Carbachol induces a form of long-term potentiation in lateral amygdala

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Received 2 February 2004; accepted 16 March 2004

DOI: 10.1097/01.wnr.0000127139.77723.b8

We examined the effects of carbachol, a muscarinic acetylcholine receptor agonist, on excitatory synaptic transmission at thalamo-amygdala synapses in rat brain slices. The application of a low concentration of carbachol (0.25 μM) produced a form of long-term potentiation (cLTP) and a transient suppression of synaptic responses, which was blocked by a muscarinic receptor antagonist, atropine (10 μM). M2 receptor agonist produced only a transient suppression, whereas M1 receptor agonist induced both a transient suppression and a long-term potentiation. Induction of cLTP required simultaneous low-frequency afferent stimulation, and was also dependent upon the activation of NMDA receptors. SQ22536 (50 μM), an adenylyl cyclase inhibitor completely blocked cLTP. Consistently, pretreatment with a maximal concentration of forskolin (10 μM) reduced cLTP. *NeuroReport* 15:1339–1343 © 2004 Lippincott Williams & Wilkins.

Key words: Amygdala; Carbachol; Forskolin; Muscarinic receptor; NMDA receptor

INTRODUCTION

The amygdala is a critical structure for emotional memory and Pavlovian fear conditioning. This form of fear conditioning is produced by the pairing of a neutral tone as a conditioned stimulus (CS) with a shock as an unconditioned stimulus (US). These two stimuli converge onto the lateral amygdala, and the coincidental presentation of CS and US is thought to induce fear conditioning by potentiating the synaptic strength of the CS pathway by a long-term potentiation (LTP)-like mechanism [1,2]. The CS arrives at the lateral nucleus of the amygdala via two routes: directly from the medial geniculate nucleus and indirectly from the auditory cortex [3,4]. *In vivo* and *in vitro* studies linking amygdala LTP to fear learning have associated the thalamic pathway with the lateral amygdala [1,2]. Multiple trains of high-frequency stimulation at thalamic input synapses to the lateral amygdala have shown that LTP is dependent on NMDA receptors and on L-type calcium channels, and mediated by protein kinase A and mitogen-activated protein kinase [5,6].

Previous experiments suggest that muscarinic receptors are involved in auditory fear conditioning, and muscarinic antagonism produces deficits in the acquisition phase of auditory fear conditioning [1]. However, no previous studies have addressed the effects of muscarinic receptor activation on thalamic input synapses onto the lateral amygdala. In the present study, we investigated the effects of muscarinic receptor activation on excitatory synaptic transmission at thalamic input synapses onto the lateral amygdala: (1) to determine whether muscarinic activation produces LTP, (2) to identify the subtype of muscarinic receptors involved, and (3) to determine whether NMDA receptors and cAMP pathways are involved in the long-term potentiation induced by the activation of muscarinic receptors as demonstrated by tetanus-induced LTP.

MATERIALS AND METHODS

Brain slices were prepared using techniques described previously [6,8]. Sprague–Dawley rats (3–5 weeks old) were decapitated under urethane anesthesia. Whole isolated brains were placed in an ice-cold modified artificial cerebrospinal fluid (aCSF) solution. The composition of modified aCSF was as follows (in mM): 175 sucrose, 20 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgCl_2$, 11 D-(+)-glucose. Coronal slices (400 μm) containing the amygdala were cut using a vibratome (Campden, UK), and incubated in aCSF continuously bubbled at room temperature with 95% O_2/5% CO_2 for $\geq$ 2 h. Just before transferring the slice to the recording chamber, the cortex overlying the amygdala was cut away with a scalpel. The recording chamber was continuously superfused with aCSF (30–32°C) at a flow rate of 1–2 ml/min. The aCSF contained (in mM): 120 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgCl_2$, 2 $CaCl_2$, 11 D-(+)-glucose. Picrotoxin (10 μM) was included in all experiments to minimize fast GABAergic transmission. To record field potentials at thalamic input synapses to the lateral amygdala, a bipolar stimulating electrode was placed in the thalamic afferent fibers innervating the lateral amygdala. The recording electrode (> 1.0 MOhm) was filled

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with aCSF and placed in the dorsal subregion of the lateral amygdala. For baseline field potential recording 50% of the maximum amplitude was used. The range of stimulus intensity and the duration of each pulse were 0.1–0.3 mA and 0.1–0.2 ms, respectively. Extracellular field potentials were amplified using a DAM differential amplifier (World Precision Instruments, Sarasota, FL, USA) and the output was digitized on-line. Digitized signals were stored and analyzed with PC using NAC (Electek, Irvine, CA). Nifedipine, oxotremorine-M, SQ22536, MCN-A-343, forskolin, atropine, methoctramine, pirenzepine and D-AP5 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Carbachol was from Tocris Cookson (Ballwin, MO, USA). Statistical analysis was performed using Student's *t*-test.

