Long-term potentiation at excitatory synaptic inputs to the intercalated cell masses of the amygdala

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Abstract
The intercalated cell masses (ITCs) of the amygdala are clusters of GABAergic interneurons that surround the basolateral complex of the amygdala. ITCs have been increasingly implicated in the acquisition and extinction of conditioned fear responses, but the underlying cellular mechanisms remain unexplored. Here, we report that repetitive stimulation of lateral amygdala (LA) afferents with a modified theta burst stimulation (TBS) protocol and induces long-term potentiation (LTP) of excitatory synapses onto medial paracapsular ITC (Imp) neurons. This TBS-induced LTP is: (1) induced and expressed post-synaptically, (2) involves a rise in postsynaptic Ca\(^{2+}\) and the activation of NR2B-containing N-methyl-D-aspartate receptors (NMDARs), (3) dependent on calcium/calmodulin-dependent protein kinase II and cAMP-dependent protein kinase activation, and (4) associated with increased exocytotic delivery of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) to the post-synaptic membrane. Remarkably, auditory fear conditioning led to a persistent increase in AMPAR/NMDAR ratio of glutamatergic synaptic currents and occluded TBS-induced LTP at LA-Imp synapses. Furthermore, extinction training rescued the effect of fear conditioning on AMPAR/NMDAR ratio and LTP induction. These results show that a prominent form of LTP can be elicited at LA-Imp synapses and suggest that this synaptic plasticity may contribute to the expression of fear conditioning.

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Introduction
The amygdala is a core brain structure underlying the acquisition and expression of conditioned fear responses (LeDoux, 2000). It comprises several anatomically and functionally distinct nuclei, including the lateral (LA) and basal (BA) nuclei (together referred to as the basolateral amygdala (BLA)), and the central nucleus (CeA). The LA has been traditionally viewed as a major sensory interface, as it receives multimodal sensory information from the thalamus and the cortex, whereas the medial subdivision of CeA (CeM) serves as a major output station, as its output neurons contact discrete structures in the brainstem and the hypothalamus to orchestrate conditioned fear responses (Ehrlich et al., 2009). As LA neurons do not project directly to the CeM, one intriguing possibility is that downstream of the LA, one or more population(s) of neurons may transmit information to the CeM. Converging lines of anatomical physiological evidence suggest that the GABAergic interneurons in the intercalated paracapsular islands are the potential relay stations between the LA and the CeM (Ehrlich et al., 2009; Paré and Duvarcì, 2012). Based on their locations, the intercalated cell clusters in the amygdala (ITCs) can be further divided into the medial (Imp) and lateral intercalated cell masses (lITC), and main intercalated nucleus (IN) (Millhouse, 1986). In addition, three types of Imp neurons have been recognized based on their different axonal projection patterns and one of them forms functional GABAergic synapses with IN neurons (Busti et al., 2011). There is evidence that ITC neurons mostly enable feed-forward control of signal flow from the cortex to the BA and the LA, whereas mITC neurons constitute an inhibitory interface gating the flow of information from the BLA to the CeA (Ehrlich et al., 2009). Recently, Paré et al., reported that fear extinction was associated with increased levels of synaptic inhibition in fear output neurons of the CeA and the increased inhibition was caused by an enhanced recruitment of the mITC by BLA inputs (Likhtik et al., 2008; Amano et al., 2010). Furthermore, neuropeptide S has been shown to facilitate extinction of previously conditioned fear when administered into the amygdala by enhancing the LA to IN glutamatergic transmission (Jüngling et al., 2008).
These findings strongly support a critical role for the mITC in the acquisition and extinction of conditioned fear responses, but the underlying cellular mechanisms remain unclear.

Long-term potentiation (LTP), a well-characterized form of synaptic plasticity, is widely considered as an important cellular mechanism of learning and memory. While LTP has been found to occur at various excitatory inputs onto LA, CeA and ITC neurons (Royer and Paré, 2002, 2003; Maren and Quirk, 2004), it remains to be delineated whether, and how, LTP occurs in the ITCs to regulate the acquisition and expression of conditioned fear responses. Here, we show that LTP can be induced in Imp neurons by theta burst stimulation (TBS) of excitatory synaptic afferents in the LA. Our results also show that fear conditioning occluded this TBS-induced LTP, which was rescued by extinction training.

