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Opposite effects of tetanic stimulation of the auditory thalamus or auditory cortex on the acoustic startle reflex in awake rats

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Abstract

The amygdala mediates both emotional learning and fear potentiation of startle. The lateral amygdala nucleus (LA) receives auditory inputs from both the auditory thalamus (medial geniculate nucleus; MGN) and auditory association cortex (AAC), and is critical for auditory fear conditioning. The central amygdala nucleus, which has intra-amygdaloid connections with LA, enhances startle magnitude via midbrain connections to the startle circuits. Tetanic stimulation of either MGN or AAC *in vitro* or *in vivo* can induce long-term potentiation in LA. In the present study, behavioural consequences of tetanization of these auditory afferents were investigated in awake rats. The acoustic startle reflex of rats was enhanced by tetanic stimulation of MGN, but suppressed by that of AAC. All the tetanization-induced changes of startle diminished within 24 h. Blockade of $GABA_B$ receptors in the LA area reversed the suppressive effect of tetanic stimulation of AAC on startle but did not change the enhancing effect of tetanic stimulation of MGN. Moreover, transient electrical stimulation of MGN enhanced the acoustic startle reflex when it lagged behind acoustic stimulation, but inhibited the acoustic startle reflex when it preceded acoustic stimulation. The results of the present study indicate that MGN and AAC afferents to LA play different roles in emotional modulation of startle, and AAC afferents are more influenced by inhibitory $GABA_B$ transmission in LA.

Introduction

The lateral nucleus of the amygdala (LA) mediates auditory fear conditioning (AFC) (Hitchcock & Davis, 1986; Romanski & LeDoux, 1992; Maren, 1996; Fendt, 2001; Goosens & Maren, 2001; Tazumi & Okaichi, 2002). Auditory inputs to LA originate from the medial geniculate nucleus (MGN) and auditory association cortex (AAC) (LeDoux *et al*., 1990; Turner & Herkenham, 1991; Mascagni *et al*., 1993; Romanski & LeDoux, 1993; Doron & LeDoux, 1999; Woodson *et al*., 2000). Although the pattern of MGN afferents to LA principal neurons and interneurons is different from that of AAC afferents (LeDoux *et al*., 1991; Li *et al*., 1995, 1996; Farb & LeDoux, 1997; Weisskopf & LeDoux, 1999; Zinebi *et al*., 2001), the detailed functional differences between the thalamic and cortical afferents remain unknown.

Long-term potentiation (LTP) in LA, which occurs during AFC (McKernan & Shinnick-Gallagher, 1997; Quirk *et al*., 1995, 1997; Rogan & LeDoux, 1995; Rogan *et al*., 1997), can be induced by tetanic electrical stimulation of MGN or AAC afferents both *in vitro* and *in vivo* (Chapman *et al*., 1990; Bauer *et al*., 2002; Clugnet & LeDoux, 1990; Rogan & LeDoux, 1995; Watanabe *et al*., 1995; Huang & Kandel, 1998; Weisskopf *et al*., 1999; Yaniv *et al*., 2001; Doyère *et al*., 2003; Bauer & LeDoux, 2004). However, patterns of tetanization-induced LTP associated with the two auditory structures are different (Yaniv *et al*., 2001; Doyère *et al*., 2003). Thus these two auditory afferents may play differential roles in mediating the neural plasticity in LA and even in AFC. Behavioural consequences of tetanization of these two auditory afferents have not been investigated before.

The acoustic startle reflex (ASR) involves rapid contractions of skeletal muscles along the whole body following a sudden and intense sound. The amygdala is essential in fear potentiation of startle (for reviews see Davis, 1992; Fendt & Fanselow, 1999; Gewirtz & Davis, 2000). Outputs from LA principal neurons are transferred through intra-amygdaloid connections toward the central nucleus (CE) of the amygdala (Pitkänen & Amaral, 1998; Paré *et al*., 2004), which has both direct projections to the startle circuits (Rosen *et al*., 1991; Koch & Ebert, 1993; Fendt *et al*., 1997) and synaptic relays in the midbrain structures projecting to the startle circuits (Rosen & Davis, 1988, 1990; Yeomans & Pollard, 1993; Fendt *et al*., 1994, 1996; Frankland & Yeomans, 1995; Meloni & Davis, 1999, 2000). Damage to the amygdala reduces the ASR (Schanbacher *et al*., 1996) and pharmacological disinhibition of the amygdala enhances the ASR (Fendt *et al*., 2000). Thus, certain changes in neural activity in LA may be reflected in the ASR.

LA principal (projection) neurons interact with local GABAergic interneurons (Lang & Paré, 1997, 1998; Mahanty & Sah, 1998). Interneurons inhibit principal neurons partially via presynaptic

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$GABA_B$ receptors (Huang & Gean, 1994; Szinyei *et al*., 2000). In the present study, we investigated effects of tetanic stimulation of either MGN or AAC on the ASR, and contributions of $GABA_B$ receptors in the LA area to the tetanic stimulation effects.

Materials and methods

Experimental subjects

Male Wistar rats (*Rattus norvegicus*), 300–450 g, obtained from Charles River Canada (St Constant, Quebec) were housed individually in a 12-h light–dark cycle (lights on at 07.00 h). The University of Toronto Animal Care Committee, following the guidelines of the Canadian Council of Animal Care, approved the use and care of the animals reported in this study.

Surgery

Detailed surgical and implantation procedures have also been described elsewhere (Li & Yeomans, 2000). Briefly, under sodium pentobarbital anaesthesia (60 mg/kg, i.p.; M.T.C. Pharmaceuticals, Cambridge, ON, Canada) following pretreatment with atropine sulphate (0.4 mg/kg i.p.), injection guide cannulae (Plastics One, Roanoke, VA, USA) and monopolar brain-stimulation electrodes (Li & Yeomans, 2000) were inserted stereotaxically (Stoelting Instruments, Wood Dale, IL, USA) into the brains of 62 rats, based on coordinates of Paxinos & Watson (1997). Referenced to bregma, bilateral cannulae were aimed at LA (AP, −2.8 to −3.8 mm; ML, ±5.4 mm, DV, −7.5 mm). Bilateral electrodes for MGN stimulation were aimed at the lateral region of the medial division of MGN (AP, −5.4 mm; ML, ±3.2 mm; DV, −5.9 to −6.2 mm). The medial division of MGN, which belongs to the nonlemniscal, thalamocortical auditory system and is involved in plasticity of frequency-receptive fields during learning (Lennartz & Weinberger, 1992), is the region that sends axonal projections to LA and mediates AFC (e.g. Doron & LeDoux, 1999). Bilateral electrodes for AAC stimulation were aimed at the temporal cortex area TE3 (AP, −5.8 mm; ML, ±6.5 mm; DV, −5.5 mm). Area TE3 in rats is the major AAC that projects to LA (Mascagni *et al*., 1993; Romanski & LeDoux, 1993; Shi & Cassell, 1997). Each stimulation electrode was connected to a male Amphenol pin connector (Electrosonics, Toronto, ON, Canada), which was inserted into a McIntyre socket (Molino & McIntyre, 1972) that was mounted on the skull with both anchoring screws and dental cement. Rats were allowed at least 1 week to recover from the general effects of surgery.

Stimulus delivery

Electrical stimuli were generated by a Grass S-88F stimulator (Grass, Quincy, MA, USA), which provided monophasic cathodal rectangular pulses (pulse duration 0.2 ms) via constant-current photoelectric stimulus-isolation units (model PSIU6). The current of single pulses used for tetanic stimulation was kept at a sufficiently low level that single-pulse stimulation of either MGN or TE3 did not evoke a bilateral whole-body response. If a single-pulse stimulation did evoke a bilateral whole body response such as the startle reflex, the current threshold was determined and the current for tetanic stimulation was first set 100 μA below the threshold and increased in 20-μA steps between stimulation series. In the present study, tetanic stimulation of MGN or TE3 had currents in the range of 180–350 μA, and consisted of four series of pulse trains (pulse frequency, 100 Hz; train length, 100 ms; train interval, 100 ms; number of trains, 10) with an interseries interval of 1 min. Low-frequency stimulation of MGN or TE3 was used as the control manipulation for tetanic stimulation. The parameters of low-frequency stimulation were the same as those of tetanic stimulation except that the pulse frequency was 5 Hz.

Acoustic stimuli were generated from a white-noise generator (model 901B, Grason-Stadler, Concord, MA, USA), gated into noise bursts with a duration of 50 ms by custom-made analogue switches, amplified by an amplifier (model C-20A; Bogen, Paramus, NJ, USA), and presented through two loudspeakers (MacBride Loudspeaker Source, Waterloo, ON, Canada) placed inside the sound-attenuating chamber. The sound level was set at 96 dB SPL (re 0.0002 dynes/cm^2) by a passive attenuator (Hewlett Packard, model 350D; Palo Alto, CA, USA) and monitored at the centre of the cage using a sound-level meter (Type 1561-A; General Radio Company, Concord, MA, USA).

