Research

Both NR2A and NR2B subunits of the NMDA receptor are critical for long-term potentiation and long-term depression in the lateral amygdala of horizontal slices of adult mice

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The lateral nucleus of the amygdala (LA) is implicated in emotional and social behaviors. We recently showed that in horizontal brain slices, activation of NMDA receptors (NMDARs) is a requirement for persistent synaptic alterations in the LA, such as long-term potentiation (LTP) and long-term depression (LTD). In the LA, NR2A- and NR2B-type NMDRs coexist in synapses of LA projection neurons. We assessed the contribution of the two NMDAR subtypes to LA-LTP and LA-LTD in adult mouse brain slices by different induction protocols and by different inputs to LA neurons in the presence of different NMDAR subunit antagonists. In general, our results indicate that both NR2A and NR2B subunits are required for the formation of LA-LTP and LA-LTD. The abolishment or reduction of plasticity changes by these compounds could be due to the reduction in calcium influx via NMDARs. We also show, to our knowledge for the first time, that paired-pulse (40-msec interstimulus interval), low-frequency stimulation of external capsule fibers causes stable LTD. Rather than resulting from exclusive roles of the NMDAR subtypes, the synaptic plasticity response in the amygdala appears to be directed by the pattern of synaptic activation and the used inputs, which recruit the major NMDAR subtypes to variable extents.

The lateral nucleus of the amygdala (LA) is the entry point for most inputs into the amygdala (Pitkaenen et al. 1997). The LA is also an essential component of the neural circuitry underlying Pavlovian fear conditioning (LeDoux 2000; Davis and Whalen 2001; Maren 2005; Sigurdsson et al. 2007). Several studies indicate that both fear conditioning–induced neuronal plasticity and long-term potentiation (LTP) at amygdaloid synapses share common mechanisms of induction and expression (McKernan and Shinnick-Gallagher 1997; Rogan et al. 1997). The phenomenon of LTP, a lasting increase in synaptic efficacy following brief, intense activation of afferences terminating on synapses in the LA, has been studied nearly exclusively in coronal brain slices. In coronal slices, synaptic responses were either elicited by stimulation of fibers from the thalamus (Weisskopf et al. 1999; Schafe et al. 2000; Fendt and Schmid 2002; Lee et al. 2002) or the external capsule (EC). The EC contains amygdala afferences from higher-order sensory cortices (De Olmos et al. 1985). In horizontal slices, EC stimulation also activates excitatory afferences from cortical structures and includes afferences from the lateral entorhinal and perirhinal cortex that course through the EC and synapse in the lateral and the basolateral nucleus of the amygdala (von Bohlen und Halbach and Albrecht 2002). Stimulation within the LA also activates local connections within the LA and afferences from other amygdaloid nuclei (von Bohlen und Halbach and Albrecht 2002). The amygdala lacks an elongated structural organization compared to other brain regions (McDonald 1984; Swanson and Petrovich 1998; Faber et al. 2001; Pitkanen et al. 2003) and is therefore not subject to anisotropic conductance (Johnston and Wu 1995; Logothetis and Wandell 2004). As a result, the field potential response in the LA is not solely

dependent on underlying dendrite alignment, allowing synaptic activity to potentially contribute to the response (Johnson et al. 2008). Field potential recordings have been used extensively in the LA to study monosynaptic plasticity not only in the behaving animal, but also in brain slices (Huang and Kandel 1998; Lamprecht et al. 2006). Studies done in coronal brain slices showed that the use of high-frequency stimulation (HFS) (Li et al. 1998) only induced persistent LA-LTP when an additional depolarization of the cells (Weisskopf et al. 1999) or GABA receptor antagonists was used (Watanabe et al. 1995; Rammes et al. 2000). In contrast, LA-LTP in horizontal brain slices can be induced either by HFS of EC-afferents (Chapman and Bellavance 1992) or of intranuclear fibers (von Bohlen und Halbach and Albrecht 1998a) without adding GABA receptor antagonists. Importantly, we also could induce stable LA-LTP by stimulation of intranuclear afferents in recordings with sharp microelectrodes (Schubert and Albrecht 2008) without adding GABA receptor antagonists and without additional depolarization of the postsynaptic site. Many studies have shown that LTP can be induced in the amygdala. However, only a few studies have indicated that long-term depression (LTD) occurs in neurons within the LA. In horizontal slices, we have shown in both intracellular and extracellular recordings that LTD can be easily induced by stimulation of intranuclear fibers (Kaschel et al. 2004). It has been suggested that LTD, as a use-dependent decrease in synaptic strength, may increase the flexibility of neuronal circuits within the amygdala.

We previously showed in rat horizontal brain slices that activation of *N*-methyl-D-aspartate subtype glutamate receptors (NMDARs) is required for LA-LTP induced either by cortical stimulation (stimulation of external capsule fibers [EC]) or by stimulation of fibers running through the LA (IN) (Drephal et al. 2006). Similar data were found in rat coronal brain slices using thalamic or cortical stimulation (Gean et al. 1993; Bauer et al. 2002; Faber et al. 2005). In most central synapses, activation of

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NMDARs is also required for inducing LTD. However, little is known about how activation of NMDARs leads to these two opposing forms of synaptic plasticity. Induction of LTD requires a relatively small amount of Ca^{2+} influx through NMDARs (Cummings et al. 1996; Nishiyama et al. 2000). We previously showed that L-type calcium channels (Tchekalarova and Albrecht 2007) and group II mGluRs are involved in the induction of LA-LTD, and that NMDARs are also crucial for the induction of LTD (Kaschel et al. 2004).