Method

Animals

Male wild-type C57BL/6 mice and 67 kDa isoform of glutamic acid decarboxylase-green fluorescence protein (GAD67-GFP) knock-in mice (Tamamaki et al., 2003) were used in our experiments. GAD67-GFP knock-in mice were generously provided by Dr. Yuchio Yanagawa and bred within our animal facility onto the C57BL/6 genetic background. Mice were group housed in a humidity- and temperature-controlled (25±1 °C) vivarium on a 12 h light/dark cycle with access to food and water ad libitum. All experiments were executed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of National Cheng Kung University.

Slice preparations and electrophysiology

Coronal brain slices containing the amygdala were prepared from 4–5-wk-old C57BL/6 mice or hemizygous GAD67-GFP mice as described previously (Huang et al., 1996). In brief, mice were anesthetized with isoflurane and decapitated, and brains were rapidly removed and placed in ice-cold sucrose artificial cerebrospinal fluid (ACSF) cutting solution [containing (in mM): sucrose 234, KCl 2.5, CaCl2 0.5, MgCl2 7, NaHCO3 25, NaH2PO4 1.25 and glucose 11 at pH 7.3–7.4 and equilibrated with 95% O2−5% CO2]. Slices (240 μm) were prepared using a vibrating microtome (VT1200S; Leica) and transferred to a holding chamber of normal ACSF [containing (in mM): NaCl 117, KCl 4.7, CaCl2 2.5, MgCl2 1.2, NaHCO3 25, NaH2PO4 1.2 and glucose 11 at pH 7.3–7.4 and equilibrated with 95% O2−5% CO2] and maintained at room temperature for at least 1 h before use.

For recording, one slice was transferred to a submersion-type recording chamber and fixed at the glass bottom of the chamber with a nylon grid on a platinum frame. The chamber was perfused constantly at 32.0±0.5 °C at 2–3 ml/min. GFP-expressing Imp neurons were first identified via fluorescence microscopy. Only neurons displaying a marked fluorescence were approached. Subsequently, infrared differential interference contrast microscopy was used to characterize their anatomical location and morphology (medium spiny, post-hoc biocytin staining revealed bipolar shape). Imp neurons were also identified by their intrinsic membrane properties (e.g., resting membrane potential was typically more negative than −65 mV and membrane resistance was more than 500 MΩ, Table 1). Whole-cell patch-clamp recordings were made from Imp neurons by using a patch-clamp amplifier (Axopatch 200B, Molecular Devices) as described previously (Huang et al., 2007). Data acquisition and analysis were performed using a digitizer (Digidata 1440A, Molecular Devices) and pCLAMP 9 software (Molecular Devices). For measurement of synaptically evoked excitatory post-synaptic potentials (EPSPs), a bipolar stainless steel stimulating electrode was placed in the LA to stimulate excitatory afferents at 0.05 Hz and the superfusate routinely contained gabazine (10 μM) to block GABA A receptor-mediated inhibitory synaptic responses. EPSPs were recorded at resting membrane potential in current-clamp mode. LTP was induced by a modified TBS protocol consisting of presynaptic activation of ten bursts (each with five pulses at 100 Hz) spaced at 200 ms and repeated three times at 10 s intervals, and post-synaptic injection of a depolarizing current pulse (1.2 nA, 40 ms) during each burst, with a 5 ms interval between the onset of pre- and post-synaptic stimulation (Lu et al., 2009). The composition of intracellular solution was (in mM): K-gluconate 115, KCl 20, HEPES 10,