Startle apparatus

Amplitudes of startle reflexes were measured using a stabilimeter that was placed in a sound-attenuating chamber (Cassella & Davis, 1986; Li & Yeomans, 2000). The output of the accelerometer was filtered at 2 Hz, amplified 10× and integrated by a signal conditioner (Endevco, model 2775A), then displayed and measured on a digital storage oscilloscope (Hitachi, model VC-6025 A; Scarborough, ON, Canada).

Drug injection

The $GABA_B$ receptor antagonist phaclofen (3-amino-2-(4-chlorophenyl)propylphosphonic acid; Qbiogene Inc., CA, USA), was dissolved in 0.9% NaCl solution whose final pH was adjusted to 7.0. The final concentration of phaclofen was 34 μM.

Injection cannulae (30-gauge; Plastics One), connected to Hamilton microsyringes by polyethylene tubings, were inserted into the guide cannulae. Hamilton microsyringes (1 μL) mounted in an infusion pump were used to infuse solutions at a constant rate of 0.25 μL/min. After bilateral injection, injection cannulae were left in place for 1 min. The injection volume was 0.8 μL on each side of the brain.

Experimental procedures

On each day before testing, rats were placed in the startle box for 30 min without being presented with startling stimuli, and then presented with 60 startling sounds during another 30 min. Formal testing (also with 30 min habituation in the startle box) was commenced on the next day if stable startle responses for 2 successive days were found. All testing was conducted during the light phase (10.00–18.00 h) of the light–dark cycle at intertrial intervals of 30 s.

Experiment 1: unilateral tetanic stimulation of MGN or TE3

ASR amplitudes of nine rats with MGN electrodes and nine rats with TE3 electrodes were measured before, immediately after, 50 min after, and 24 h after unilateral tetanic stimulation. ASR amplitudes of five rats with MGN electrodes and five rats with TE3 electrodes were measured before, immediately after, 50 min after and 24 h after low-frequency stimulation.

Experiment 2: bilaterally blocking $GABA_B$ receptors in LA

Twelve rats with MGN electrodes and LA cannulae were assigned to two groups. The first group ($n = 7$) received phaclofen injection into

LA, followed by tetanic stimulation of MGN. The second group ($n = 5$) received saline injection into LA, followed by tetanic sitmulation of the MGN. Twelve rats with TE3 electrodes and LA cannulae were also assigned to two groups. The first group ($n = 7$) received phaclofen injection into LA, followed by tetanic stimulation of TE3. The second group ($n = 5$) received saline injection into LA, followed by tetanic stimulation of TE3. Three rats with MGN electrodes and three rats with TE3 electrodes in phaclofen-injection groups of this experiment were randomly selected from the rats tested in Experiment 1. These six rats' startle responses had returned to the pretetanization level before they were used in this experiment. Because bilateral phaclofen injection led to more effective blockade of $GABA_B$ receptors in the LA area than unilateral injection, both bilateral injection and bilateral stimulation were used in Experiment 2 to keep bilateral balance between the pharmacological treatment and electrical stimulation.

Following baseline testing for 20 min, these rats were removed from the startle box and injected with phaclofen or saline bilaterally. Immediately after injection, rats were placed back in the startle box for bilateral tetanic stimulation and acoustic startle testing. The testing procedure was the same as that used in Experiment 1. Three additional rats were used for examining the effect of phaclofen injection on the ASR. After these rats were injected and placed back in the startle box, no electrical stimulation was given. Amplitudes of their ASRs were measured at the time points associated with those immediately after, 1 h after, and 24 h after tetanic stimulation in other rats.

Experiment 3: effects of tetanic stimulation on startle induced by pairing acoustic stimulation with electrical stimulation of MGN or TE3

In this experiment, we wanted to know (i) whether transient stimulation of MGN or AAC had any modulating effects on auditory startle; (ii) if yes, whether the modulating effects could be changed by tetanic stimulation.

Acoustic stimulation was paired with subthreshold, transient (single-pulse) electrical stimulation of MGN or TE3 with different interstimulus intervals (ISIs), including −25, −20, −15, −10, −5, 0, 5, 10, 15, 20 and 25 ms (Li *et al*., 1998, 1999; Li & Yeomans, 2000). Positive ISI values were defined as those for which the acoustic stimulus led the electrical stimulus. The current of single-pulse electrical stimulation was in the range 230–340 μA. Eight rats with MGN electrodes and eight rats with TE3 electrodes were used.

Startle responses were measured at each of these ISIs before, immediately after and 24 h after bilateral tetanic stimulation of MGN or TE3 to assess changes in startle responses resulting from tetanic stimulation. Five trials were assigned to each ISI with the presenting order arranged in a pseudo-random manner.

Statistical analyses

Amplitudes of startle responses were normalized for each animal with respect to the mean values of baseline startle before tetanic stimulation. Statistical analyses applied to the data were ANOVA, with the significance level set at $P < 0.05$.

Histology

At the end of testing, the rats were killed with an overdose of sodium pentobarbital. Lesions were made by an anodal DC current (500 μA for 10 s) via the electrodes to mark the stimulation sites. The brains were removed, stored in 10% formalin with 30% sucrose until they sank, and then sectioned at 40 μm in the frontal plane in a cryostat (−20 °C). Sections were examined to determine locations of cannula and electrode tips.

Results

Histology

The sites of stimulation electrodes in the three experiments are presented in Fig. 1. The injection sites used in Experiment 2 are presented in Fig. 2. Correct placements of stimulation sites in MGN or TE3 were based on coordinates of Paxinos & Watson (1997). In Experiment 1, correct placements of electrodes used for stimulating MGN or TE3 were found in 23 rats (Fig. 1A). In Experiment 2, correct placements of cannulae used for injection and electrodes used for stimulating MGN or TE3 were found in 19 rats (Figs 1B and 2). In Experiment 3, correct placements of electrodes used for stimulating MGN or TE3 were found in 11 rats (Fig. 1C). Behavioural results are presented only for the rats with correct placements of electrodes used in the three experiments, and for the rats with correct placements of injection cannulae used in Experiment 2.

Experiment 1: effects of unilateral tetanic stimulation of MGN or TE3

The amplitudes of ASR recorded before, immediately after, 1 h after and 24 h after tetanic stimulation of MGN were significantly different ($F_{3,28} = 5.420$, $P < 0.05$) (Fig. 3A). *Post hoc* tests indicate that, compared to the baseline ASR before tetanic stimulation, startle responses both immediately after and 1 h after unilateral tetanic stimulation of MGN were significantly enhanced ($P < 0.05$). However, the startle enhancement was not significant 24 h after tetanic stimulation ($P > 0.05$).

The amplitudes of ASR before, immediately after, 1 h after and 24 h after tetanic stimulation of TE3 were also significantly different ($F_{3,20} = 3.800$, $P < 0.05$; Fig. 3B). However, in contrast to the effects of tetanic stimulation of MGN, the ASR amplitude was suppressed after unilateral tetanic stimulation of TE3. *Post hoc* tests indicate that the ASR amplitude was significantly reduced immediately following unilateral tetanic stimulation of TE3 ($P < 0.05$). This reduction in ASR amplitude lasted to 1 h after tetanic stimulation ($P < 0.05$) but had disappeared 24 h later ($P > 0.05$).

There were no effects of low frequency stimulation of either MGN ($F_{3,12} = 0.841$, $P > 0.05$) or TE3 ($F_{3,16} = 0.566$, $P > 0.05$) on the ASR amplitude (Fig. 4), even though a slight reduction in the ASR occurred immediately and 1 h after stimulation of MGN, and immediately after stimulation of TE3.

Experiment 2: effects of bilaterally blocking $GABA_B$ receptors in the LA area

For rats with bilateral tetanic stimulation of MGN, two-way ANOVA indicate that there was no significant difference in the ASR amplitude between the rats with saline injection and those with phaclofen injection ($F_{1,35} = 0.383$, $P = 0.542$). Also, there was no significant interaction between testing time and saline or phaclofen in these rats ($F_{3,35} = 0.968$, $P = 0.424$).