NMDARs are heteromers composed of NR1 and NR2 subunits. NR1 subunits, which exist in one of eight splice variants, contain the binding site for the coagonist glycine, whereas four types of NR2 subunits exist, each of which contains a binding site for glutamate (Dingledine et al. 1999; Erreger et al. 2005). Large numbers of individual subunit combinations of NR1 and NR2A-D subunits are possible, each of which could have a different functional role within the synapse or, indeed, outside of it, in the extrasynaptic regions. Combinations of NR1 with one or more of the NR2 subunits generate receptors with functional properties (Kutsuwada et al. 1992). It has been demonstrated that NR2A and NR2B subunits dominate in the forebrain and that the NR2A subunit kinetics are more rapid compared with those of NR2B (Monyer et al. 1994).

Recent work dissecting the role of NR2 subunits in synaptic plasticity indicates that the NR2A subunit is involved in the induction of LTP, whereas the NR2B subunit contributes to the formation of LTD in the hippocampus and cortex (Liu et al. 2004; Massey et al. 2004). The mechanisms underlying amygdala LTP have been studied most extensively in the thalamo- and cortico-LA pathways. In these pathways, it has been shown that the NR2B subunit antagonist ifenprodil blocked LA-LTP (Bauer et al. 2002). In addition, systemic injections of ifenprodil before training led to a dose-dependent impairment in the acquisition of auditory and contextual fear conditioning, whereas injections before testing had no effect (Rodrigues et al. 2001). It has been shown recently that blockade of NR2B-containing NMDA receptors impairs fear acquisition while sparing fear expression. Conversely, blockade of NR2A-containing NMDA receptors impairs both fear acquisition and expression (Walker and Davis 2008). However, to our knowledge, no data are available regarding whether NR2A- or NR2B-containing NMDARs contribute to the induction of LTD in the LA. Here, we show that NR2A and NR2B are involved in amygdaloid plasticity in a pathway-specific manner, and that both NR2A and NR2B are involved in the induction of LA-LTP and LA-LTD.

Results

Effects of the NR2A- and NR2B-specific antagonists on input/output curves and PP facilitation

Examination of the contribution of the NR2B subunit to baseline activity showed that insignificant changes were obtained in input/output curves when we independently tested Co 101244 (1 μM) and Ro 04-5595 (10 μM) in the horizontal brain slices. We stimulated either IN fibers, which also involve afferents from other amygdaloid nuclei (von Bohlen und Halbach and Albrecht 1998b), or EC fibers, which mainly contain afferents from the entorhinal and perirhinal cortex (Fig. 1A; von Bohlen und Halbach and Albrecht 2002). A typical example of a field potential recorded in the LA is shown on the left side. The amplitudes increase by increasing the current intensities. The observed independence of the input/output curves of the NMDA antagonists is in agreement with our previous results in adult rats (Drephal et al. 2006), which showed that APV did not change the basal transmission. Figure 1B shows that the input/output curves remain uninfluenced by washing-in of the NR2B antagonist. We

found similar results using the intranuclear stimulation site (IN) (Fig. 2A). No significant changes in input/output curves were also found with the NR2A-specific antagonist NVP-AAM077 at different concentrations (0.05 μM , 0.1 μM , and 0.4 μM ; Figs. 1C,D, 2B–D).

Next, we studied the effect of NR2B subunit antagonists on PPF. PPF is thought to be a presynaptic process but is also dependent on inhibitory mechanisms (Zinebi et al. 2001). In the presence of Co 101244 (1 μM) and Ro 04-5595 (10 μM), PPF was unchanged using either the IN or the EC stimulation site (Figs. 1E, 2E). These results suggest that these drugs do not affect glutamate release, at least not in adult mice. In the presence of NVP-AAM077 at different concentrations, PPF was also not changed significantly (see, e.g., Figs. 1F, 2F). These results are also in accordance with findings in adult rats, in which APV did not influence PPF (data not shown).

