Distinct molecular components for thalamic- and cortical-dependent plasticity in the lateral amygdala

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INTRODUCTION
Synaptic plasticity, a property of neuronal connections characterized by a change in synaptic strength following neuron activation, is essential for memory formation but also for forgetting. Whether presynaptic stimulation increases or decreases synaptic strength depends on the magnitude of post-synaptic calcium elevation (Citri and Malenka, 2008). Long-term potentiation (LTP), a form of synaptic strengthening, is induced by a high rise in intracellular calcium concentration leading to activation of protein kinases. In contrast, long-term depression (LTD), a form of synaptic weakening, requires a moderate rise in intracellular calcium concentration that activates protein phosphatases including PP2B (calcineurin) and subsequently PP1 (Mulkey et al., 1993, 1994; Jouvenceau et al., 2001), in particular, post-synaptic NMDAR and AMPAR subunits, leading to NMDAR downregulation and AMPAR endocytosis, ultimately resulting in synaptic depression [for review, see (Mansuy and Shenolikar, 2006)].

In the lateral amygdala (LA), LTP is associated with the formation of fear memory (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997; Tsvetkov et al., 2002), while LTD is thought to underlie the extinction of fear memory (Kim et al., 2007; Liu et al., 2009; Dalton et al., 2012). PP1 at both pathways, it requires NR2B-containing NMDARs at the thalamic pathway, but NR2C/D-containing NMDARs at the cortical pathway. LTD appears to be induced post-synaptically at the thalamic input but presynaptically at the cortical input, since post-synaptic calcium chelation and NMDAR blockade prevent thalamic but not cortical LTD. These results highlight distinct molecular features of LTD in LA that may be relevant for traumatic memory and its erasure, and for pathologies such as post-traumatic stress disorder (PTSD).

Keywords: LTD, NR2B, NR2C/D, amygdala, phosphatase, mice, PP1

MATERIAL AND METHODS

ANIMALS
For all experiments, adult male mice C57Bl/6 (8–12 weeks old) were used. Animals were housed in standard housing conditions in a temperature- and humidity-controlled facility on a 12 h reversed light/dark cycle. Mice had free access to food and water. All procedures were carried out in accordance with the guidelines of the Veterinary Office of the Canton of Zurich, Switzerland.

N-methyl-D-aspartate receptor (NMDAR)-dependent long-term depression (LTD) in the lateral nucleus of the amygdala (LA) is a form of synaptic plasticity thought to be a cellular substrate for the extinction of fear memory. The LA receives converging inputs from the sensory thalamus and neocortex that are weakened following fear extinction. Combining field and patch-clamp electrophysiological recordings in mice, we show that paired-pulse low-frequency stimulation can induce a robust LTD at thalamic and cortical inputs to LA, and we identify different underlying molecular components at these pathways. We show that while LTD depends on NMDARs and activation of the protein phosphatases PP2B and PP1 at both pathways, it requires NR2B-containing NMDARs at the thalamic pathway, but NR2C/D-containing NMDARs at the cortical pathway. LTD appears to be induced post-synaptically at the thalamic input but presynaptically at the cortical input, since post-synaptic calcium chelation and NMDAR blockade prevent thalamic but not cortical LTD. These results highlight distinct molecular features of LTD in LA that may be relevant for traumatic memory and its erasure, and for pathologies such as post-traumatic stress disorder (PTSD).
and approved by its Commission for Animal Research (License numbers 150/2006 and 105/2008).

**SLICES PREPARATION**

Mice were anesthetized with isoflurane 99.9% (AttaneTM) and rapidly decapitated. Immediately after decapitation, the brain was extracted and sectioned in coronal slices (400 µm thick for extracellular field recordings, 300 µm for whole-cell patch clamp recordings) in ice-cold modified artificial cerebrospinal fluid (aCSF) containing 175 mM sucrose, 20 mM NaCl, 3.5 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 1.3 mM MgCl2, and 11 mM D-(-)-glucose, and gassed with 95% O2/5% CO2 using a vibratome (VT 1000S; Leica Microsystems, Bannockburn, IL, USA). Coronal slices were placed in a holding chamber at 34°C and incubated in normal aCSF containing 119 mM NaCl, 2.5 mM KCl, 1.3 mM NaH2PO4, 26 mM NaHCO3, 1.3 mM MgCl2, 2.5 mM CaCl2, and 11 mM D-(-)-glucose, and continuously bubbled with 95% O2/5% CO2 at 34°C for at least 2.5 h prior to recording. For recording, slices were transferred to a superfusion (1.5–2.5 ml/min flow rate) chamber (Warner Instruments) heated at 33.5-34°C and held below a platinum wire.