RESULTS

The field potential in our experimental condition reliably had a peak latency of about 5 ms, followed high frequency (50 Hz) stimulation, and could be blocked by kynurenic acid (5 mM), a non-selective glutamate receptor antagonist (data not shown). These findings suggest that the field potential measured in the present study reflects glutamatergic, monosynaptic responses at thalamic input synapses to the lateral amygdala [6].

In order to examine whether the activation of muscarinic acetylcholine receptors has any effects on synaptic transmission at thalamic input synapses to the lateral amygdala, we applied carbachol, a muscarinic receptor agonist. Application of 0.25 μM carbachol for 20 min initially produced a transient suppression of the field potential amplitude. The amplitude of the field potentials gradually increased over the baseline and reached a steady state within 20 min. If the magnitude of the potentiation was $>120\%$ of the baseline response, and if the potentiation persisted for >60 min, the carbachol-induced potentiation was referred to cLTP. The average transient suppression was $69.9 \pm 9.5\%$ (n=5, $p<0.05$) of control. The averaged potentiation measured 120 min after carbachol washout was $149.9 \pm 13.3\%$ (n=5, $p<0.01$) of control (Fig. 1b). A higher concentration of carbachol (5 μM) produced only a transient suppression ($73.9 \pm 6.3\%$ of control, n=6, $p<0.01$; Fig. 1a). Atropine (10 μM) blocked both cLTP and this transient suppression