Both NR2A and NR2B subunit antagonists decrease isolated NMDAR EPSCs

Since other authors described an influence of NR2A and/or of NR2B subunits on baseline activity (Shin et al. 2006), we performed additional recordings in the whole-cell patch-clamp mode. We recorded whole-cell NMDAR EPSCs in the presence of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM) at a holding potential of +40 mV to relieve the Mg^{2+} block of NMDAR channels. In horizontal slices derived from adult male mice in the presence of 0.05 μM , as well as of 0.1 μM NVP-AAM077, isolated NMDAR EPSC declines in the course of repetitive presynaptic EC stimulation (0.1 Hz) (Fig. 3A,C). NVP-AAM077 (0.05 μM) moderately reduced the magnitude of the NMDAR EPSC by $24.5\% \pm 5.0\%$ ($n = 4$) of its baseline value. The increase in NVP-AAM077 concentration (0.1 μM) induced a stronger reduction of NMDAR EPSCs. Subsequent addition of D-APV (50 μM) nearly completely blocked the NMDAR EPSC ($81.7\% \pm 1.7\%$ reduction, $n = 4$; Fig. 3E). To analyze the effect of NR2B blockade, we used ifenprodil to get a comparison to the recent data obtained in the LA in juvenile coronal slices (Shin et al. 2006). Application of ifenprodil (10 μM) depressed the isolated NMDAR EPSC recorded in the presence of CNQX at a holding potential of +40 mV. In the presence of the NR2B antagonist ifenprodil, the NMDAR EPSC was reduced by $46.6\% \pm 7.1\%$ ($n = 4$) of its baseline value (Fig. 3B,D). Subsequent addition of D-APV (50 μM) increased the suppression of NMDAR EPSCs (reduction by $79.6\% \pm 3.2\%$; $n = 4$). These results show that the effect of NR2B subunits on baseline activity seems to be stronger than that of NR2A subunits.

Both NR2A and NR2B subunits are involved in the induction of LA-LTP

We used the IN stimulation site to induce LA-LTP, and then we tested two different NR2B subunit antagonists. As shown in Figure 4, A and C, both NR2B antagonists provoked a reduction in LA-LTP (control: $145.3\% \pm 7.2\%$ [$n = 6$] vs. 10 μM Ro 04-5595: $121.9\% \pm 5.2\%$ [$n = 7$] and 1 μM Co 101244: $119.1\% \pm 6.1\%$ [$n = 8$]; $P = 0.03$). The effects of Ro 04-5595 and Co 10244 did not differ significantly. In the presence of NVP at different concentrations (Fig. 4B), the amount of LTP suppression induced by NVP did not differ (control: $151.8\% \pm 6.7\%$ [$n = 7$] vs. 0.1 μM NVP: $127.5\% \pm 2.5\%$ [$n = 7$] and 0.4 μM NVP: $125.1\% \pm 4.1\%$ [$n = 12$]).

In summary, because HFS-triggered LA-LTP is reduced but not blocked in the presence of NR2A or NR2B subunit antagonists, more than one subtype seems to be involved during induction.

To further substantiate that both NMDAR subtypes are capable of inducing LTP, further experiments were carried out with EC stimulation in the presence of NR2B antagonists. Figure 4,

Figure 1. Basal excitability and paired-pulse facilitation induced by stimulation of fibers running through the external capsule (EC). (A) Example of a rodent brain horizontal section. Location of recording (1 and 2) and stimulation electrodes (EC, 3; within the LA, 4) are schematically shown. If EC stimulation was used, then field potentials could be easily recorded in position 1. If the IN site was used, then the recording electrode was always located in the dorsoventral part of the LA (2). If both stimulation sites were used in one slice, the position of the recording electrode was frequently located in position 1. On the left site a representative example of original traces of a field potential recorded in the LA is shown (six averages). The amplitude of field potentials increases with increasing current intensity. (B) EC-induced input/output curves recorded in drug-free conditions and in the presence of 1 μ M Co 101244 ($n = 10$). (C,D) Input/output curves before and after application of 0.4 μ M ($n = 9$) and 0.05 μ M NVP-AAM077 ($n = 12$), respectively ($P \geq 0.05$). (E) PP facilitation of the same sample of recordings (black columns) before and (white columns) after wash-in of Co 101244 ($P \geq 0.05$). (F) PPF (black columns) before and (gray columns) after application of 0.05 μ M NVP. PP ratio: The relationship of the second-response amplitude with respect to the first-response amplitude was calculated and plotted as the PP ratio against the interstimulus interval. X-axis, interstimulus interval; EC, External capsule stimulation.

D and F, demonstrates that LTP was still induced in the presence of 1 μ M Co 101244; however, the magnitude of LA-LTP was significantly reduced (control: $153.3\% \pm 7.2\%$ [$n = 7$] vs. Co 101244: $118.4\% \pm 11.6\%$ [$n = 10$]; $P = 0.03$; Fig. 4D). This result correlates with recent data showing that the NR2B antagonist ifenprodil is able to suppress LA-LTP induced by cortical or thalamic stimulation in rat coronal brain slices (Bauer et al. 2002). Next, we investigated the role of the NR2A subunit in the formation of EC-induced LA-LTP. Blockade of the NR2A subunit with NVP also caused a reduction in LTP magnitude (control: $149.5\% \pm 5.7\%$ [$n = 7$] vs. 0.05 μ M NVP: $139.5\% \pm 8.7\%$ [$n = 11$] and 0.4 μ M NVP: $128.8\% \pm 10.4\%$ [$n = 10$]; $P = 0.03$; Fig. 4E). The different drug-free controls did not differ significantly. Moreover, the effects of used NMDA subunit antagonists did not differ significantly when we compared IN versus EC stimulation site.

Therefore, our results indicate that both NR2A and NR2B subunits are required for the formation of LA-LTP, independent of the afferents that were used. Figure 4F shows that the effect of the NR2B blockade on the magnitude of LA-LTP appeared to be stronger than that of NR2A, at least when the cortical fibers were stimulated.