**ELECTROPHYSIOLOGY**

The recording electrode was placed in the dorsal part of the LA, and the stimulation electrodes were placed close to the internal capsule and externally to the capsule to stimulate fibers originating from the thalamus or auditory cortex, respectively (see Figure 1A). Extracellular field excitatory post-synaptic potentials (fEPSPs) were recorded from the dorsal part of the LA, while basal single-electrical stimuli at 0.05 Hz were applied at both pathways. After 10 min of stable baseline fEPSPs recording, paired pulse low-frequency stimulation [ppLFS, 900 pulses at 1 Hz, interstimuli interval (ITI) of 40 ms] was used to induce LTD D (Müller et al., 2009). To test input specificity, ppLFS was about 200–300 times to allow irreversible binding of MK-801 dialysis (charge transfer reduced by 28.6 ± 9.5% n = 3 for the thalamic pathway, and 17.2 ± 6.7% n = 3 for the cortical pathway). Cells were clamped again at −70 mV for another 10 min showing no significant change in the peak amplitude of AMPAR-mediated responses.

**DATA ANALYSIS**

Data analysis was performed using Clampfit software (v10.2, Molecular Devices, Sunnyvale, DA, USA), GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA), and Excel (Microsoft). For all recordings, fEPSP slope, and EPSP and EPSC amplitude were normalized to the average of baseline slope and amplitude, respectively. To improve the signal-to-noise ratio, data were averaged into 1 min bins. For each experiment, two to three slices per animal were recorded, one was always used as control slice and one or two slices received drug-treatment. For statistical analyses, individual animals (not slices) were considered biological replicates. For both extracellular field and whole-cell recordings, data are expressed as mean ± SEM. Statistical comparisons were monitored by measuring the passive current transients induced by 10 mV hyperpolarizing voltage steps from a holding potential of −60 mV. Accepted deviations from this parameter in current transients recorded over the time-windows used for statistical analysis were <10% (Brandalise et al., 2012). Data were recorded using an Axopatch 200B amplifier, sampled with a Digidata-1440 interface (sampling time = 250 msec for current-clamp recording, 10 ms for voltage-clamp recordings) and analyzed with P-CLAMP software (Axon Instruments, Foster City, CA, USA) and Origin software (Microsoft Software, Northhampton, MA, USA).
FIGURE 1 | Input-specific NMDAR-dependent LTD at the thalamic and cortical pathways in the lateral amygdala. (A) Schematic illustration of electrode placement for ppLFS and control pathway recording of the thalamic pathway (left) and the cortical pathway (right). (B) Robust, long-lasting LTD was specifically induced at the pathway receiving ppLFS (thalamic, \( n = 18 \); cortical, \( n = 18 \)) but not at the control pathway (thalamic: \( n = 11 \); cortical: \( n = 14 \)). Insets show representative traces of extracellular field potentials averaged across 10 min before ppLFS (black line) and the last 10 min of recording after ppLFS (gray line). (C) D-APV (50 μM) prevents LTD at thalamic afferents (control: \( n = 6 \); D-APV: \( n = 9 \)) and at cortical afferents (control: \( n = 5 \); D-APV: \( n = 9 \)). Insets show I/O curves on top and below representative traces of extracellular field potentials averaged across 10 min before ppLFS (black line) and the last 10 min of recording after ppLFS (gray line). Data represent mean ± SEM. *** \( p < 0.001 \).
performed using Student’s unpaired t-tests when two groups were compared. One-way ANOVAs were used when more than two groups were compared. If significant, ANOVAs were followed using Duncan’s post hoc test. Significance was set to \( p < 0.05 \).