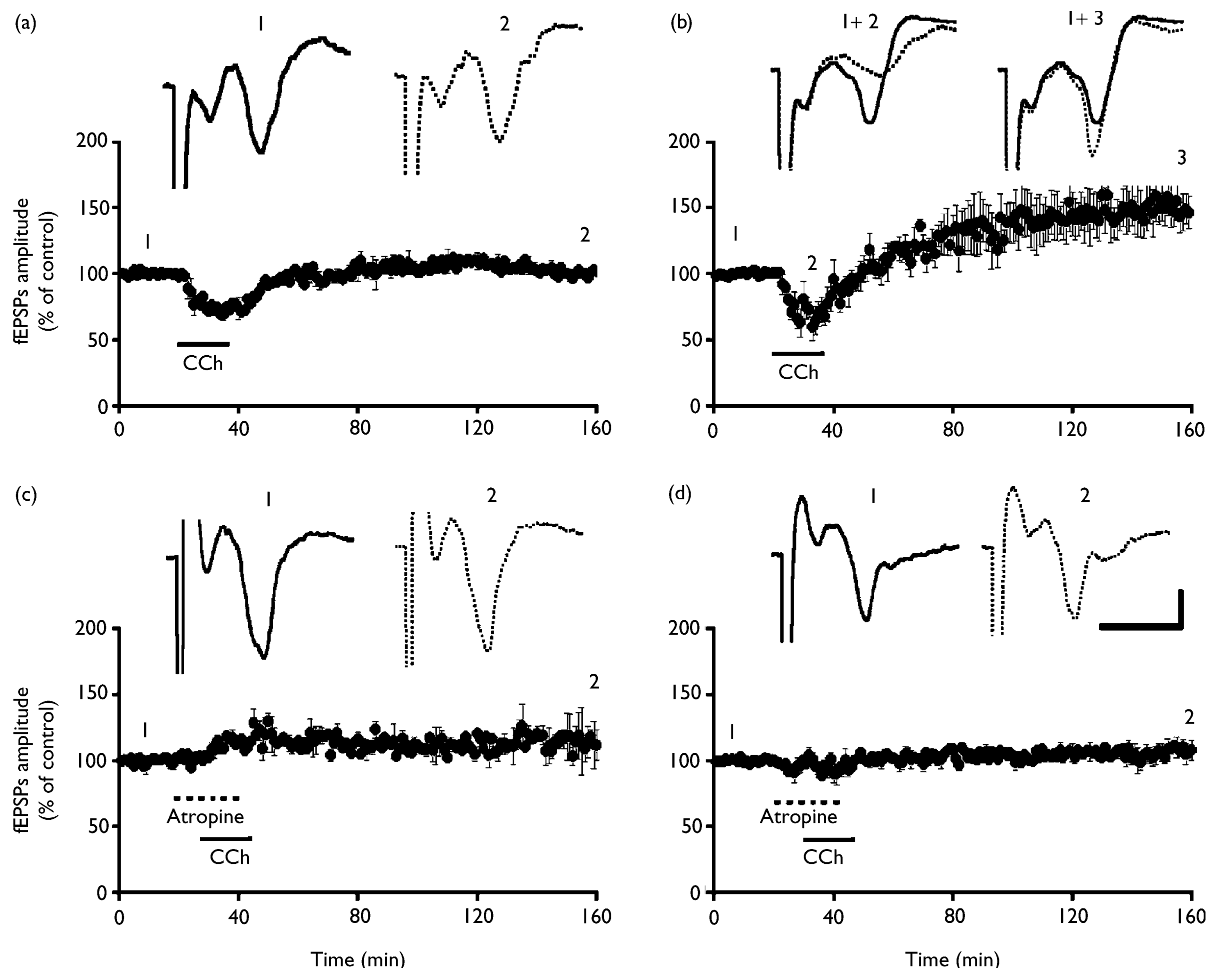

Fig. 1. Carbachol produces changes in excitatory synaptic transmission at amygdala synapses. (**a**) A high concentration of carbachol (5 μM) induced a transient suppression of field potentials (n=6). (**b**) A low concentration of carbachol (0.25 μM) produced cLTP and a transient suppression in field EPSPs (n=5). (**c**,**d**) Both the transient suppression and cLTP, produced by 5 μM (c, n=4) or 0.25 μM (d, n=5) of carbachol, were completely blocked by 10 μM atropine, a muscarinic receptor antagonist. Solid and dotted bars represent carbachol and atropine application, respectively. Representative traces of field EPSPs were shown at the indicated times.

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(Fig. 1c,d), suggesting that the carbachol effect is dependent only on activation of the muscarinic receptors.

In order to determine the subtypes of muscarinic receptors mediating the carbachol effect, we used subtype-specific muscarinic agonists. Like carbachol, the muscarinic M1 receptor agonist, MCN-A-343 (0.25 μM), produced both transient suppression (84.5 ± 5.5%, n=6, p < 0.01) and cLTP (138.5 ± 5.7%, n=6, p < 0.01; Fig. 2a). By contrast, application of oxotremorine-M (0.25 μM), a muscarinic M2 receptor agonist, produced only a transient suppression of the field potentials (41.2 ± 11.5%, n=5, p < 0.01; Fig. 2c), suggesting that the carbachol effect on cLTP is mediated through M1 receptors. Consistently, cLTP was blocked only by the M1 receptor antagonist, pirenzepine (0.5 μM; 107.3 ± 7.3%, n=5, p > 0.1), but not by the muscarinic M2 receptor antagonist, methoctramine (0.25 μM; 128.1 ± 9.5%, n=5, p < 0.01). By contrast, the suppressive effect of carbachol was completely blocked by M1 antagonist, but only partially by M2 antagonist (81.9 ± 7.8%, n=5, p < 0.05; Fig. 2b,d). Therefore, our findings suggest that cLTP is generated by the activation of M1 receptors, and that transient suppression is induced by either M1 or M2 receptors.

When no afferent stimuli were delivered during its application, carbachol failed to induce cLTP (101.3 ± 5.2%, n=7; Fig. 3a), suggesting that the induction of cLTP requires concomitant afferent stimulation. The NMDA receptor antagonist, D-AP5 (50 μM), also blocked cLTP induction (93.6 ± 5.9%, n=6; Fig. 3b). We then tested whether cLTP requires elevated of cAMP levels, since late-phase LTP at thalamic input synapses to the lateral amygdala has been shown to depend upon the cAMP pathway [5]. Pretreatment with SQ22536 (50 μM), an adenylyl cyclase inhibitor, blocked only cLTP without affecting transient suppression by carbachol (cLTP: 110.2 ± 15.1%, n=6, p > 0.1; suppression: 47.5 ± 9.7%, n=6, p < 0.01; Fig. 3c).