Figure 5A shows the effects of bilateral tetanic stimulation of MGN in rats receiving either saline or phaclofen injection into LA. Similar to unilateral tetanic stimulation of MGN, bilateral tetanic stimulation of MGN in rats with saline injection significantly increased the ASR

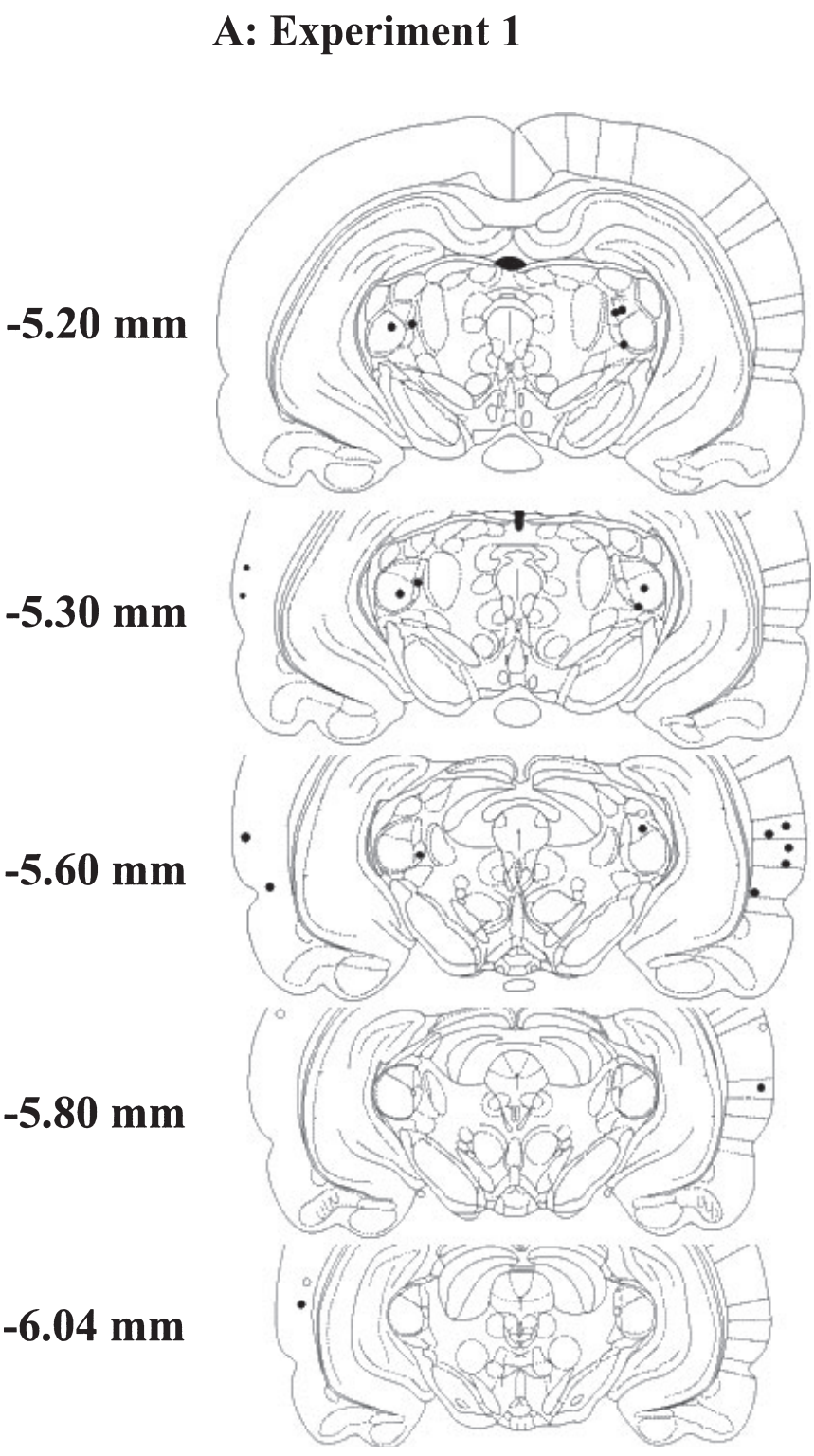

FIG. 1. Locations of electrode tips for tetanic stimulation of the regions of the medial geniculate nucleus (MGN) and that of the auditory association cortex (AAC, area TE3) for the three experiments (Panel A, Experiment 1; Panel B, Experiment 2; Panel C, Experiment 3); ●, electrode locations within the target areas; ○, electrode locations outside the target areas. Different panels in this and the next figure are frontal brain sections based on Paxinos & Watson (1997) with distances to bregma shown in the left column.

($F_{3,16} = 4.832$, $P < 0.05$). *Post hoc* tests indicate that bilateral tetanic stimulation of MGN in the rats with saline injection into LA immediately enhanced the ASR ($P < 0.05$). However, the tetanization effect was not significant either 1 or 24 h later ($P > 0.05$). In the rats with phaclofen injection into LA, the effect of bilateral tetanic stimulation of MGN was also significant ($F_{3,16} = 4.706$, $P < 0.05$). *Post hoc* tests indicate that, immediately following bilateral tetanic stimulation of MGN, the ASR was significantly enhanced ($P < 0.05$).

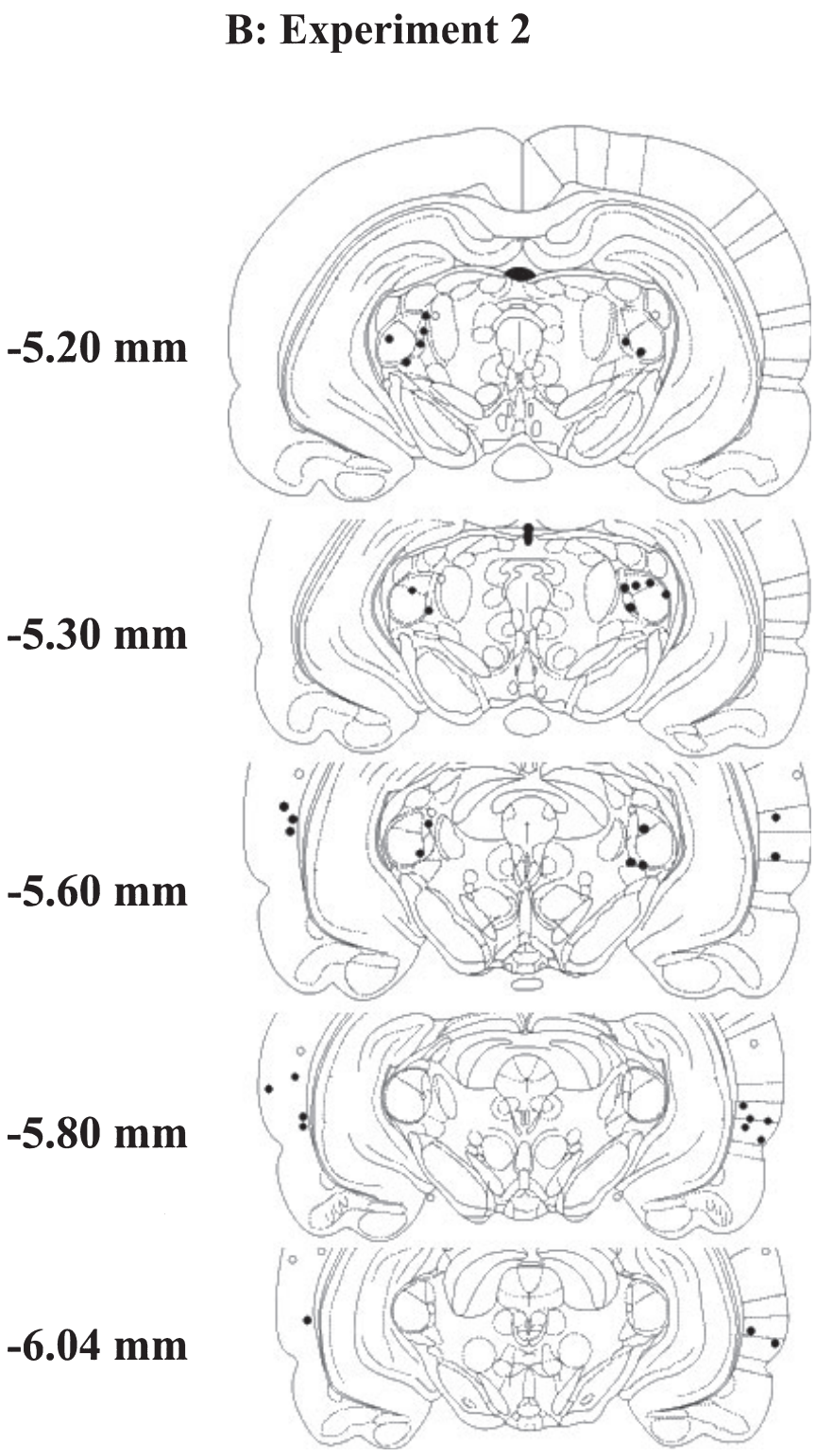

FIG. 1. Continued

However, the enhancing effect was not significant either 1 or 24 h later ($P > 0.05$). There were no statistically significant differences between the rats that were also used in Experiment 1 for unilateral tetanic stimulation of MGN and the rats that were used only in this experiment with phaclofen injection ($F_{1,12} = 0.055$, $P > 0.05$).

For rats with bilateral tetanic stimulation of TE3, two-way ANOVA indicates that there was a significant difference between the rats with saline injection and those with phaclofen injection ($F_{1,31} = 14.128$, $P < 0.05$). Also, there was a significant interaction of testing time and saline or phaclofen in these rats ($F_{3,35} = 4.593$, $P < 0.05$).