NR2A and NR2B subunits are involved in the induction of LA-LTD

It is known that inhibitory mechanisms in horizontal slices are weaker than in coronal slices (Samson et al. 2003; Samson and Pare 2006). This could account for the fact that we can induce LA-LTD by IN-LFS in both intracellular and extracellular recordings in adult rats (Kaschel et al. 2004) and mice (Tchekalarova and Albrecht 2007), whereas the LFS of the EC pathway did not result in LA-LTD.

To analyze the involvement of NR2B subunits in the induction of LFS-induced LA-LTD, we stimulated IN fibers in the presence of 1 μ M Co 101244. As shown in Figure 5A, LTD was not completely blocked by the NR2B antagonist, but was significantly weaker than in drug-free control recordings (control: $76.6\% \pm 4.1\%$ [$n = 8$] vs. 1 μ M Co 101244: $88.3\% \pm 4.5\%$ [$n = 11$]; $P = 0.03$). Although LA-LTD was still induced in the presence of the NR2B antagonist, it was abolished at 0.4 μ M and at 0.1 μ M NVP (control: $83.9\% \pm 4.5\%$ [$n = 11$] vs. 0.1 μ M NVP: $95.9\% \pm 2.3\%$ [$n = 10$] and 0.4 μ M NVP: $95.3\% \pm 4.8\%$ [$n = 16$]; $P = 0.03$; Fig. 5B,C).

Figure 2. Basal excitability and paired-pulse facilitation induced by stimulation of fibers running within the LA (IN). (A) IN-induced input/output curves recorded in drug-free conditions and in the presence of 10 μ M Ro 04-5595 ($n = 18$); $P \geq 0.05$. (B–D) Input/output curves before and after application of 0.05 μ M ($n = 10$), 0.1 μ M ($n = 14$), or 0.4 μ M NVP-AAM077 ($n = 30$), $P \geq 0.05$. (E) PP facilitation of the same sample of recordings (black columns) before and (white columns) after wash-in of 10 μ M Ro 04-5595, $P \geq 0.05$. (F) PPF (black columns) before and (white columns) after application of NVP. PP ratio: The relationship of the second response amplitude with respect to the first response amplitude was calculated and plotted as the PP ratio against the interstimulus interval. X-axis: interstimulus interval.

To test whether EC stimulation and IN stimulation activated two independent pathways, we delivered paired pulses to the two stimulation sites (one pulse to IN, 5 sec later the other to EC) and compared the results. The successive application of LFS caused a persistent LA-LTD in the IN pathway, whereas single-pulse LFS of EC fibers provoked a late LA-LTP (IN: $77.7\% \pm 7.1\%$ vs. EC: $124.6\% \pm 8.7\%$; $n = 8$; Fig. 5E). A late EC-induced LA-LTP in response to low-frequency stimulation of EC fibers was described previously in coronal slices (Huang and Kandel 2007). These results show that EC and IN stimulation activate different afferents to the LA. Similar results were found in the BLA of coronal slices (Aroniadou-Anderjaska et al. 2001).

Prolonged repetitive synaptic stimulation at 1 Hz is a typical protocol for inducing LTD in the brain. However, in the adult brain, LFS is less effective at inducing LTD (Wagner and Alger 1995; Norris et al. 1996). To induce a higher rate of calcium entry postsynaptically, we tested paired-pulse low-frequency stimulation (PP-LFS) with different interstimulus intervals (ISI); this is

known to be a reliable stimulation protocol in the hippocampus of adult animals (Kemp and Bashir 1999). We tested different paired-pulse (PP) intervals of low-frequency stimulation of fibers within the LA: 30, 40, and 60 msec. Whereas ISIs from 30–40 msec provoke a reliable and persistent reduction of field potential amplitudes (30 msec: $90.6\% \pm 4.6\%$ [$n = 9$]; 40 msec: $82.7\% \pm 4.9\%$ [$n = 8$]), the interval of 60 msec resulted in insignificant changes in field potential amplitude 1 h after LFS, in comparison with pre-LFS levels ($101.2\% \pm 4.3\%$ [$n = 10$]; see also Fig. 6A).

To test whether the involvement of NR2A and NR2B subunits in LA-LTD induction is dependent on the induction protocol, we next applied PP-LFS to IN fibers with an ISI of 40 msec during drug application. Using this protocol in the presence of 10 μ M Ro 04-5595, LA-LTD did not differ significantly from drug-free control recordings (control: $82.7\% \pm 4.9\%$ [$n = 8$] vs. $84.5\% \pm 5.8\%$ [$n = 8$]; $P \geq 0.05$; Fig. 5D). In contrast, in the presence of 0.05 μ M NVP, LA-LTD was significantly reduced by PP-LFS (0.05 μ M NVP: $91.0\% \pm 2.6\%$ [$n = 10$]; 0.4 μ M NVP: $94.8\% \pm 5.2\%$ [$n = 11$]; $P = 0.03$; Fig. 5D). There was no difference in the effects on field potential amplitude between the two concentrations of NVP. Thus, as summarized in Figure 5, C and D, the NR2A antagonist seemed to have a stronger effect on the induction of LA-LTD when IN fibers were stimulated.