<table>
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<tr>
<th>Parameter</th>
<th>Average value</th>
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<tr>
<td>Rm (MΩ)</td>
<td>610±23</td>
<td>42</td>
</tr>
<tr>
<td>RMP (mV)</td>
<td>−76.1±1.7</td>
<td>42</td>
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<tr>
<td>Cm (pF)</td>
<td>77.4±2.9</td>
<td>42</td>
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<tr>
<td>Membrane t (ms)</td>
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<tr>
<td>Sag ratio</td>
<td>0.86±0.05</td>
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<tr>
<td>AP amplitude</td>
<td>83.2±3.2</td>
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<tr>
<td>AP width (ms)</td>
<td>0.84±0.07</td>
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<tr>
<td>AHP (mV)</td>
<td>−10.5±0.4</td>
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<tr>
<td>Adaptation index</td>
<td>0.57±0.03</td>
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<tr>
<td>Max firing (Hz)</td>
<td>28.9±2.3</td>
<td>42</td>
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AHP=after hyperpolarization; AP=action potential; Cm=membrane capacitance; max firing=maximal firing; Tin=input resistance; RMP=resting membrane potential; Error bars indicate standard error of the mean. The total number of neurons examined is indicated by n.
MgCl₂ 2, EGTA 0.2, Na₂ATP 3, Na₃GTP 0.3 and 0.4% w/v biocytin, osmolarity 280 mOsm/l without biocytin, pH 7.3 with KOH. Biocytin (0.5%) was routinely included in the intracellular solution to allow post-hoc staining of the recorded neurons. An AMPAR/NMDAR ratio was computed by dividing the peak amplitude of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-mediated excitatory post-synaptic currents (EPSCs) recorded at −70 mV by the peak amplitude of the N-methyl-D-aspartate receptor (NMDAR)-mediated EPSCs recorded at +40 mV. The NMDAR-mediated component of EPSCs was calculated as the difference between the EPSCs measured in the absence and presence of D-2-amino-5-phosphonovalerate (APV, 50 μM). Series resistance (Rₛ) and input resistance were monitored on-line throughout the whole-cell recording with a 5 mV depolarizing step given after every afferent stimulus. Neurons were discarded for analysis if initial resting membrane potential was positive to −65 mV or if Rₛ changed by more than 20% during the course of the recordings.

Asynchronous EPSCs were recorded from Imp neurons by substituting calcium with strontium (4 mM) in the ACSF. Neurons were voltage clamped at −70 mV in the presence of gabazine (10 μM). During TBS, neurons were held in current-clamp mode and bathed in calcium-containing ACSF. After TBS, the recordings were performed in strontium-containing ACSF under voltage-clamp mode again. For asynchronous EPSC analysis, the first evoked EPSC was discarded and the subsequent EPSCs were analysed using a time window of 200 ms from the stimulus artifact as described previously (Oliet et al., 1996).

**Auditory fear conditioning**

Six-week-old C57BL/6 or hemizygous GAD67-GFP mice were fear conditioned, extinguished and tested in an operant-conditioning chamber (15.9×14.0×12.7 cm; ENV-307A, MED Associates) that was equipped with a shock floor and placed into a ventilated sound-attenuating isolation cubicle. Mice were randomly divided into the following three experimental groups: naive, conditioned (Cond) and extinction (Ext). On day one, mice in all three groups were habituated to the training chamber (context A) for 20 min. On day two, mice of the Cond and Ext groups received one habituation trial (tone alone) and then received three presentations of an auditory conditioning stimulus (CS; 90 dB sound at 2 kHz for 20 s) paired with an aversive foot-shock unconditioned stimulus (US; 0.6 mA for 2 s), separated by a 30 s interval. On days three and four, the Ext group was placed in a different conditioning chamber (context B) and received ten extinction trials (tone alone) while the Cond group remained in their home cages. On day five, mice in all three groups were presented with a tone in a novel-conditioning chamber (context C), their freezing recorded, and then slice recordings were performed. The conditioned response (CR) was scored as the total time the mouse spent ‘freezing’ during a 3-min test session. The behaviour data were analysed by differential subtraction of two consecutive images captured at 7.5 Hz to calculate the significant motion pixels (SMP). A freezing behaviour was defined as the value of SMP<20 at any indicated timepoint.