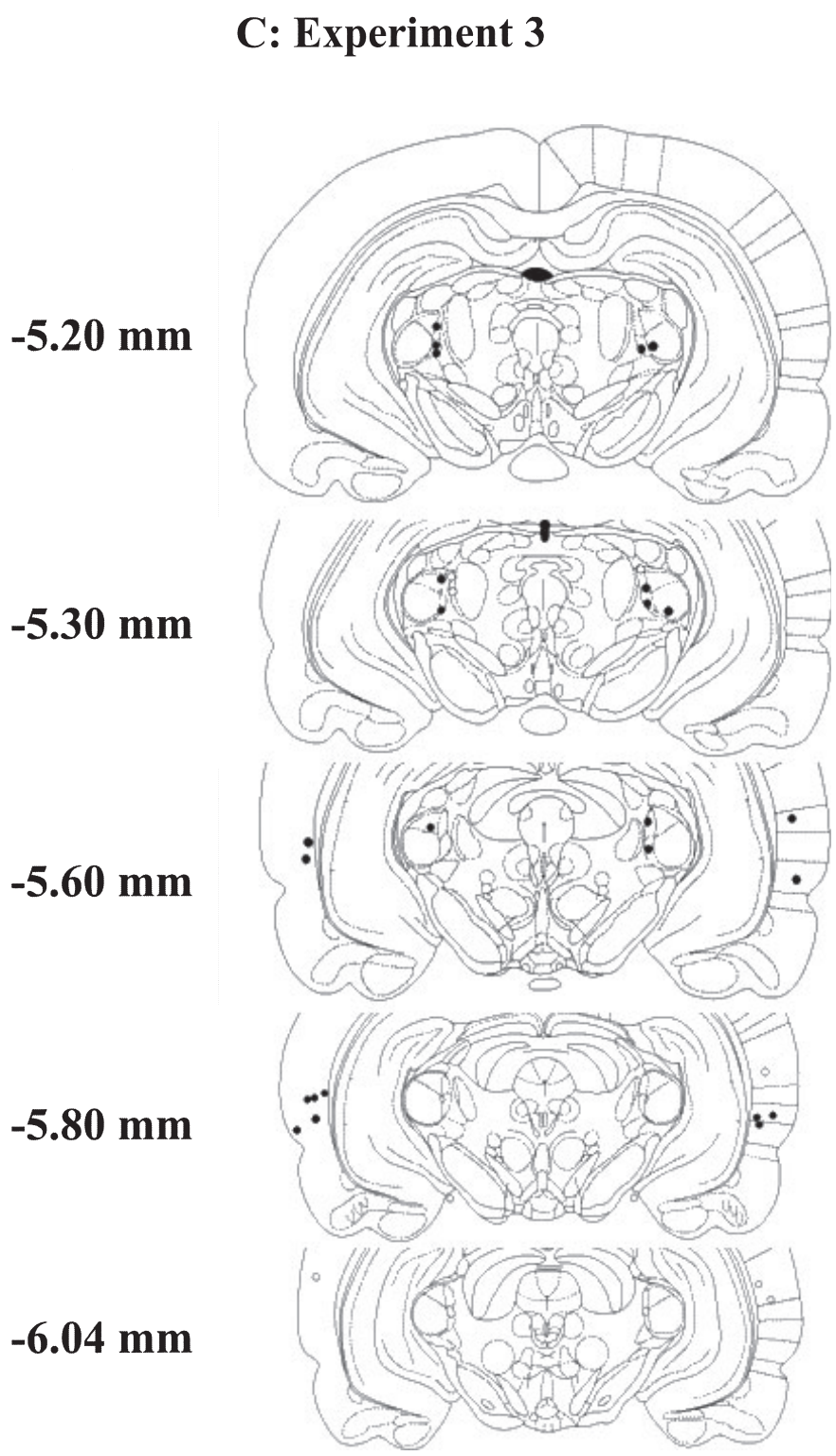

FIG. 1. Continued

Figure 5B shows the effects of bilateral tetanic stimulation of TE3 in rats receiving either saline or phaclofen injection into LA. Similar to unilateral tetanic stimulation of TE3, bilateral tetanic stimulation of TE3 in rats with saline injection significantly decreased the ASR ($F_{3,12} = 4.570$, $P < 0.05$). *Post hoc* tests indicate that the ASR amplitudes were significantly suppressed both immediately ($P < 0.05$) and 1 h ($P < 0.05$) after tetanic stimulation of the TE3. The suppressing effect was not significant 24 h later ($P > 0.05$). Interestingly, in rats with injection of phaclofen into the LA, the ASR was significantly enhanced ($F_{3,16} = 4.130$, $P < 0.05$). The enhancement was significant both immediately ($P < 0.05$) and 1 h ($P < 0.05$) after bilateral tetanic stimulation of TE3. The ASR returned to the baseline

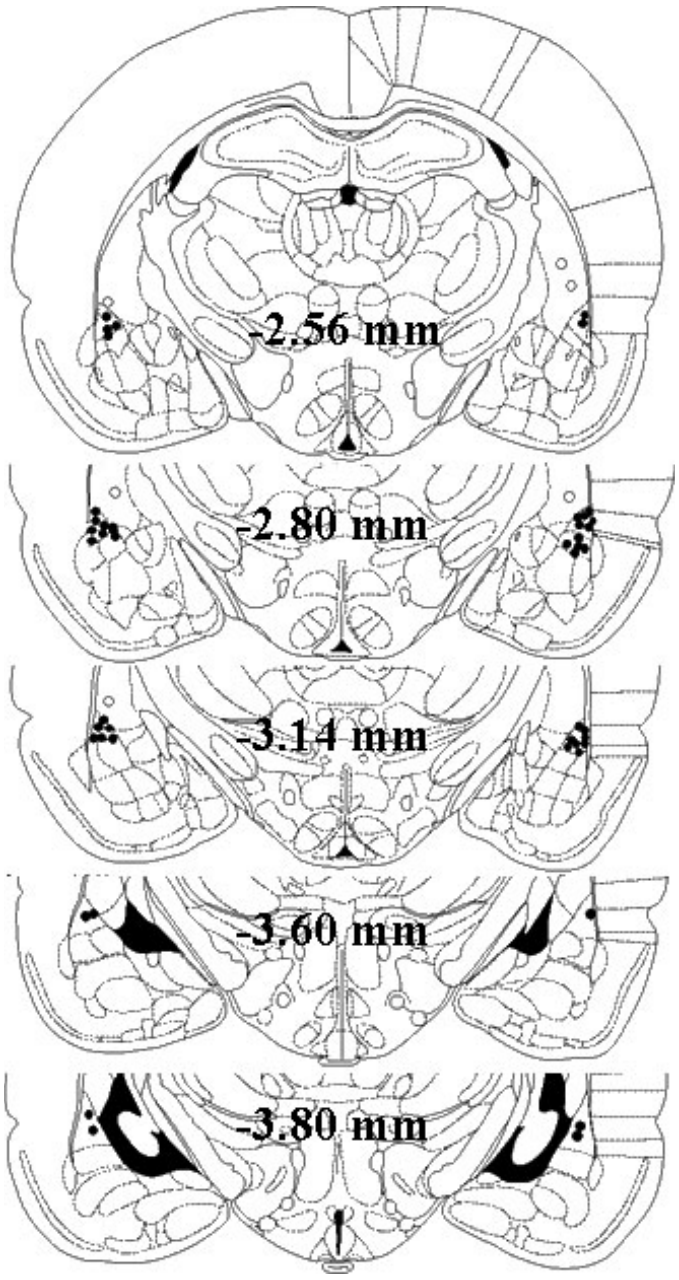

FIG. 2. Locations of cannula tips around the regions of the lateral nucleus of amygdala (LA) for 24 rats in Experiment 2; ●, cannula locations within the target areas; ○, cannula locations outside the target areas.