To our knowledge, EC-induced LA-LTD has not been demonstrated previously. After single-pulse, low-frequency stimulation of IN and EC afferents, we could record LTD in response to stimulation of fibers running through the LA, whereas simultaneously in response to EC stimulation LA neurons developed a late LTP (Fig. 5E). Therefore, we tested PP-LFS of EC-fibers to prove whether this protocol will be suited to induce LA-LTD.

As shown in Figure 5F, EC-induced PP-LFS with an interstimulus interval of 40 msec induced stable LA-LTD. This persistent decrease in field potential amplitude was completely blocked in the presence of 10 μ M Ro 04-5595 (control: $77.8\% \pm 4.2\%$ [$n = 9$] vs. Ro 04-5595: $121.3\% \pm 6.4\%$ [$n = 7$]; $P < 0.03$). Blockade of the NR2A subunit with NVP also abolished LA-LTD (0.1 μ M NVP: $101.8\% \pm 2.6\%$ [$n = 5$], 0.4 μ M NVP: $111.9\% \pm 6.6\%$ [$n = 7$]; Fig. 5G,H). Thus, PP-LFS administered in the presence of either NR2A- or NR2B-subtype-specific antagonists fails to reveal a preferred NMDAR subtype for LA-LTD induction.

In addition, we observed that PPF was increased 1 h after LFS in comparison with pre-LFS PPF (40 msec ISI: $P = 0.001$; 30 msec ISI: $P = 0.0005$; Fig. 6B,C), suggesting a presynaptic involvement in LA-LTD induction. It should be noted that changes in PPF were not significant 1 h after HFS (data not shown).

Taken together, our results indicate that both NR2A and NR2B subunits are required for the formation of LA-LTP and LA-LTD, and that the abolishment of plasticity changes by these

Figure 3. NR2A and NR2B subunits contribute to the evoked EPSC induced by EC stimulation. (A) Representative responses of a LA pyramidal neuron to repetitive (0.1 Hz) EC stimulation under control conditions, in the presence of NVP (0.05 μ M) and after application of APV (50 μ M) recorded at +40 mV. (B) Representative responses of a LA pyramidal neuron to repetitive (0.1 Hz) EC stimulation under control conditions, in the presence of ifenprodil (10 μ M) and after application of APV (50 μ M) recorded at +40 mV. The mean amplitude under control conditions was 78 ± 7 pA ($n = 7$). Current and time calibration at A also applied to B. (C) Time course of NMDA-mediated EPSC amplitude under control conditions, after bath application of 0.05 μ M NVP and subsequent bath application of 50 μ M APV. (D) Time course of NMDA-mediated EPSC amplitude under control conditions, after bath application of 10 μ M ifenprodil and subsequent bath application of 50 μ M APV. (E) Histogram showing the effect of NMDA receptor subunit blockade. EPSC amplitudes were reduced by NVP (0.05 μ M) and ifenprodil (10 μ M).

compounds could be due to a reduction in calcium influx via NMDARs.

Discussion

Using whole-cell voltage-clamp recordings from pyramidal neurons of the LA at room temperature, we show that both NR2A- and NR2B-subunit-containing NMDARs are functionally expressed at cortico-amygdala synapses not only in coronal slices derived from juvenile rats (Shin et al. 2006) but also in horizontal slices derived from adult mice. Addition of the selective NR2A antagonist NVP-AAM077 also in a highly specific concentration of 0.05 μ M as well as of the selective NR2B antagonist ifenprodil (10 μ M) to the external solution produced progressive decreases in the amplitude of the isolated NMDAR EPSC recorded at a holding potential of +40 mV in the presence of CNQX and bicuculline at least in the cortical pathway. The stronger decrease in NMDAR EPSC in the

presence of 0.5 μ M NVP-AAM077 might be due to the higher concentration used in the study of Shin et al. (2006), which partly also may block NR2B subunits of the NMDA receptor. In addition, it has been demonstrated in whole-cell patch-clamp recordings in coronal mice brain slices that ifenprodil significantly inhibited NMDA EPSCs in principal LA cells induced by thalamic stimulation (Miwa et al. 2008). AMPA and NMDA receptor-mediated responses were shown to coexist at different inputs to the LA (Mahanty and Sah 1999; Weisskopf and LeDoux 1999). In contrast to our patch-clamp recordings and other results in coronal slices also obtained in patch-clamp recordings (Mahanty and Sah 1999; Weisskopf and LeDoux 1999), we did not find significant changes in I/O curves in the presence of NR2A or NR2B antagonists in extracellular recordings. Extracellular recordings were performed under nearly the same experimental conditions as patch-clamp recordings (horizontal slice, same age, same stimulation electrodes). However, in field potential recordings, AMPA and kainate receptors were not blocked. The single-pulse stimulation in extracellular recordings does not seem to be able to release Mg^{2+} blockade. As expected in coronal slices at the resting membrane potential, EPSPs evoked either the EC or thalamic inputs were unaffected by application of the NMDA receptor antagonist APV (Mahanty and Sah 1999). In extracellular recordings, we have not observed any NMDAR involvement in basal LA transmission in adult rats (Drephal et al. 2006). The lack of any effects of the NMDAR antagonist APV on normal synaptic transmission in the LA of adult rodents has also been observed in coronal slices during EC stimulation (Huang and Kandel 1998; Schroeder and Shinnick-Gallagher 2004). Therefore, the degree of NMDAR involvement in basal transmission might be explained not only by

differences in which inputs are stimulated, by species differences, or in experimental paradigms such as temperature, but rather by the methods used. In all patch-clamp recordings, the blockade of the NMDA receptor by Mg^{2+} is removed by the positive holding potential.