**Drug treatment**

All drugs were applied by manually switching the superfusate. Drugs were diluted from stock solutions just before application. 4-[(2S)-2-[(5-isoquinolinesulfonyl)-methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl] phenyl isouquinolinesulfonic acid (KN-62), N-[2-[[3-(4-chlorophenyl)-2-propan-yl]methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulphonamide (KN-93), 2-N-(4-methoxy-benzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzy lamine (KN-92), bisindolylmaleimide I (Bis-I), nimodipine and raclopride were dissolved in dimethylsulfoxide stock solutions and then diluted to their final concentration in ACSF just before application. APV, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), (5S,10R)-(+)5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801), ifenprodil, (arβS)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol (Ro 25-6981) and (R)-[S-1-(4-bromo-phenyl)-ethylamino]-[2,3-dio xo-1,2,3,4-tetrahydroquinoxalin-5-yl]-methyl]-phosphonic acid (NVP-AAM077) were dissolved in distilled water. Autocamtide-2-related inhibitory peptide (AIP), 1,2-Bis(2-amino-ethane-N,N,N’,N’-tetraacetic acid (BAPTA), pep1-TGL and brefeldin A were dissolved in intracellular solution. The respective drug concentrations were selected on the basis of both previously published and our pilot studies (Huang et al., 2005; Baxter and Wyllie, 2006; Babayan et al., 2012; Inoue et al., 2013). Nimodipine, BAPTA, KN-92 were purchased from Sigma-Aldrich, KN-62, KN-93, Ro 25-6981, ifenprodil, MK-801, Bis-I, pep1-TGL, brefeldin A, gabazine, APV and CNQX were obtained from Tocris Cookson Ltd. NVP-AAM077 was a generous gift from Novartis Pharma (Basel, Switzerland).

**Statistical analysis**

Because no significant differences in LTP magnitude and electrophysiological properties of Imp neurons between wild-type and hemizygous GAD67-GFP mice were found, the data were pooled. The data for each experiment were normalized relative to baseline, and are presented as means ±S.E.M. The number of neurons or animals examined is indicated by n. For LTP experiments, statistical analysis was performed using the non-parametric Mann-Whitney U test. The significance of the difference between the groups was calculated by ANOVA with Bonferroni’s post-hoc analyses or Student’s t test with a significance level of p<0.05.
Results

TBS induces LTP at LA-Imp synapses

For the identification of Imp neurons, we used hemizygous GAD67-GFP knock-in mice, in which GABAergic neurons are specifically labelled with GFP fluorescence under the control of the GAD67 promoter (Tamamaki et al., 2003). With the help of these mice, Imp neurons that are located at the border between the LA and the CeA could be distinguished in the amygdala (Fig. 1a).

In accordance with previous observations (Marowsky et al., 2005; Busti et al., 2011; Mariko et al., 2011), Imp neurons were medium-sized spiny neurons (Fig. 1b) that exhibited high input resistance exceeding 600 MΩ, maximal firing frequency up to 29 Hz with limited adaptation, moderate action potential width with relatively small after hyperpolarization (Table 1). Synaptic responses were induced by stimulating the LA, which, in the presence of gabazine (10 μM), evoked EPSPs in post-synaptic Imp neurons in current-clamp mode at resting membrane potential. The EPSPs were completely abolished by CNQX (20 μM) plus APV (50 μM), indicating that they were mediated by ionotropic glutamate receptors (Fig. 1c).

LTP was induced by one to three trains of TBS with an inter-train interval of 10 s (Fig. 1d) that corresponded to the firing patterns of LA neurons during fear conditioning trials (Paré and Collins, 2000). As shown in the representative example of Fig. 1e, three trains of TBS (3-TBS) induced a robust and persistent increase in the amplitude of EPSPs in Imp neurons. LTP (>20% potentiation measured 40 min after TBS) was observed in five neurons of nine (55.6%) neurons by one train of TBS (1-TBS) and in eight neurons of nine (89.9%) neurons by two trains of TBS (2-TBS), whereas 3-TBS induced LTP in all nine neurons. The mean synaptic potentiation measured 30–40 min after 1–3 trains of TBS were 46.8±9.2% (n=5), 68.9±8.6% (n=8) and 75.2±7.8% (n=9), respectively (Fig. 1f). Likewise, 3-TBS-induced
LTP was observed in five of six (83%) neurons in the absence of gabazine (Supplementary Fig. S1). These results suggest that LTP can occur at LA-Imp synapses. Because 3-TBS consistently induced LTP in the vast majority of Imp neurons, we chose this protocol for all subsequent experiments designed to identify the cellular mechanisms underlying LTP.