In order to corroborate the finding that adenylyl cyclase activation may be required for cLTP induction, we utilized an adenylyl cyclase activator, forskolin. The application of forskolin (10 μM) for 20 min produced a long-lasting increase in field potentials (142.7 ± 9.3% of control measured 40 min after drug washout; n=6, p < 0.01). In order to avoid any possible ceiling effects due to the enhanced field responses, the stimulus intensity was adjusted to reduce the amplitude of the potentiated field potentials to the

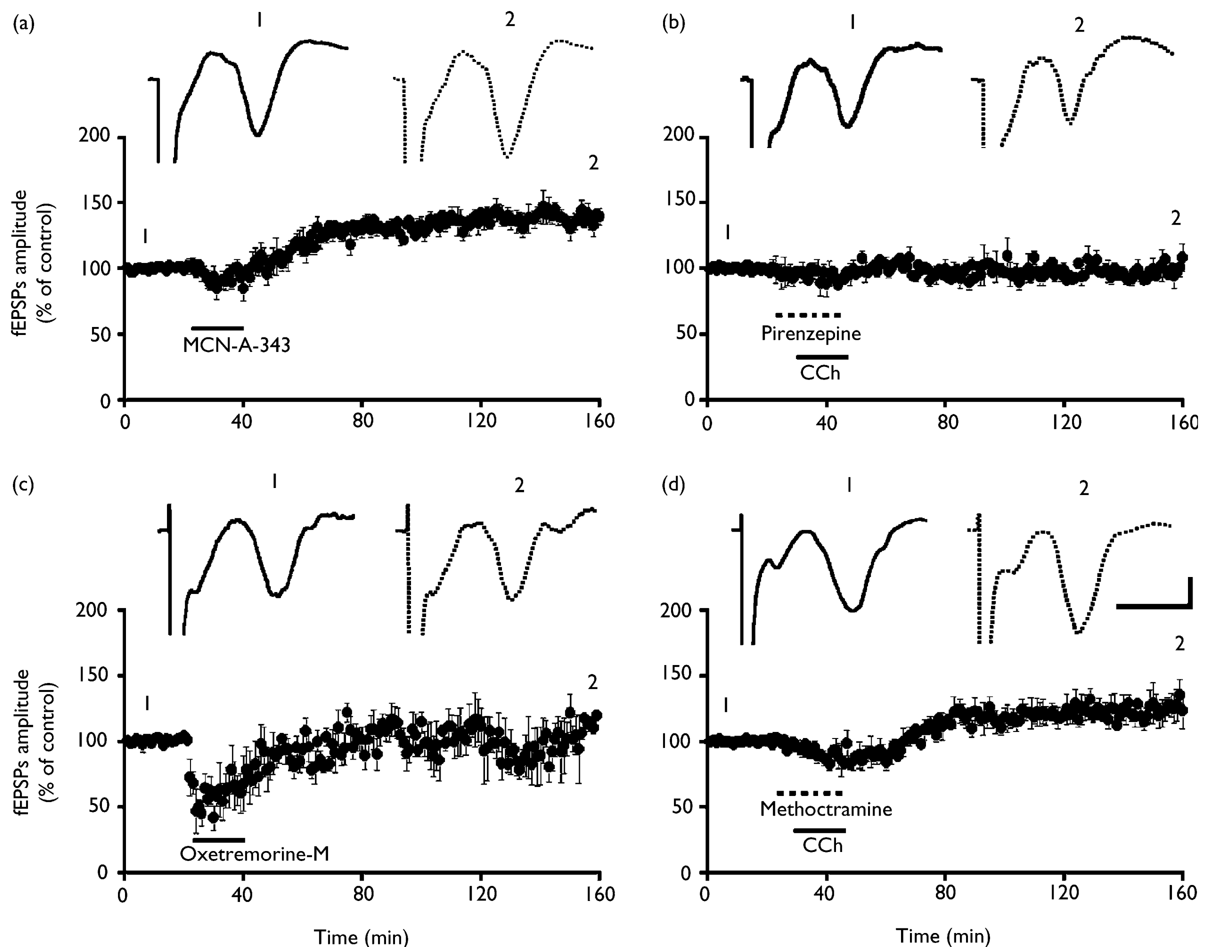