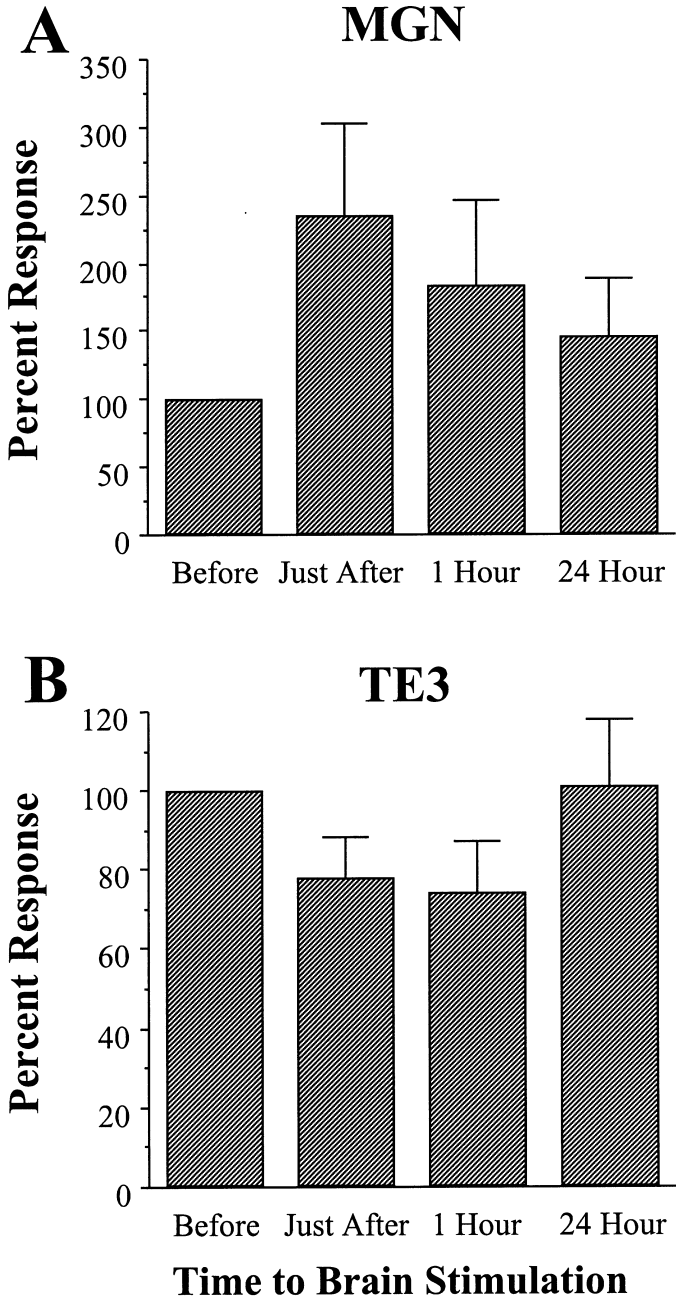

FIG. 3. Normalized amplitudes of the acoustic startle reflex (ASR) before, immediately after, 1 h after and 24 h after unilateral tetanic stimulation of MGN (Panel A) or TE3 (Panel B). In this and the following figures, the baseline ASR before tetanic stimulation is 100%, and the error bars are SEM.

level 24 h later. There were no statistically significant differences between the rats that were also used in Experiment 1 for unilateral tetanic stimulation of TE3 and the rats that were used only in this experiment with phaclofen injection ($F_{1,12} = 1.228$, $P > 0.05$).

Figure 6 shows the normalized amplitudes of the ASR for the rats receiving phaclofen injection but no tetanic stimulation. Injection of phaclofen in LA seemed to slightly reduce the ASR; however, the change was not significant ($F_{3,8} = 1.416$, $P > 0.05$).

Experiment 3: effects of tetanic stimulation on startle induced by pairing acoustic and electrical stimulation

When the current of transient electrical stimulation was below the threshold of the startle-like responses, the amplitude of the startle response to paired acoustic stimulation and bilateral electrical stimulation of MGN was significantly determined by both the ISI between the two types of stimuli ($F_{10,185} = 14.892$, $P < 0.05$) and tetanic stimulation ($F_{2,185} = 8.402$, $P < 0.05$). However, the interaction between ISI and tetanization was not significant ($F_{20,185} = 0.345$, $P > 0.05$; Fig. 7, upper panel). When the electrical stimulus preceded the acoustic stimulus, the startle response was suppressed, as with electrically induced prepulse inhibition (Li *et al*., 1998). When the electrical stimulus lagged a few ms behind the acoustic stimulus, the summation between the two stimuli was evident (Rosen & Davis, 1988). There was also a significant effect of tetanic stimulation.

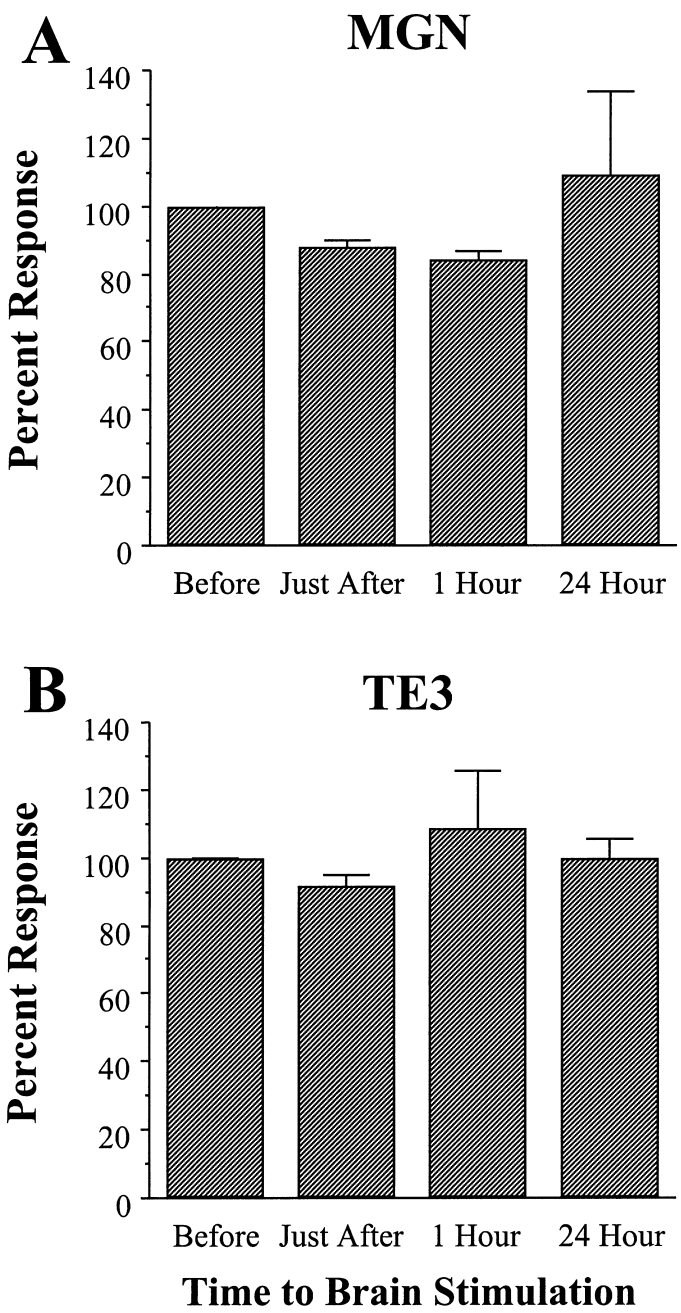

FIG. 4. Normalized amplitudes of the acoustic startle reflex (ASR) before, immediately after, 1 h after and 24 h after unilateral low-frequency sustained stimulation of MGN (Panel A) or TE3 (Panel B).

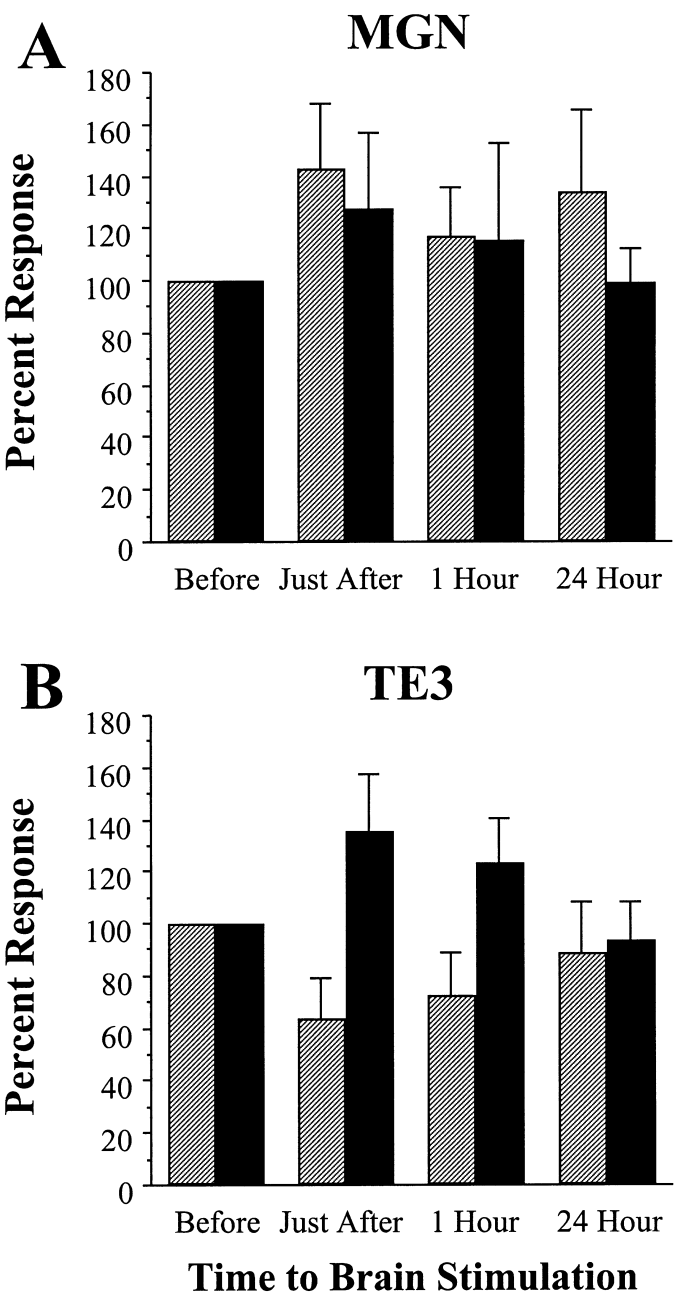

FIG. 5. Normalized amplitudes of the ASR before, immediately after, 1 h after and 24 h after bilateral tetanic stimulation of MGN (Panel A) or TE3 (Panel B). Light bars indicate the ASR of rats receiving saline injection; dark bars indicate the ASR of rats receiving phaclofen injection.