**Mechanisms of LTP induction**

To determine whether activation of NMDARs is required for LTP induction, LTP was attempted in the presence of NMDAR antagonist APV (50 μM). APV completely blocked LTP induction (106.3±5.5% of baseline, n=6, p<0.05 compared with control; Fig. 2a and e). In addition, post-synaptic loading of the NMDAR open channel blocker MK-801 (1 mM) also blocked the induction of LTP (105.5±3.5% of baseline, n=6, p<0.05 compared with control; Fig. 2b and e), suggesting a pivotal role for post-synaptic NMDAR activation in LTP induction. Given that the presence of different NR2 subunits may confer distinct gating and pharmacological properties to heteromeric NMDARs (Monyer et al., 1994) and couple them to different cytoplasmic signalling cascades (Sala et al., 2000), we also discerned which NMDAR sub-populations are required for the induction of LTP at LA-Imp synapses. To this end, an NR2A-preferring antagonist NVP-AAM077 (Auberson et al., 2002), two NR2B-selective antagonists, Ro 25–6981 (Fischer et al., 1997) and ifenprodil (Williams, 1993), were used. At the NR2A-selective concentration of 0.1 μM, NVP-AAM077 had no effect on LTP (164.5±8.6% of baseline, n=8, p=0.37 compared with control; Fig. 2c and e). In contrast, 3-TBS failed to induce LTP in the presence of Ro 25–6981 (0.3 μM, 108.7±4.6% of baseline, n=8, p<0.05 compared with control; Fig. 2d and e). These results suggest that the induction of LTP at LA-Imp synapses relies on the activation of post-synaptic NR2B-containing NMDARs.

To test whether Ca\(^{2+}\) influx through the NMDARs is required for LTP induction, we chelated intracellular Ca\(^{2+}\) with the post-synaptic application of membrane-impermeant Ca\(^{2+}\) chelator, BAPTA. Neurons were dialysed for at least 20 min before TBS application to ensure complete dialysis of BAPTA (10 mM) into the neurons. 3-TBS failed to induce LTP in neurons loaded with BAPTA (110.4±3.4% of baseline; n=6, p<0.05 compared with control; Fig. 3a), indicating that a post-synaptic [Ca\(^{2+}\)]\(\text{rise}\) is crucial for LTP induction. Furthermore, control BAPTA-loaded neurons did not show a significant change in synaptic response when held for the identical period of time (n=5). However, normal levels of LTP were observed in the presence of L-type Ca\(^{2+}\) channel blocker nimodipine (20 μM; 168.2±7.2% of baseline, n=6,

![Fig. 2. NMDAR activation is required for LTP induction.](image-url)

(a) Summary of experiments showing that APV (50 μM) prevented the induction of LTP by 3-TBS. (b) Summary of experiments showing that intracellular dialysis of post-synaptic cells with MK-801 (1 mM) blocked LTP induction. (c, d) Summary of experiments showing the effects of NVP-AAM077 (0.1 μM) and Ro 25–6981 (0.3 μM) on the induction of LTP. (e) Bar graph comparing the effects of different NMDAR antagonists on LTP induction. The magnitude of potentiation at 30–40 min was calculated as the percentage change in synaptic response when held for the identical period of time (n=6) and is expressed as the mean±SEM. *p<0.05 compared with control; Fig. 2.
p=0.55 compared with control; Fig. 3b), excluding the contribution of L-type Ca\(^{2+}\) channels.