**RESULTS**

**PATHWAY-SPECIFIC LTD IN LA**

Using extracellular field recording, we first assessed whether a paired-pulse low frequency stimulation protocol (ppLFS) induces stable and input-specific LTD at thalamic and cortical afferents to the LA in slices from adult mouse (for electrode placement see Figure 1A). A robust LTD that lasted over 1hr was specifically induced at the pathway receiving ppLFS but not at a control pathway, both at thalamic (ppLFS pathway: 53.3 ± 4.3%, \( n = 18 \) vs. control pathway: 106.2 ± 10.9%, \( n = 11, p < 0.001 \), Figure 1B) and cortical input (ppLFS pathway: 52.7 ± 3.6%, \( n = 18 \) vs. control pathway: 108.6 ± 12.2%, \( n = 14, p < 0.001 \), Figure 1B). The magnitude of fEPSP suppression was comparable between thalamic and cortical ppLFS (\( p > 0.9 \)). These results indicate that the ppLFS protocol leads to a strong and input-specific induction of LTD (LA–LTD) at both thalamic and cortical pathways to LA.

**NMDAR-DEPENDENT LTD IN LA DEPENDS ON PROTEIN PHOSPHATASES**

In the hippocampus, the most common form of LTD requires post-synaptic rise in calcium that depends on NMDARs, and is associated with activation of a PP2B/PP1 signaling cascade (Collingridge et al., 2010). Both PP2B and PP1 are known as memory suppressors (Mansuy et al., 1998; Malleret et al., 2001). We thus first tested whether LA–LTD and cortical ppLFS (control: 55.2 ± 5.9%, \( n = 5 \); ifenprodil: 48.2 ± 5.2%, \( n = 6 \); Co101244: 52.5 ± 12.2%, \( n = 5, p > 0.8 \), Figure 3) did not affect L TD at the cortical pathway (control: 55.2 ± 5.9%, \( n = 5 \); ifenprodil: 48.2 ± 5.2%, \( n = 6 \); Co101244: 52.5 ± 12.2%, \( n = 5, p > 0.8 \), Figure 3). Ifenprodil and Co101244 did not affect I/O curves, suggesting no effect on basal synaptic transmission (Figure 3A, insets). These results demonstrate that LTD at the thalamic pathway is NR2B-dependent, while LTD at the cortical pathway is not.

To determine which other NR2 subunits may be implicated in LTD at the cortical pathway, we next tested the contribution of NR2C/D subunits (NR2A was previously reported not to be involved in L TD (Dalton et al., 2012)). We used PPDA, a potent and dose-dependent selective NR2C/D antagonist (Hrabetova et al., 2000; Feng et al., 2004). We used PPDA at low concentration (0.25 \( \mu \)M) to preferentially antagonize NR2C/D subunits, and at high concentration (1 \( \mu \)M) to antagonize all NR2 subunits (Feng et al., 2004). At 0.25 \( \mu \)M, PPDA fully blocked LA–LTD specifically at the cortical input, but had no effect at the thalamic pathway (Figure 4). In contrast, 1 \( \mu \)M of PPDA abolished LA–LTD at both pathways (thalamic, control: 45.2 ± 8.5%, \( n = 7 \); PPDA 0.25 \( \mu \)M: 44.3 ± 9.1%, \( n = 5 \); PPDA 1 \( \mu \)M: 113.6 ± 20.6%, \( n = 6, p < 0.01 \). Cortical, control: 41.6 ± 9.8%, \( n = 6 \); PPDA 0.25 \( \mu \)M: 113.3 ± 14.1%, \( n = 6 \); PPDA 1 \( \mu \)M: 107.1 ± 22.2%, \( n = 6, p < 0.05 \), Figure 4). I/O curves were not affected by PPDA at either concentration (Figure 4A, insets). Overall, these results indicate that LTD at the thalamic pathway depends on NR2B-containing NMDARs, whereas LTD at the cortical pathway depends on NR2C/D-containing NMDARs.