Following bilateral tetanic stimulation of MGN, the startle response to paired stimulation had similar enhancement across various ISIs. Thus the shape of the response curve was not changed by tetanic stimulation. Twenty-four hours later, the response amplitude had returned to the pretetanization level.

Unlike that for paired acoustic stimulation and bilateral electrical stimulation of MGN, the amplitude of the startle response to paired acoustic stimulation and bilateral electrical stimulation of TE3 was not influenced by the ISI ($F_{10,152} = 1.312$, $P > 0.05$; Fig. 7, lower panel), and the interaction between ISI and tetanization was not significant ($F_{20,185} = 0.339$, $P > 0.05$). However, there was a significant main effect of tetanic stimulation ($F_{2,152} = 14.659$, $P < 0.05$). Following bilateral tetanic stimulation of TE3, the startle response to paired stimulation had a generally even reduction across various ISIs. Thus the shape of the response curve stayed flat. Twenty-four hours later the response amplitude had returned to the pretetanization level.

Discussion

Behavioural consequences of tetanic stimulation of the two auditory afferents

Doyère *et al.* (2003) reported that in awake rats the pattern of LTP induced by tetanic electrical stimulation of MGN afferents was

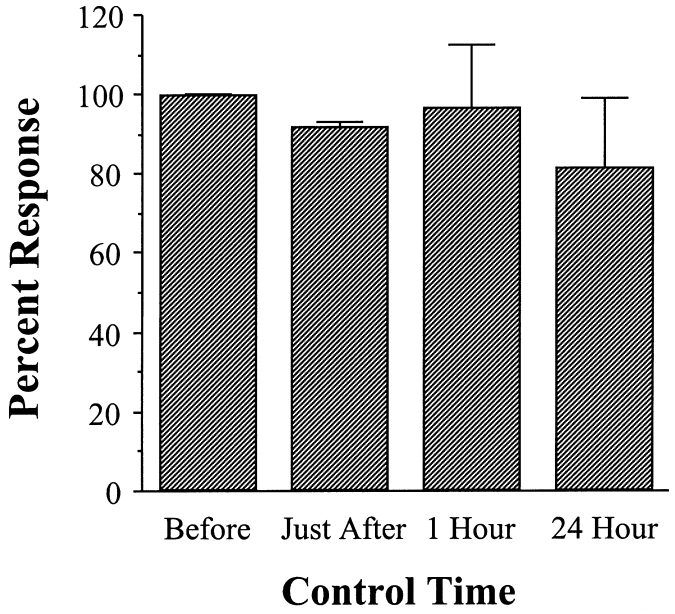

FIG. 6. Normalized amplitudes of the ASR for rats who received phaclofen injection but no tetanic stimulation, at the time points associated with those immediately after, 1 h after and 24 h after tetanic stimulation in other rats.

different from that induced by tetanic electrical stimulation of AAC afferents. Although both auditory afferents are important for AFC (for review see LeDoux, 2000), Doyère *et al*. (2003) suggested that these two afferents rely on different mechanisms for inducing LTP in LA and have differential functions that may not be observed with an *in vitro* preparation. The present results extend the electrophysiological observations on the ASR of Doyère *et al*. (2003) by showing that tetanic stimulation of MGN and AAC had opposite effects: the former enhanced the ASR while the latter suppressed it. These opposite effects were observed in each of the three experiments of the present study with tetanic stimulation being applied either unilaterally or bilaterally. Surprisingly, bilateral tetanic stimulation applied in Experiments 2 and 3 produced less robust effects on startle than unilateral tetanic stimulation applied in Experiment 1. The weaker bilateral effect suggests that unilateral tetanic stimulation alone is sufficient to modulate the ASR, and the startle modulation by bilateral tetanic stimulation is somehow limited by unknown mechanisms.

The enhanced startle reflex has been widely used as a behavioural index of fear in animals since the study by Brown *et al*. (1951), and the amygdala has been found to play an important role in fear potentiation of startle (for reviews see Davis, 1992; Fendt & Fanselow, 1999; Gewirtz & Davis, 2000). Previous studies have also shown that electrical or chemical activation of the amygdala enhances the ASR (Koch & Ebert, 1993; Koch, 1993; Li *et al*., 1999; Fendt *et al*., 2000; Lin *et al*., 2002), and damage to the amygdala reduces the ASR (Schanbacher *et al*., 1996). Thus the ASR can be used as a behavioural model for disclosing certain conditions of the amygdala. The opposite behavioural consequences reported here suggest that tetanization of MGN afferents is more associated with excitation of CE output neurons; tetanization of AAC afferents is more associated with excitation of inhibitory interneurons and, consequently, more associated with suppression of CE output neurons.

In the present study, we also used two control groups (MGN-stimulation control and AAC-stimulation control) with lower-frequency sustained stimulation. The results show that immediately following low-frequency stimulation of MGN or TE3, the amplitudes of acoustic startle responses were slightly reduced. Although the startle reduction was not statistically significant, the possibility of certain inhibitory consequences of low-frequency stimulation of either auditory afferent should not be ruled out.

Involvement of $GABA_B$ transmission from the AAC afferent

Activity of principal neurons is modulated by GABAergic interneurons, via both feedforward and feedback inhibition (Li *et al*., 1996; Lang & Paré, 1997, 1998; Szinyei *et al*., 2000; Bauer & LeDoux, 2004). For both *in vivo* and *in vitro* preparations, inhibitory transmission at principal neurons, which is induced by paired-pulse or primed-pulse stimulation, is reduced by application of antagonists of $GABA_B$ receptors (Huang & Gean, 1994; Li *et al*., 1996; Szinyei *et al*., 2000), suggesting that, in addition to postsynaptic $GABA_A$ receptors, presynaptic $GABA_B$ receptors are involved in feedforward and/or feedback inhibition of principal neurons.

In the present study, compared to injection of saline, injection of the $GABA_B$ receptor antagonist phaclofen into the LA area had no significant influence on the effect of tetanic stimulation of MGN on the ASR, but significantly reversed the suppressive effect of tetanic stimulation of AAC on the ASR. Injection of phaclofen into LA in the rats receiving no tetanic stimulation did not change the ASR significantly, though it did slightly reduce it. The results suggest that tetanic stimulation of AAC produces sustained $GABA_B$ receptor-mediated inhibition of principal neurons and consequently reduces the ASR. Blockade of $GABA_B$ receptors decreases the inhibitory influence from interneurons on LA principal neurons, increases the possibility of inducing LTP in principal neurons following tetanic stimulation of AAC and consequently enhances the ASR. Figure 5 also appears to show a diminution between the effect of MGN tetanization and that of AAC tetanization following blockage of $GABA_B$ receptors in LA.

Difference between the effect of transient stimulation of MGN and that of AAC on startle

As mentioned in the Introduction, expression of AFC in the startle reflex is mediated by the pathways that originate from LA principal neurons, go through CE via intra-amygdaloid connections and then descend to the startle circuits both directly and indirectly. A useful method for probing the time course of signal transfer in a pathway that modulates startle is to apply a transient electrical stimulation in the pathway and then study both the shape and position of the ISI curve resulting from the transient stimulation (Li & Yeomans, 2000). Our recent studies have shown that startle-like EMG responses evoked by electrical stimulation of the trigeminal nucleus can be modulated by transient electrical stimulation of LA in anaesthetized rats (He *et al*., 2005). The present study extended this line of research from anaesthetized rats to awake rats and further studied effects of tetanic stimulation on ISI curves.