We next explored the possibility that an NMDAR-mediated activation of calcium/calmodulin-dependent protein kinase II (CaMKII) is required for LTP induction. Application of the selective CaMKII inhibitors, KN-62 (10 \(\mu\)M) or KN-93 (10 \(\mu\)M), but not its inactive structural analogue KN-92 (10 \(\mu\)M), prevented LTP induction (KN-62: 108.5±5.9% of baseline, \(n=8\), \(p<0.05\) compared with control; KN-93: 104.9±7.7% of baseline, \(n=6\), \(p<0.05\); KN-92: 164.8±6.7% of baseline, \(n=4\), \(p=0.43\) compared with control; Fig. 3c and d). Furthermore, post-synaptic loading of neurons with the specific CaMKII inhibitor AIP (1 \(\mu\)M) abolished LTP (108.5±5.9% of baseline, \(n=6\), \(p<0.05\), Fig. 3e). In contrast, inhibition of protein kinase C (PKC) activity with Bis-I (2 \(\mu\)M) failed to affect LTP induction (162.9±8.6% of baseline, \(n=6\), \(p=0.31\) compared with control; Fig. 3f). Taken together, these results indicate that the induction of LTP by TBS in Imp neurons depends on NMDAR activation and CaMKII.

**Post-synaptic expression of LTP**

To explore whether the expression of LTP in Imp neurons was pre- or post-synaptic in origin, three different approaches were used. We first examined the paired-pulse ratio (PPR) before and during LTP expression. If the expression of LTP involved a pre-synaptic mechanism of action, it would be associated with a decrease in the PPR. Two consecutive stimuli with a 50 ms inter-stimulus interval elicited a pair of EPSPs with the second EPSP significantly larger than the first EPSP. Under control conditions, the ratio of the amplitude of second EPSP divided by the first EPSP was 2.16±0.09 (\(n=7\)). The expression of LTP was not associated with a change in PPR (2.08±0.08 measured 30–40 min after 3-TBS, \(p=0.10\), paired Student’s \(t\) test; Fig. 4a).

To further confirm a post-synaptic locus of LTP expression, we measured quantal currents by replacing calcium with strontium in the ACSF to desynchronize transmitter release (Oliet et al., 1996). In eight neurons examined, 3-TBS significantly increased the mean amplitude of asynchronous EPSCs from 15.24±0.54 pA to 24.72±1.91 pA (\(p<0.05\); paired Student’s \(t\) test; Fig. 4b and c), whereas no significant change in the frequency of asynchronous EPSC was observed after 3-TBS (before: 9.91±0.72 Hz; 30–40 min after 3-TBS: 11.03±0.91 Hz, \(p=0.35\); paired Student’s \(t\) test; Fig. 4c and d).

There is considerable evidence that AMPAR trafficking to the post-synaptic membrane represents an important mechanism contributing to LTP expression (Song and Huganir, 2002). To assess the role for AMPAR trafficking in LTP, we performed experiments in which Imp neurons were loaded with GluR1 subunit C-terminal peptide analogue (pep-1-TGL; 50 \(\mu\)M), which has been shown to block synaptic delivery of AMPARs (Yang et al., 2008), before
3-TBS application. We found that post-synaptic loading with pep-1-TGL significantly reduced the magnitude of LTP (112.5±5.6% of baseline; \(n=6\), \(p<0.05\) compared with control; Fig. 4a). We also tested whether exocytosis process contributes to LTP expression by introducing brefeldin A, an inhibitor of protein trafficking between the Golgi apparatus and cell membranes (Klausner et al., 1992), into Imp neurons. As shown in Fig. 4f, post-synaptic application of brefeldin A (10 \(\mu\)M) resulted in a significant reduction in LTP magnitude (113.5±5.2% of baseline, \(n=6\), \(p<0.05\) compared with control). These findings suggest that the expression locus of LTP by TBS in Imp neurons is post-synaptic and AMPAR trafficking to the post-synaptic membrane contributes to LTP expression.