**DISTINCT LOCI OF LTD INDUCTION AT THALAMIC AND CORTICAL PATHWAYS**

Although LTD is generally thought to be induced post-synaptically, it is known that NMDAR-dependent LTD can also occur presynaptically in several brain regions (Casado et al., 2002; Sjostrum et al., 2003; Rodriguez-Moreno and Paulsen, 2008; Rodriguez-Moreno et al., 2010). NR2B-containing NMDARs are mostly localized post-synaptically (Loftis and Janowsky, 2003; Mameli et al., 2005; Miwa et al., 2008; Yu et al., 2010) and NR2C/D-containing NMDARs are mostly presynaptic (Thompson et al., 2002; Mameli et al., 2005; Grilli et al., 2009) and have been
FIGURE 2 | PP2B and PP1 involvement in LTD at the thalamic and cortical pathways in the lateral amygdala. (A) The PP2B antagonist FK-506 (100 μM) blocks LTD induced at thalamic afferents (left panel, control: n = 5; FK-506: n = 5) and at cortical afferents (right panel, control: n = 5; FK-506: n = 5). (B) The PP1 antagonist tautomycetin (4 nM) blocks LTD induced at thalamic afferents (control: n = 5; tautomycetin: n = 10), and at cortical afferents (control: n = 7; tautomycetin: n = 8). Insets show I/O curves on top and below representative traces of extracellular field potentials averaged across 10 mins before ppLFS (black line) and the last 10 min of recording after ppLFS (gray line). Data represent mean ± SEM. ***p < 0.001, **p < 0.001, *p < 0.05.

implicated in presynaptic LTD in the somatosensory cortex (Banerjee et al., 2009). Because NR2B and NR2C/D subunits are differentially involved in LTD at thalamic and cortical pathways, we hypothesized that LTD may have different loci of induction at thalamic and cortical pathways. We tested this hypothesis using whole-cell patch clamp recording in LA pyramidal neurons. The recorded cells (n = 32) showed a firing pattern and spike frequency adaptation characteristic of LA pyramidal neurons (Figure 5A; Weisskopf and LeDoux, 1999; Faber et al., 2001). The average resting potential of these neurons was −67.6 ± 4.3 mV. We observed a mono-exponential relationship between current transients and voltage steps, indicating that excitatory cells in LA behave as single electrical compartments (τ1 = 40.65 ± 0.1 ms). Transients were also used to estimate series resistance (15.3 ± 4.23 MΩ) and membrane capacitance (67.7 ± 16.8 pF), all typical values
for LA excitatory cells (Weisskopf and LeDoux, 1999; Faber et al., 2001).

Before assessing the locus of LTD induction, we examined whether LTD can be induced in individual excitatory LA neurons with the ppLFS protocol in current clamp configuration, and whether it depends on NMDARs. ppLFS induced a robust and persistent LTD in LA neurons, which was similar at thalamic and cortical inputs (thalamic: 47.38 ± 9.74%, n = 4; cortical: 56.2 ± 4.6%, n = 5, p > 0.3, Figure 5B). LTD was blocked by D-APV, confirming that it is NMDAR-dependent (thalamic: D-APV: 112.5 ± 3.0%, n = 3, p < 0.001; cortical: D-APV: 108.1 ± 6.2%, n = 3, p < 0.01, Figure 5B). Because post-synaptic plasticity depends on changes in post-synaptic intracellular calcium concentration, we examined whether LTD is post-synaptic by preventing calcium increase at the post-synaptic site using the membrane impermeable calcium chelator BAPTA (100 mM, dialyzed for 20 min before ppLFS). LTD at the thalamic pathway was fully blocked by BAPTA (control: 47.6 ± 6.7%, n = 5; BAPTA: 46.9 ± 6.1%, n = 5, p > 0.5, Figure 6A). These results suggest that the induction of LTD requires a post-synaptic rise in calcium at thalamo-LA synapses but not at cortico-LA synapses. To further assess the synaptic locus of LTD at thalamic and cortical synapses, we selectively blocked post-synaptic NMDARs before LTD induction by intracellular dialysis of the activity-dependent NMDAR antagonist MK-801 (40 μM) into the pyramidal-like
LA neuron. In the presence of MK-801, LTD was fully blocked at thalamo-LA synapses (control: 37.9 ± 13.5%, n = 3, MK-801: 104.3 ± 5.4%, n = 3, p < 0.05) but was not affected at cortico-LA synapses (control: 52.5 ± 14.2%, n = 3, MK-801: 54.8 ± 11.6%, n = 3, p > 0.9, Figure 6B), suggesting that LTD requires the activation of post-synaptic NMDARs at thalamic but not cortical synapses. Together, these results support a post-synaptic locus of LA–LTD at the thalamic pathway that likely depends on post-synaptic NMDARs, but a mechanism independent of post-synaptic NMDARs and independent of changes in post-synaptic calcium at the cortical pathway.