Combined startling stimuli with short ISIs can evoke startle with larger amplitude, a phenomenon called summation (Marsh *et al*., 1973; Blumenthal & Berg, 1986; Yeomans *et al*., 1989; Li & Yeomans, 1999; Li *et al*., 2001; for a recent review see Yeomans *et al*., 2002). On the other hand, a weak nonstartling sound that is presented 10–500 ms before the startling sound can inhibit the ASR, a phenomenon called prepulse inhibition (Graham, 1975; Hoffman & Ison, 1980; Ison & Hoffman, 1983; Geyer *et al*., 1990; Fendt *et al*., 2001; Li & Yue, 2002). In the present study, transient (single-pulse) electrical stimulation of MGN with currents below the threshold for eliciting startle-like responses had dual effects: (i) enhancing the ASR

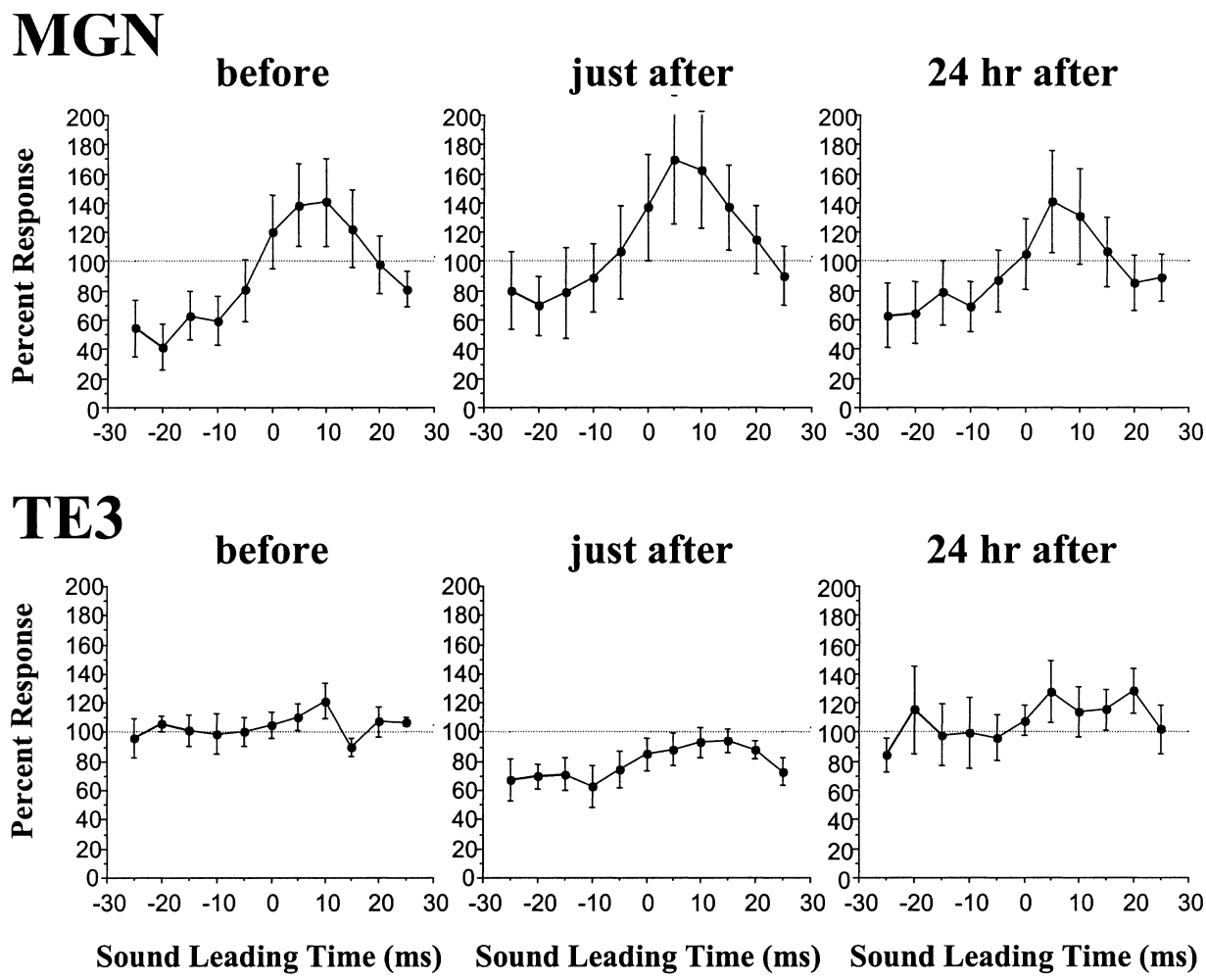

FIG. 7. Normalized amplitudes of the startle responses to paired acoustic stimulation and bilateral transient electrical stimulation of MGN (upper graphs) or TE3 (lower graphs) before, immediately after and 24 h after bilateral tetanic stimulation of MGN or TE3. Straight dotted lines indicate the baseline ASR before tetanic stimulation.

when the electrical stimulus lagged slightly (peaking at 5–10 ms) behind the acoustic startling stimulus; and (ii) suppressing the ASR when the electrical stimulus led (peaking at −20 ms in Fig. 7) the acoustic startling stimulus. Moreover, tetanic stimulation of MGN enhanced the startle responses equally across various ISIs without changing the shape of the ISI function. The fast and enhancing effect of transient stimulation of MGN may be partially mediated by the amygdala (He *et al*., 2005) while the slow and suppressing effect of transient stimulation of MGN must involve more synaptic relays.

Unlike transient stimulation of MGN, transient stimulation of AAC with similar currents had no effect on the ASR. Also unlike tetanic stimulation of MGN, tetanic stimulation of AAC reduced the startle responses equally across various ISIs. The lack of effects of transient stimulation of AAC further suggests a functional difference between MGN and AAC in modulating startle.

MGN and AAC afferents to LA principal neurons and interneurons

The LA contains principal neurons and interneurons with different morphological, immunohistochemical and physiological characteristics (McDonald, 1982; Millhouse & de Olmos, 1983; Rainnie *et al*., 1991; Washburn & Moises, 1992; McDonald & Augustine, 1993; Sugita *et al*., 1993; Lang & Paré, 1998; Mahanty & Sah, 1998). Both principal neurons and interneurons receive excitatory afferents from both MGN and AAC (Li *et al*., 1996; Lang & Paré, 1998; Mahanty & Sah, 1999; Szinyei *et al*., 2000; Bauer & LeDoux, 2004; Tsvetkov *et al*., 2004).

Although MGN afferents also innervate inhibitory interneurons directly (Farb & LeDoux, 1997; Woodson *et al*., 2000), the vast majority of excitatory MGN–LA synapses, however, occur on dendritic spines, which contain both the R1 subunit of *N*-methyl-D-aspartate receptors (NMDARs) and GluR1–3 subunits of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors (LeDoux *et al*., 1991; Farb & LeDoux, 1997). Because dendritic spines occur mainly on LA principal neurons but not interneurons (McDonald, 1982; Millhouse & de Olmos, 1983; Nitecha & Ben-Ari, 1987) it is reasonable to propose that the direct impact of MGN afferents is stronger on principal neurons than on interneurons.

Similar to MGN afferents, the vast majority of AAC axonal terminals innervate dendritic processes containing the R1 and GluR1–3 units (Farb & LeDoux, 1999). However, for some reason, NMDARs contribute mainly to excitatory transmissions at MGN afferents, but to a lesser extent to those at AAC afferents (Li *et al*., 1995, 1996; Weisskopf & LeDoux, 1999; Zinebi *et al*., 2001). Interestingly, Sah and colleagues (Mahanty & Sah, 1998; Sah & de Armentia, 2003) found that glutamatergic inputs onto LA principal neurons form

synapses with both NMDA and AMPA receptors while for glutamatergic inputs onto LA interneurons the contribution of NMDARs is very small or negligible. We thus assume that a large direct impact of AAC afferents is on interneurons. As hypothesized by Doyère *et al*. (2003), the difference in the ratio of excitatory AMPA vs. NMDA receptors between MGN and AAC afferents to LA may be one potential mechanism underlying the different features of LTP induced by the two afferent sources.

Some physiological studies support this assumption. *In vitro,* tetanic stimulation of the external capsule, which contains axons projecting from AAC to LA, induces NMDAR-independent LTP in LA interneurons and augments inhibitory inputs to LA principal neurons (Mahanty & Sah, 1998). In anaesthetized cats, electrical stimulation of the perirhinal and entorhinal cortical regions produces much larger inhibitory effects on principal neurons than on interneurons (Lang & Paré, 1998). In particular, the predominant response of principal neurons to high-current cortical stimuli is a large-amplitude hyperpolarization lasting hundreds of milliseconds, while only a narrow range of low currents can evoke orthodromic spikes. On the other hand, short-latency excitatory responses of interneurons to cortical stimulation continue to increase with stimulation currents over a large range. Moreover, the excitatory response profile of interneurons corresponds with the inhibitory response profile of principal neurons. These results support the assumption that tetanic stimulation of AAC mainly induces LTP in interneurons. This provides the first explanation of the suppressive effect of tetanic stimulation of AAC on the ASR (for the other explanation see the discussion below of the new model proposed by Paré *et al*., 2004).

Tetanic stimulation and fear conditioning

Tetanic stimulation is an artificial way of inducing prolonged neural depolarization and is quite different from fear conditioning in which more natural stimuli are applied. Tetanic stimulation of MGN or AAC with sufficiently high currents usually causes prolonged excitation of both presynaptic fibers and postsynaptic cells in LA at the same time, simulating an integration of inputs of conditioned stimulus (CS) and inputs of unconditioned stimulus (US). However, associative fear conditioning requires the concurrent activation of weak presynaptic CS inputs to LA and strong depolarization of the same neurons by the US. Thus although tetanic stimulation of MGN or AAC follows the Hebbian rule, the LTP patterns that occur during tetanic stimulation in LA may not be the same as the natural LTP patterns that occur during fear conditioning.