**Auditory fear conditioning increases AMPAR/NMDAR ratio and occludes LTP induction at LA-Imp synapses**

To investigate whether fear conditioning induces changes in synaptic strength at LA-Imp synapses, three groups of mice were tested. On day one, mice in all three groups were habituated to the training chamber. On day two, two groups were exposed to auditory fear conditioning consisting of three tone-shock pairing. On days three and four, the Ext group \((n=8)\) received ten tone-alone trials while the Cond group \((n=8)\) remained in their home cages. A third group \((Naive, n=8)\) remained in their home cages until day five when they received test trials and were killed. As shown in Fig. 5a, the Cond and Ext groups behaved similarly during habituation and conditioning. On day five, the Cond group froze more than either the naive or Ext groups. In addition, the Ext group showed good recall of extinction. One-way ANOVA showed a significant main effect \(F_{2,31}=49.8, p<0.01\), and post-hoc comparisons indicated that the Cond group froze more than the Ext group \((p<0.01)\) and the naive group \((p<0.01)\). To assess synaptic strength, the ratio of AMPAR-mediated EPSCs to NMDAR-mediated EPSCs was calculated. We observed a significant increase in the AMPAR/NMDAR ratio at synapses onto Imp neurons of Cond group mice \((3.14±0.13, n=8, p<0.01)\) when compared with either naive group \((1.84±0.07, n=8)\) or Ext group mice \((1.94±0.08, n=8;\) Fig. 5f). However, there was no significant difference between Ext group and naive group in AMPAR/NMDAR ratio \((p=0.36)\). Moreover, a strong positive correlation was found in individual mice between the AMPAR/NMDAR ratio and the percentage freezing at test \(r^2=0.74, p<0.01;\) Fig. 5c).

Finally, we tested whether fear conditioning would occlude LTP at LA-Imp synapses. As shown in Fig. 5d, the slices from either naive or Ext group mice showed robust LTP of EPSCs \((naive: 172.5±7.6% of baseline, n=5, p<0.01; Ext: 159.9±9.5% of baseline, n=6, p<0.01)\). However, the slices from Cond group mice showed
significantly impaired LTP (106.5±6.7% of baseline, n=7) when compared with either naive group (p<0.01) or Ext group mice (p<0.01), consistent with the idea that fear conditioning leads to an LTP-like phenomenon that shares one or more steps with LTP at LA-Imp synapses.

Discussion

This study identifies a novel form of TBS-induced LTP at LA-Imp synapses. This LTP is predominantly induced and expressed post-synaptically. Its induction is dependent on NR2B-containing NMDAR activation, requires a rise in post-synaptic [Ca\(^{2+}\)], and subsequent activation of CaMKII. The expression of LTP involves increasing AMPAR trafficking to the post-synaptic membrane. Our result also demonstrates that fear conditioning results in a long-lasting increase in the synaptic strength of the LA-Imp pathway and occludes electrically induced LTP.

Imp neurons receive excitatory inputs from the LA and provide feed-forward inhibition onto the lateral division of CeA (CeL) neurons, whereas IN neurons receive excitatory inputs from the BA and project to the CeM (Ehrlich et al., 2009; Paré and Duvarci, 2012). Previous studies have focused mainly on the synaptic plasticity occurring at BLA-IN synapses (Likhtik et al., 2008; Amano et al., 2010). Here we have extended these observations by showing that LTP also occurs at excitatory synapses from LA inputs onto Imp neurons, although the study of excitatory synaptic transmission in the absence of synaptic inhibition may result in a relatively non-physiological condition. Our findings that LTP was prevented by application of APV during induction and by dialysis of Imp neurons with MK-801 or BAPTA support the view that it is induced post-synaptically and requires an increase in Ca\(^{2+}\) influx through NMDARs for its induction. Our results also demonstrate that the NR2B-selective antagonists, Ro 25-6981 and ifenprodil specifically blocked the induction of LTP, whereas an NR2A-selective antagonist, NVP-AAM077, had no significant effect on LTP. This suggests that the activation of NR2B-containing NMDARs is specifically involved in
TBS-induced LTP at LA-Imp synapses. Before our work, two previous studies have provided evidence that repetitive high-frequency trains of BLA stimuli paired with post-synaptic depolarization also induced a NMDAR-dependent form of LTP at BLA-IN synapses of guinea pigs (Royer and Paré, 2002, 2003). There are three main differences between the studies by Royer and Paré (2002) and the present study. First, in the present study, the GAD67-GFP transgenic mice were used to specifically identify Imp neurons, while Royer and Paré (2002, 2003) mainly used electrophysiological and morphological criteria to distinguish IN neurons from cells in neighbouring nuclei. Secondly, a TBS protocol corresponding to the firing patterns of LA neurons during fear conditioning trials (Paré and Collins, 2000) was used in the present study to induce LTP. However, Royer and Paré (2002, 2003) used high-frequency trains of stimuli paired with post-synaptic depolarization to induce LTP. Finally, we conducted LTP experiments at LA-Imp synapses of mice, whereas Royer and Paré (2002, 2003) identified LTP at BLA-IN synapses of guinea pigs. These observations strongly suggest that both Imp and IN neurons are not just passive relay stations between the BLA and the CeA but can indeed undergo activity-dependent synaptic plasticity.

