the highest levels of Cu-sensitive, α<sub>1</sub>-subunit-containing GABA<sub>A</sub> receptors (Kim and Macdonald 2003; McDonald and Mascagni 2004). Based on our previous electrophysiological study and the increased sensitivity of PAM+/− mice to the anxiolytic effects of benzodiazepines (Gaier et al. 2010), this result was particularly interesting. The synaptic plasticity experiments were performed in the presence of PTX, whereas the Cu bath-application experiment was performed without any pharmacological 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 CuSO4 to amygdala slices was sufficient to potentiate wild-type synapses without LTP induction (Fig. 4F). In addition, the potentiating effect of Cu is mediated through an increase in presynaptic 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 should focus on PAM+/− 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. 2003, 2009; 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_A receptors to inhibit Cu-induced LTP. 

Fig. 6. Synaptic model of Cu homeostasis. The schematic diagram is of an afferent synapse in the lateral amygdala 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 potentiate amygdalar synapses in Wt 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 GABA_B 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 Cu^{2+} influx through voltage-gated Cu^{2+} channels or NMDA receptors and inhibited by GABA receptor activation. Although the evidence that Cu is important to LTP at this synapse is compelling, the underlying mechanism remains to be uncovered.
observe amygdalar but not hippocampal LTP (Bauer et al. 2002; Gaier et al. 2010; Ma et al. 2008; Tully et al. 2007). This difference adds supporting evidence to the connection between Cu and GABAergic signaling.

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. 2013a). Synaptic Cu levels rise as high as 100–250 μM (Kardos et al. 1989), whereas low concentration levels of Cu bind to and modulate GABA_A receptors (Fisher and Macdonald 1998; McGee et al. 2013), AMPA receptors (Doreulee et al. 1997), NMDA receptors (Schlief et al. 2006; Vlachova et al. 1996), L-type voltage-gate calcium channels (Castelli et al. 2003; Korte et al. 2003), and others (Mathie et al. 2006; Tamano and Takeda 2011).

Synaptic Role for Endogenous Copper

Estimates of Cu concentration vary between studies, brain regions, and method of measurement (Gaier et al. 2013a). Cu has been reported to be concentrated in the amygdala and hippocampus (Akatsu et al. 2012; Jackson et al. 2006). ATP7A and Cu are present in biochemically isolated synaptic fractions from cortical and hippocampal neurons, and ATP7A localizes to synapses in cultured cortical neurons (Gaier et al. 2013b). Therefore, ATP7A and Cu are present at synapses in the mammalian forebrain, in key position to influence synaptic transmission. To assess the role of endogenous Cu in LTP in amygdalar slices, we used BCS, a Cu(I)-specific, cell-impermeant chelator (Hawkins and Perrin 1963; Rae et al. 1999).

The affinity of BCS for Cu(I) is 10^3-fold greater than its affinity for Cu(II), Zn(II), or Fe(II) (Hawkins and Perrin 1963), many orders of magnitude more specific than histidine, which has been used to chelate Cu (McGee et al. 2013). We used BCS at 50 μM; other studies using higher concentrations (200–360 μM) still found that BCS was specific for Cu(I) (Lynch and Frei 1995; Rae et al. 1999; Verhaegh et al. 1997). Since we applied BCS to slices only a few minutes before testing LTP, it is presumably working by binding Cu(I) released into the medium. To observe LTP in PAM^{+/−} slices, these experiments were conducted in the presence of both PTX and CGP, blocking essentially all GABAergic transmission. For both wild-type and PAM^{+/−} slices, LTP was eliminated following brief addition of BCS to the perfusate, arguing that endogenous secreted Cu is necessary for LTP at these synapses.

Potential Mechanism

GABAergic inhibition presumably acts upstream to suppress Cu release, which is essential for amygdalar LTP. Neuronal activation stimulates Ca^{2+}-dependent release of Cu (Dodani et al. 2011; Schlief et al. 2006); PAM^{+/−} mice, with enhanced GABAergic inhibition and reduced Cu stores, require a higher stimulation threshold to achieve sufficiently increased synaptic Cu levels. This threshold is normalized when GABA_B receptors are blocked or Cu is supplemented. The model is supported by the need for higher shock intensities to achieve wild-type levels of fear-potentiated startle memory and added suppression of GABAergic signaling to achieve amygdalar LTP in PAM^{+/−} mice (Gaier et al. 2010).

Interactions Between PAM and Cu

Several aspects of the behavioral response to dietary Cu deficiency resemble the effects of Pam heterozygosity (Bousquet-Moore et al. 2010). PAM^{+/−} animals have increased susceptibility to 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 (Bousquet-Moore et al. 2010; De et al. 2007). Dietary Cu supplementation has dramatic effects on PAM^{+/−} 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^{+/−} mice (Bousquet-Moore et al. 2010), but a clear deficit was found in the amygdala (Fig. 1), whereas 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. 2005). 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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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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