At the molecular level, however, it has become evident that both tetanus-induced LTP and fear-conditioning-induced LTP in LA share similar mechanisms. In LA, LTP induced by tetanic stimulation of the MGN or AAC is abolished by the antagonist of NMDAR, APV (Huang & Kandel, 1998; Bauer & LeDoux, 2004). Moreover, the specific role of NMDARs in synaptic plasticity in LA following fear conditioning was tested by Rodrigues *et al*. (2001). In their study, infusion of the specific antagonist of the NR2B subunit of NMDARs, ifenprodil, into LA during the acquisition phase of AFC impaired conditioning when rats were tested after training. Infusion of ifenprodil after the acquisition phase did not impair the expression of previously learned fear conditioning. Thus, NMDARs that incorporate the NR2B subunit may be particularly important for AFC.

Behavioural significance of the different effects of thalamic and cortical stimulation

NMDARs mainly contribute to glutamatergic excitatory transmissions from MGN afferents to LA principal neurons. In other words, glutamatergic inputs from MGN, but not from AAC, are mainly associated with NMDARs at principal neurons in LA. Thus tetanic stimulation may provide a way of differentiating the functions of MGN and AAC afferents.

LA contains at least two populations of principal neurons; one associated with early initial plasticity following acquisition and the other associated with long-term memory storage (Repa *et al*., 2001). As proposed by Blair *et al*. (2001), combined calcium entry through both NMDARs and voltage-gated calcium channels (VGCCs) is necessary to trigger mechanisms underlying long-term fear memory, which is associated with both early and later phases of LTP. However, tetanization of presynaptic inputs to LA mainly produces NMDAR-dependent but not VGCC-dependent LTP in LA (Huang & Kandel, 1998; Bauer *et al*., 2002), and calcium entry through NMDARs but not VGCCs is essential to support short-term memory, which is associated with early phase of LTP. Because tetanization of presynaptic inputs to LA mainly produces NMDAR-dependent but not VGCC-dependent LTP in LA (Huang & Kandel, 1998; Bauer *et al*., 2002), the stimulation protocols used in the present study and by Doyère *et al*. (2003) in awake rats might not trigger the VGCC-dependent late phase of LTP, but mainly induce short-term physiological or startle changes. We therefore hypothesize that during fear conditioning, MGN and AAC make different contributions to short-term memory, which are associated with short-term synaptic changes not involving gene transcription or synthesis of new proteins.

Because AAC and MGN occupy different levels of signal processing in the central auditory system, the AAC input to the LA would be more sophisticated than the MGN input in term of signal complexity. During AFC, MGN may play the major role in inducing the early phase of LTP in LA principal cells and only part of the early phase of LTP can be upgraded to the late phase of LTP. Due to the flood of sensory inputs that enter the brain with the CS, MGN-dependent short-term memory is not well tuned. The AAC afferents play an important 'gating' role to refine the short-term memory by activating LA interneurons, making the relationship between the CS and short-term memory more specific. In addition, the basolateral nucleus of the amygdala, which receives axonal projections from AAC (Shi & Cassell, 1997), also sends axons to the auditory cortex (Sripanidkulchai *et al*., 1984). The auditory cortex, in turn, sends massive axonal projections to subcortical auditory structures, including MGN (e.g. Diamond *et al*., 1969). Signals from the amygdala to MGN by way of the auditory cortex are essential for the development of discriminative training-induced neuronal activity in the medial division of MGN (Duvel *et al*., 2001). However, direct axonal projections from the amygdala to MGN have not been reported. The lack of reciprocal projections between MGN and the amygdala further suggests that certain types of signal integration between the amygdala and the auditory cortex do not exist in the interaction between the amygdala and the auditory thalamus. Therefore, during formation of short-term memories, MGN afferents mainly play the 'bottom-up' role while AAC afferents mainly play the 'top-down' role in refining AFC. This hypothesis appears to be consistent with the assumption that the shorter and faster thalamic pathway is limited in its processing capacity relative to the longer and slower cortical pathway (LeDoux, 1995).

A new model and the inhibitory effect of tetanic stimulation of AAC on startle

Recently, Paré *et al*. (2004) proposed a new neural model of AFC. One key component in this model is that GABAergic intercalated (ITC) cell clusters, which are interposed between the basolateral complex and CE of the amygdala, bridge the connection gap between LA and CE. One ITC cell cluster produces unidirectional feed-forward

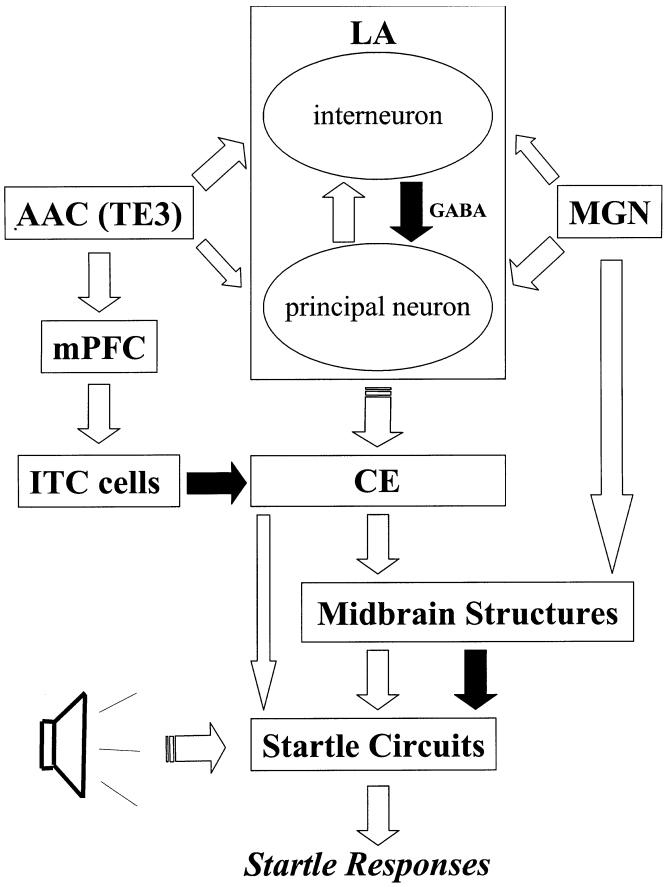

FIG. 8. A model of neural pathways modulating the ASR as a result of tetanic stimulation of MGN or AAC. The open arrows without a broken tail represent direct excitatory axonal projections; the open arrows with a broken tail represent indirect excitatory pathways. The solid arrows represent direct inhibitory axonal projections.

inhibitory influence to another ITC cell cluster in the direction from the lateral to the medial region (Royer *et al*., 2000), and the most medially located ITC cell clusters generate sustained inhibition of CE cells. Outputs from LA excite laterally located ITC cell clusters which in turn inhibit medially located ITC cell clusters and consequently disinhibit CE cells. The final result is that an increase in activation in LA causes an increase in CE outputs.

Interestingly, the infralimbic region (IL) of the medial prefrontal cortex (mPFC), which receives projections from AAC (Barbas *et al*., 1999) and processes information associated with AFC (Baeg *et al*., 2001), strongly projects to ITC cell clusters (Sesack *et al*., 1989; McDonald *et al*., 1996; Freedman *et al*., 2000). Damage to IL impairs reversal learning (Li & Shao, 1998) and extinction (Morgan *et al*., 1993; Quirk *et al*., 2000). Electrical stimulation of IL reduces both responsiveness of CE output cells (Quirk *et al*., 2003) and conditioned fear (Milad *et al*., 2004). Thus an additional possible explanation of the suppressive effect of tetanic stimulation of AAC on the ASR is that tetanic stimulation of AAC triggers the following chain reactions: increase in excitation of IL output cells, increase in excitation of amygdaloid ITC cells that inhibit CE cells, increase in inhibition of CE output cells and eventually suppression of the ASR. Further investigation is needed to test this hypothesis and, particularly, to investigate whether the inhibition of CE output cells by ITC cells is mediated by $GABA_B$ transmission.

Summary

We have used the ASR as a behavioural method to reveal the effects of tetanic stimulation of MGN or AAC. The results indicate that tetanization of MGN enhances the ASR but tetanization of AAC suppresses the ASR. The suppressive effect of tetanization of AAC is mediated via inhibitory $GABA_B$ transmission in the LA area. Thus MGN and AAC may play different roles in mediating AFC. Based on this and previous studies, a model of neural pathways mediating the effects of tetanic stimulation of MGN or AAC on startle is shown in Fig. 8.

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Abbreviations

AAC, auditory association cortex; AFC, auditory fear conditioning; AMPA, a-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; ASR, acoustic startle reflex; CE, central nucleus of the amygdala; CS, conditioned stimulus; IL, infralimbic region of medial prefrontal cortex; ISI, interstimulus interval; ITC, intercalated; LA, lateral nucleus of the amygdala; LTP, long-term potentiation; MGN, medial geniculate nucleus; NMDAR, *N*-methyl-D-aspartate receptor; VGCC, voltage-gated calcium channel.

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