Since we did not find changes in PP facilitation in the presence of NR2A or NR2B subunit antagonists, the presynaptic involvement in mediation of plasticity changes as shown recently (Tsvetkov et al. 2002; Humeau et al. 2003) owing to the existence of presynaptic NMDARs (Farb et al. 1995) seems to play a smaller role in adult rodents.

Using a pharmacological approach, our results also show that LTP and LTD induction at lateral amygdala synapses does not require a particular NMDAR subtype. However, our results, which were obtained using different LTD induction protocols, also reveal that there exists, to a certain degree, an input specificity. In the presence of NR2B subunit antagonists, EC-induced LA-LTD was

Figure 4. NR2A and NR2B subunits are involved in the induction of LA-LTP. IN stimulation caused a reduction in the magnitude of LA-LTP in the presence of different NR2B subunit antagonists (A) and in the presence of NR2A subunit antagonist NVP at different concentrations (B). Recordings in D and E were performed during stimulation of EC fibers. (D) In the presence of the NR2B subunit antagonist Co 101244, the magnitude of LA-LTP was reduced compared with drug-free controls. (E) Low concentrations of NVP suppress LA-LTP to a lower extent than do higher concentrations, when compared with controls. (HFS) High-frequency stimulation. Data points represent averaged amplitudes (mean $\pm$ SEM) normalized with respect to baseline values. Representative traces were recorded (solid lines) 5 min prior to tetanus and (dashed lines) 60 min after tetanus in the lateral nucleus of the amygdala. (C,F) Bar histograms of data points averaged 57 to 60 min after HFS and normalized with respect to baseline (mean $\pm$ SEM). Significant differences are indicated. * $P \leq 0.05$.

completely blocked, whereas using the same induction protocol, stimulation of fibers within the LA LTD was dependent to a greater extent on NR2A NMDARs. In contrast, using different inputs to LA neurons, LTP induction was not prevented by the NR2A- and NR2B-specific antagonists. Similar results were described in young mouse hippocampal slices (Berberich et al. 2005; Bartlett et al. 2007) and in the anterior cingulate cortex (Zhao et al. 2005). Our findings that NR2B-type NMDARs are involved in the induction of LTP are consistent with those obtained in genetically modified mice, in which NR2B expression was up-regulated (Tang et al. 1999; Wong et al. 2002) or NR2A signaling was impaired (Kiyama et al. 1998; Kohr et al. 2003). Our findings also correspond to results found in coronal slices of the lateral amygdala, in which stimulation of thalamic and/or cortical fibers in the presence of ifenprodil did not elicit LA-LTP (Bauer et al. 2002; Miwa et al. 2008). In addition, we have shown, for the first time to the best of our knowledge, that NR2A subunits are also involved in the induction of LA-LTP. Since NVP-AAM (0.5 μ M) had no effect on

spike timing-dependent LTP at amygdala synapses (Shin et al. 2006), the involvement of NMDA NR2A subunits seems to depend on LTP induction paradigms. Thus, LA-LTP elicited by HFS in adult mice appears to involve more than a single NMDAR subtype. In contrast, LTP induction in NR2A^{-/-} mice relies solely on NR1/NR2B receptors and requires repeated tetanization (Sakimura et al. 1995; Kiyama et al. 1998).

The first study to document LA-LTD in coronal slices of the lateral amygdala (Heinbockel and Pape 2000) used theta-pulse stimulation (TPS) (8 Hz for 150 sec) of thalamic input fibers. This stimulation resulted in LA-LTD in 21% of the tested neurons. We found that TPS of afferents within the LA caused a weaker LTD in horizontal slices than did low-frequency stimulation (900 pulses, 1-Hz LFS) (Schubert et al. 2005). We have shown in the present study that LA-LTD cannot be induced by using single-pulse LFS of EC fibers. This result has been also documented previously in horizontal slices derived from rats or mice (Kaschel et al. 2004; Tchekalarova and Albrecht 2007) or in coronal brain slices (Heinbockel and Pape 2000). It is most likely due to inadequate protocols and not to a reduced ability for decreased synaptic transmission. It is known that EC fibers innervate GABAergic interneurons besides projection neurons, and NR2B subunits contribute to NMDA receptor-mediated responses associated with thalamic and cortical input fibers not only in projection neurons but also in LA GABAergic interneurons (Szinyei et al. 2003). The cortical innervation of GABAergic interneurons seems to be stronger in comparison with intranuclear stimulation sites. Thus, projection neurons may be slightly more hyperpolarized by low-frequency EC stimulation, with no relief of the Mg²⁺ block of NMDAR, as would be the case during LFS of IN fibers. The present

results show that by using PP-LFS of EC fibers with an interstimulus interval of 40 msec, LA-LTD can be provoked. PP-LFS seemed to cause a higher increase in intracellular calcium, thereby facilitating the induction of LA-LTD. A partial restriction of the NR2B pathway after PP-LFS became only evident when cortical fibers running through the external capsule were stimulated, which gave the NR2B-subtype an advantage over NR2A. Recently it has been shown that NR2A- and NR2B-subunit antagonists block LTD in cingulate neurons (Toyoda et al. 2005).