Fig. 2. M1 receptors, but not M2 receptors, are involved in cLTP. (**a**) MCN-A-343 (0.25 μM), an M1 receptor agonist, produced both transient suppression and cLTP (n=6). (**b**) Pirenzepine (0.5 μM), an M1 receptor antagonist, blocked a transient suppression and cLTP by carbachol (n=5). Solid and dotted lines indicate carbachol and pirenzepine treatment, respectively. (**c**) Oxotremorine-M (0.25 μM), an M2 receptor agonist induced only a transient suppression (n=5). (**d**) Methoctramine (0.25 μM), an M2 receptor antagonist failed to block a transient suppression or cLTP (n=5). Antagonist treatment was started 10 min before agonist treatment.

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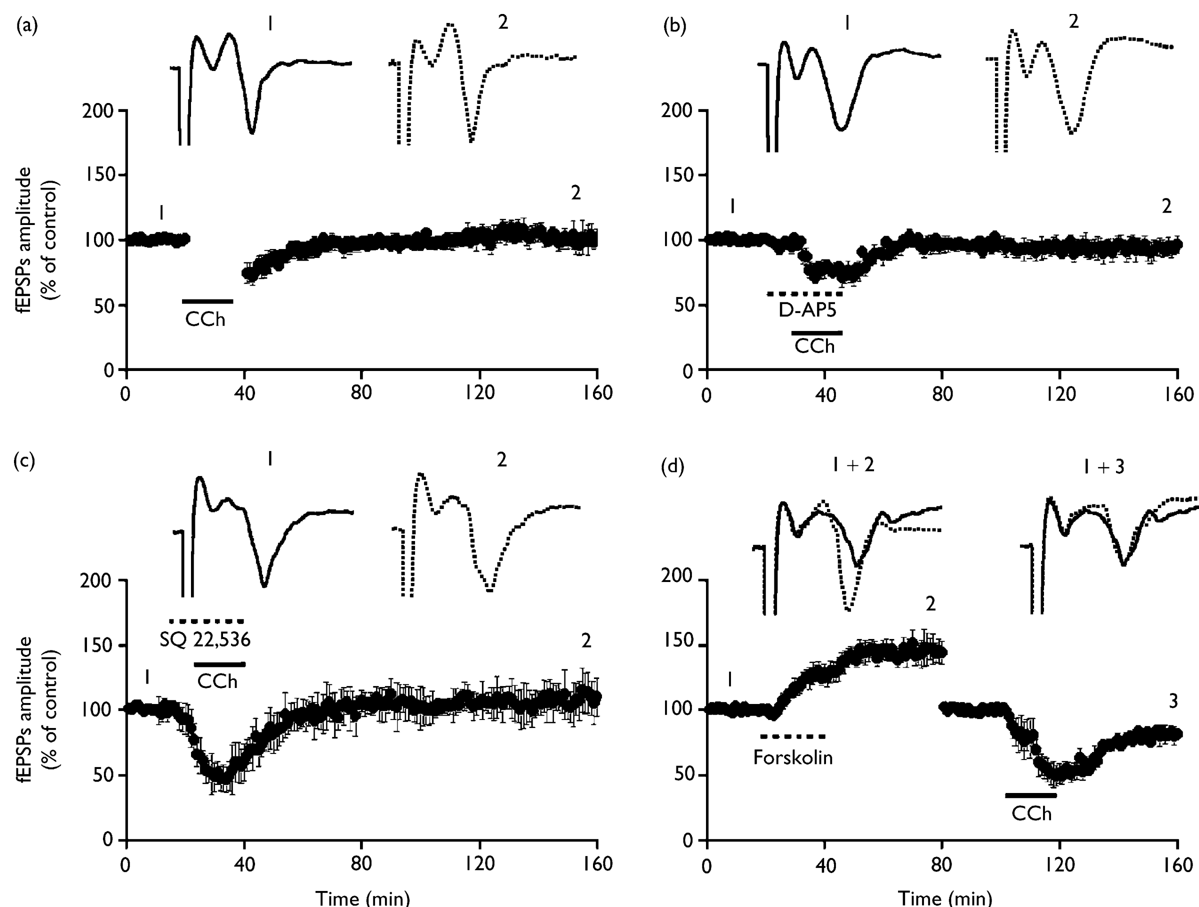