**DISCUSSION**

The protein phosphatases PP2B and PP1 are key players in the regulation of synaptic strength, and in the formation and the maintenance of memory traces (Lisman and Zhabotinsky, 2001; Mansuy and Shenolikar, 2006; Baumgärtel and Mansuy, 2012). Activation of PP2B/PP1 signaling is known to be necessary for LTD in different brain regions (Kirkwood et al., 1993; Mulkey et al., 1994; Morishita et al., 2001; Lin et al., 2003b; Yasuda et al., 2003). This study provides novel evidence that these phosphatases are also involved in the induction of LTD in LA at both thalamic and cortical pathways. This finding is in line with previous results showing that depotentiation at the cortical pathway in LA requires PP2B.
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FIGURE 5 | NMDAR-dependent LTD in single pyramidal cells. (A) On top, response of a LA cell to current injection of $-0.10$ and $+0.15$ nA and below, hyperpolarizing voltage steps of $10$ mV from a holding potential of $-60$ mV were used to measure series resistance.

(B) D-APV (50 μM) blocks LA–LTD induced at the thalamic pathway (left panel, control: $n = 4$; D-APV: $n = 3$), and at the cortical pathway (right panel, control: $n = 5$; D-APV: $n = 3$). Insets show the average of 10 sweeps of a single cell recorded 10 min before (black) and 30 min after (gray) ppLFS. Data represent mean ± SEM. ***$p < 0.001$, **$p < 0.01$.

(Lin et al., 2003a), and that PP2B and PP1 play an important role in memory tasks that depend on the amygdala including conditioned taste aversion and extinction of fear memory (Lin et al., 2003b; Baumgärtel et al., 2008; Oberbeck et al., 2010; Koshiibu et al., 2011). They also complement findings in the hippocampus that PP2B or PP1 inhibition enhances hippocampal LTP and memory performance in hippocampus-dependent tasks (Malleret et al., 2001; Genoux et al., 2002) but impairs LTD (Jouveauceau et al., 2006). Taken together, these findings support the concept that PP2B/PP1 are key regulators of synaptic plasticity, and that their inhibition favors LTP and memory acquisition, but impairs LTD and memory extinction in both hippocampus and amygdala.

Our finding that LTD at the thalamic LA pathway is NR2B-dependent is consistent with previous studies (Müller et al., 2009; Dalton et al., 2012; Park et al., 2012). NR2B is present in postsynaptic densities (PSD) in LA (Miwa et al., 2008), and LTD at the thalamic pathway depends on NR2B-dependent postsynaptic AMPAR endocytosis (Yu et al., 2010). Surprisingly, we observed that LTD induced at the cortical pathway is independent of NR2B signaling, since NR2B antagonists do not block LTD induction. Instead, we observed that blocking NR2C/D subunits fully prevents LTD at the cortical pathway, but does not affect LTD at the thalamic pathway. The observation that LTD at the cortical pathway is NR2B-independent contrasts with a previous report showing that antagonizing NR2B blocks LTD at both pathways in horizontal slices from adult mice (Müller et al., 2009). This apparent discrepancy likely results from a different orientation of the slices leading to different sites of stimulation and recording. Specifically, placing the stimulating electrode laterally to the internal capsule in coronal slices primarily activates cortical afferents to LA, but in horizontal slices, it also activates afferents from the entorhinal and perirhinal cortex (von Bohlen und Halbach and Albrecht, 2002; Müller et al., 2009). The spatial organization of excitatory and inhibitory connections within the LA depends as well on slice orientation (Samson et al., 2003; Samson and Pare, 2006). It thus needs to be determined whether LTD differentially relies on NR2B or NR2C/D-containing receptors in the cortical pathway depending on the slice orientation. Given our clear finding that thalamic and cortical input to LA rely on different molecular and postsynaptic mechanisms, we postulate that projections to LA from
FIGURE 6 | Distinct locus of LTD induction at thalamic and cortical pathways. (A) Dialysis of BAPTA blocks LA–LTD induced at the thalamic pathway (left panel, control: \(n = 4\); BAPTA: \(n = 4\)), but not at the cortical pathway (right panel, control: \(n = 5\); BAPTA: \(n = 5\)). Insets show averaged traces of 10 sweeps taken 10 min before (black) and 30 min after (gray) ppLFS. (B) Dialysis of MK-801 (40 \(\mu\)M) blocks LA–LTD induced at the thalamic pathway (left panel, control: \(n = 3\); MK-801: \(n = 3\)), but not at the cortical pathway (right panel, control: \(n = 3\); MK-801: \(n = 3\)). Insets show averaged traces of 10 sweeps taken 10 min before (black) and 30 min after (gray) ppLFS. Data represent mean ± SEM. **\(p < 0.01\), *\(p < 0.05\).