Three findings point to post-synaptic locus of LTP expression. First, the expression of LTP was not accompanied by a significant change in the PPR, a reciprocal indicator of pre-synaptic release probability. Secondly, an increase in sEPSC amplitude after TBS was not accompanied by any change in the frequency, which is generally considered to indicate a post-synaptic mode of action. Lastly, post-synaptic infusion of either pep-1-TGL or brefeldin A into Imp neurons to disrupt AMPAR trafficking to the post-synaptic membrane blocked LTP expression. A pressing question that follows these observations is how NMDAR activation leads to increased expression of post-synaptic AMPA receptors. Our finding that KN-62, KN-93 and AIP blocked LTP induction supports a mechanism involving CaMKII. Indeed, CaMKII has been reported to enhance AMPAR-mediated synaptic transmission by phosphorylating stargazin, causing stargazin binding to PSD-95, thereby increasing the number of AMPARs at synapses (Lisman et al., 2012). However, it cannot be excluded that the enhancement of AMPAR-mediated synaptic transmission during LTP is due to increased CaMKII-mediated phosphorylation of the AMPAR GluR1 subunit at Ser831 site, which may increase single channel conductance (Kristensen et al., 2011). These findings seems to contradict the idea that CaMKII expression is restricted to excitatory glutamatergic neurons and absent from GABAergic interneurons (Benson et al., 1992; Jones et al., 1994) but are consistent with early observations that both α isoform CaMKII (αCaMKII) and phosphorylated αCaMKII (pαCaMKII) are present in the mITCs of C57Bl/6j mice and fear retrieval and extinction increase pαCaMKII levels in the mITC (Meins et al., 2010). Furthermore, our immunofluorescence analysis also confirmed the presence of αCaMKII protein in Imp neurons (Supplementary Fig. S2).

Recent evidence suggests that different fear states may induce the activation of distinct ITC clusters of the amygdala; for example, Imp neurons are preferentially activated during fear expression, whereas extinction training and extinction retrieval activate IN neurons (Busti et al., 2011). We have extended these findings by showing that fear conditioning induces a lasting potentiation of AMPAR-mediated synaptic transmission and occludes TBS-induced LTP at LA-Imp synapses, suggesting that fear conditioning-induced enhancement shares one or more steps with electrically-induced LTP. These findings provide further evidence that the mechanisms of LTP are recruited behaviourally in learned fear.

In conclusion, the results of the present study provide the first description of TBS-induced LTP at LA-Imp synapses and confirm that LTP in the slice preparation and fear conditioning share a common set of synaptic mechanisms. Based on these findings, we propose that LTP induction at LA-Imp synapses may powerfully enhance the inhibitory drive to GABAergic neurons in the CeL and, consequently, cause a disinhibition of CeM output neurons and therefore increase fear responses.

Supplementary material
For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145714000133.

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Statement of interest
The authors declare no conflict of interest.

References