LTD within the amygdala could enhance the relative effect of LTP at neighboring synapses by increasing the signal-to-noise ratio, as shown recently in the amygdala (Royer and Pare 2003). The true role of LTD in the amygdala remains to be determined. It might be an adaptive response in which stress or anxiety resets previously induced plastic changes to facilitate the generation of new information through LTP (Diamond et al. 2005). Taken together with recent evidence that extrasynaptic spillover of glutamate is detected exclusively by NR2B-containing NMDARs

In summary, our results do not support the model that only NR2A-type NMDARs are responsible for LTP induction. Moreover, our data regarding LFS of IN fibers also cast doubt on the proposed preponderance of NR2B in LTD induction (Collingridge et al. 2004). Sufficient calcium entry during different induction protocols seems to be more important than the presence of a distinct NMDAR subtype. Thus, rather than resulting from exclusive roles of NMDAR subtypes, the synaptic-plasticity response in the amygdala appears to be directed by the pattern of synaptic activation and the inputs used, which recruit the major NMDAR subtypes to variable extents, and trigger distinct signaling cascades (Berberich et al. 2005, 2007).

We used adult male C57BL/6 mice (8–12 wk) for electrophysiological experiments. Animals were housed in standardized conditions with an artificial 12-h dark-light cycle and a room temperature of

Figure 6. PPF increased after induction of LA-LTD. (A) Bar histogram of data points as averaged 57 to 60 min after PP-LFS with different interstimulus intervals and normalized with respect to baseline (mean $\pm$ SEM). Induction of LTD was associated with an increase in PPF. (B, C) PPF before (pre; open bars) and 60 min after (post; filled bars) PP-LFS-induced LTD. PP-LFS with an ISI of 30 msec (B) and 40 msec (C). The relationship of the second-response amplitude with respect to the first-response amplitude was calculated and plotted as a PP ratio against the interstimulus interval. X-axis, interstimulus interval; *** $P < 0.0005$ (Wilcoxon test); IN, intranuclear stimulation.

22°C. Mice had free access to food and water. All of the experimental protocols were approved by government authorities and conformed to the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Preparation and recording

Detailed methods for slice preparation have been previously described (Drephal et al. 2006). The positioning of the recording and stimulation electrodes is shown in Figure 1A. The mice were anesthetized and decapitated. For extracellular recordings, the brains were quickly removed and placed in ice-cold artificial

cerebrospinal fluid (ACSF) (129 mM NaCl, 3 mM KCl, 1.6 mM CaCl_2 , 1.8 mM MgSO_4 , 1.25 mM NaH_2PO_4 , 10 mM glucose, and 21 mM NaHCO_3). Hemisected horizontal slices (400 μm) were prepared with a vibroslicer (Campden Instruments). Slices were placed in an interface chamber and allowed to equilibrate for 120 min at 35°C. They were superfused continuously with ACSF (1.5 mL/min). The pH was maintained at 7.4 (95% O_2 and 5% CO_2).

Glass microelectrodes (Science Products) were filled with ACSF (tip resistance 1–3 M Ω) and placed in the LA to record field potentials. Bipolar stimulation electrodes were used to stimulate either EC fibers or IN fibers or both.

Stimulation parameters

An input/output (I/O) response curve was constructed by varying the intensity of single-pulse stimulation and averaging six responses per intensity. The stimulus intensity that evoked a mean field potential equal to 50% of the maximum response was then used for all subsequent stimulations. To evaluate short-term synaptic interactions, we delivered paired-pulse (PP) stimuli with interstimulus intervals ranging from 10 to 500 msec. Six consecutive impulses were averaged off-line for each interval. The relative amplitude of the second response with respect to that of the first was calculated and plotted as the PP ratio against the interstimulus interval. Following PP stimulation, single stimuli were applied for at least 30 min, and responses were monitored. Single stimuli (duration of 100 μsec) were presented every 10 sec. Once a stable baseline of responses was obtained for at least 20 min, we either (1) recorded input/output curves and PP facilitation (PPF) in the presence of NR2A or NR2B subunit antagonists and then continued single-pulse stimulation for 15 min; or (2) in drug-free conditions, directly delivered high-frequency stimulation (HFS) as two trains at 100 Hz (duration: 1 sec, 30 sec apart) or low-frequency stimulation (LFS). The HFS paradigm has been chosen since theta burst stimulation did not produce consistent and reliable induction of LA-LTP. Different LFS protocols (single-pulse stimulation or PP stimulation [PP-LFS] with different interstimulus intervals; 1 Hz, 15 min) were used to induce LTD. In some cases, we tried to record field potentials in the LA if single-pulse stimulation and LFS were alternatively applied either to EC or to afferents running through the LA (IN). Test stimulus pulses were applied alternately to IN and EC, with an interval of 5 or 10 sec between an EC pulse and an IN pulse. However, in many slices, we detected either field potentials in response to EC stimulation or in response to intranuclear stimulation site. Therefore, not in all cases do both afferents converge at those LA neurons whose activity was recorded extracellularly. Drug-induced changes in baseline activity were considered. Subsequent responses to single stimuli were recorded for at least 60 min, and their amplitudes were quantified as percent change with respect to baseline.