Our observation that different NR2 subunits mediate the effects of ppLFS-induced LTD at both input pathways to the LA are in agreement with previous studies reporting differences in the molecular cascades at these pathways in LTP and depotentiation (Humeau et al., 2003, 2005; Hong et al., 2009; Jung et al., 2010; Meis et al., 2012). Although the distribution of NMDAR subunits in the amygdala remains largely unknown, the receptor kinetics at resting membrane potential is known to be different at cortical and thalamic pathways (Weisskopf and LeDoux, 1999). NMDARs at cortical inputs are less sensitive to magnesium blockade than at thalamic inputs, and the kinetic properties are akin to NR2C/D-containing NMDARs at the cortical pathway, but resemble NR2A/B-containing NMDARs at the thalamic pathway (Monyer et al., 1994; Weisskopf and LeDoux, 1999; Cull-Candy et al., 2001). This is in agreement with our observation that NR2C/D-containing receptors seem to mediate LTD at the cortical pathway, but not at the thalamic pathway.
Presynaptic NR2C/D-containing NMDARs are believed to be involved in spike-timing dependent LTD in the cortex (Banerjee et al., 2009). This prompted us to investigate the site of LTD induction at both LA pathways by whole cell patch-clamp recording. We observed that LTD induction occurs post-synaptically at the thalamic pathway, but is independent of post-synaptic calcium influx or post-synaptic NMDARs at the cortical pathway. These findings for LTD complement previous reports for LTP in the amygdala showing that LTP engages different pre- and post-synaptic mechanisms at thalamic and cortical pathways (Huang and Kandel, 1998; Humeau et al., 2003, 2005, 2007; Tsvetkov et al., 2004; Rumpel et al., 2005; Shaban et al., 2006).

Although distinct NMDAR subunits and post-synaptic mechanisms are involved at thalamic and cortical afferents to the LA, both pathways converge onto a PP2B/PP1 signaling cascade. In the hippocampus, calcium influx through NMDARs, rather than other calcium channels, is specifically required for PP2B and PP1 activation (Unoki et al., 2012). It is possible that presynaptic calcium influx through NR2C/D-containing NMDARs, and post-synaptic calcium influx through NR2B-containing NMDARs, lead to the activation of PP2B/PP1 at the cortical and thalamic pathway, respectively, a possibility that will need to be tested in future experiments. Whether NR2C or NR2D subunits are localized presynaptically at cortical but not at thalamic afferents to LA will also need to be determined, as well as the molecular mechanisms downstream of PP2B/PP1 activation at both pathways. In the hippocampus, PP2B and PP1 have presynaptic and post-synaptic targets (Strack et al., 1999; Nakano-Kobayashi et al., 2007; Baumgärtel and Mansuy, 2012). In hippocampal and cortical neurons, PP1 can dephosphorylate NR2B (Farinelli et al., 2005; Prabhu Ramya et al., 2012), resulting in a downregulation of postsynaptic GABA(B) receptors induces disinhibition of the Golgi cell-granule cell synapse in rat cerebellum. PLoS ONE 7:e43417. doi: 10.1371/journal.pone.0043417

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