Patch-clamp recordings

Whole-cell recordings were performed under submerged recording conditions in horizontal slices (300 μm) derived from 8-wk-old mice. The internal patch solution contained 130 mM Cs-gluconate, 8 mM CsCl, 10 mM HEPES, 10 mM EGTA, 2 mM NaCl, and 2 mM Mg-ATP (pH 7.3). Whole-cell recordings were made using a Multiclamp 700B (Axon Instruments, Molecular Devices) and standard techniques (Gebhardt and Cull-Candy 2006). Patch pipettes pulled from thick-walled borosilicate glass had a resistance of 6–8 M Ω when filled with solution. LA projection neurons were identified visually. The access resistance was checked during the recording, and the experiments were stopped if the access resistance had changed more than 10%. NMDAR-isolated evoked EPSCs were measured after EC stimulation. Data were recorded by Clampex 9 with a sampling rate of 10 kHz and filtered at 2 kHz.

Drug application

All of the drugs were bath-applied at the indicated concentrations, starting at least 30 min before HFS or LFS and continuing throughout the train stimulation for a total period of 45–60 min. We alternated between control and treatment experiments

to account for potential day-to-day and time-of-day differences. We used two different NR2B antagonists that were obtained from Tocris Bioscience. It is known that the Co 101244 hydrochloride (1-[2-(4-hydroxy-phenoxy)-ethyl]-4-(4-methyl-benzyl)-piperidin-4-ol) IC₅₀ values are 0.043 μ M, >100 μ M, and >100 μ M for the NR1A/2B, NR1A/2A, and NR1A/2C subunit combinations, respectively (alternative names: Ro 63-1908 and PD 174494). In addition, the NR2B-specific antagonist Ro 04-5595 (1-[2-(4-chlorophenyl)ethyl]-1,2,3,4-tetrahydro-6-methoxy-2-methyl-7-isoquinolinol hydrochloride; K_i = 31 nM) was used. The NR2A-specific antagonist NVP-AAM077 ([R-[(S)-1-(4-bromo-phenyl)-ethylamino]-2,3-dioxo-1,2,3,4-tetrahydro-quinolin-5-yl]-methyl]-phosphonic acid; NVP) was a kind gift from Novartis Pharma. NVP-AAM077 is ~14-fold selective for NR2A versus NR2B receptors against recombinant receptors in HEK293 cells (Bartlett et al. 2007). To separate the effects on NR2A from those on NR2B with regard to plasticity, we tested different concentrations (0.05, 0.1, and 0.4 μ M NVP). Recent studies indicated that, at a high concentration (0.4 μ M), NVP-AAM077 also antagonizes NR2B-containing NMDA receptors (Berberich et al. 2005; Weitlauf et al. 2005; Frizelle et al. 2006), whereas considering the uncertainties of the concentrations of NVP-AAM077 reaching synaptic sites, it can be suggested that at concentrations of 0.05 and 0.1 μ M, NVP-AAM077 will block predominantly NR2A subunits (Bartlett et al. 2007; Wu et al. 2007). Ifenprodil hemitartrate (10 μ M), CNQX (10 μ M), APV (50 μ M), and bicuculline methochloride (30 μ M) were from Tocris.

Data analysis

Data were collected and averaged with the custom-made software Signal 2 (Cambridge Electronic Design). We defined the field-potential amplitude as the absolute DC voltage of a vertical line running from the minimal point of the field potential to its intersection with a line running tangential to the points of field-potential onset and offset. It is assumed that the recorded negative wave reflects a summation of both excitatory postsynaptic potentials (EPSPs) and synchronized action potentials (population spike component) (Watanabe et al. 1995; Doyere et al. 2003). We therefore analyzed the amplitude of field potentials in the present study. Additionally, the slope measure in the lateral amygdala is more sensitive to variability and signal noise, making it more difficult to analyze (Doyere et al. 2003).

Whole-cell recordings were analyzed with Clampfit 9. Data are given as mean $\pm$ SEM.

Significance of differences within groups was calculated by Wilcoxon test. Significance of differences between groups was calculated by Mann-Whitney test (Software GraphPad Prism 5). $P < 0.05$ was considered to be significant. Analyses between the groups were performed for the whole input/output curves and for all intervals together after determination of PP ratios. To express and compare changes of field potential amplitudes between the animal groups, we averaged responses from the 57–60 min period after HFS or LFS.

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Both NR2A and NR2B subunits of the NMDA receptor are critical for long-term potentiation and long-term depression in the lateral amygdala of horizontal slices of adult mice

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