Fig. 3. Failure of cLTP induction in the presence of NMDA receptor antagonist or adenylyl cyclase inhibitor. (**a**) When electrical stimuli were not applied during carbachol treatment, cLTP was not induced (n=7). (**b**) D-AP5 (50 μM), an NMDA receptor antagonist blocked cLTP induction, but not transient suppression (n =6). (**c**) cLTP was not induced in the presence of SQ22536 (50 μM), a PKA inhibitor (n=6). Solid and dotted lines indicate the treatment period of carbachol and SQ22536, respectively. (**d**) Forskolin-induced long-term potentiation occluded cLTP (n=6). Solid and dotted lines indicate the carbachol and forskolin treatment periods, respectively. Representative traces of field EPSPs were shown at the indicated times. Calibration=5 ms, 0.1 mV.

baseline level before the forskolin treatment. Under this condition, the application of 0.25 μM carbachol failed to induce cLTP (81.4±7.5% of control, n=6, 40 min after drug washout; Fig. 3d). The failure of carbachol to induce cLTP after the forskolin pretreatment further suggests that the induction and/or expression of cLTP is dependent on the cAMP signal pathway.

DISCUSSION

Our experiments show, for the first time, that the activation of muscarinic acetylcholine receptors by carbachol produces a form of long-term potentiation (cLTP) at thalamic input synapses to the lateral amygdala. M1 subtype receptors appeared to play a major role in the generation of cLTP, and this was found to be dependent on the activation of the NMDA receptors and cAMP signal pathways. To the best of our knowledge, no previous examples of an action of carbachol on excitatory synaptic transmission at thalamic input synapses onto the lateral amygdala have been published. Carbachol has been shown to produce a depolarization of neurons and to depress excitatory glutamatergic synaptic transmission in the basolateral amygdala, though carbachol actions in the basolateral amygdala were short-lasting [9,10]. Therefore, cholinergic activation in the basolateral amygdala failed to show any long-term modification that might represent a cellular mechanism underlying the cholinergic modulation of fear memory.

Previous studies [5,6] have reported that repeated tetanic stimulation produced late-phase LTP (L-LTP). Since L-LTP has an enduring phase, it may be more relevant to study L-LTP as a cellular substrate for conditioned fear memory. This enduring form of LTP (L-LTP) has been shown to be dependent on activation of NMDA receptors, L-type calcium channels, and metabotropic glutamate receptor 5 [6,11,12]. In addition, L-LTP has been shown to be dependent on protein synthesis, and to be mediated by protein kinase A and mitogen-activated protein kinase [5]. Interestingly, the cLTP studied here resembles critical aspects of L-LTP: cLTP is dependent on NMDA receptors, and is mediated by protein kinase A. Therefore, it is plausible that cholinergic activation with low-frequency afferent stimulation recruits the same mechanism as L-LTP induction. In addition, cholinergic activation appears to lower the LTP induction threshold since low-frequency afferent stimulation with cholinergic activation was enough to induce cLTP in the present study [13].

The terminal field of the cholinergic projection from the basal forebrain is distributed within the amygdaloid complex and includes the lateral amygdala [14]. In addition,

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muscarinic receptors are abundantly expressed in higher brains regions, including the cerebral cortex, hippocampus, amygdala and striatum [15,16]. Thus, muscarinic receptors in the lateral amygdala are likely to be activated by endogenously released acetylcholine.

It is not clear why a higher concentration of carbachol failed to induce cLTP. One possibility is that a lower carbachol concentration preferentially activates M1 and/or M2 receptors. In contrast, a higher carbachol concentration could activate other unknown G-protein coupled receptors that may prevent the proper functioning of M1 receptors. In support of this suggestion, some evidence indicates that one subtype of a given G-protein-coupled receptor modulates other subtypes [17].

CONCLUSIONS

Our findings demonstrate that the activation of muscarinic receptors in combination with basal afferent stimulation produces a form of long-term potentiation, the induction of which depends upon the activation of NMDA receptors and adenylyl cyclases. The series of experiments performed using M1 and M2 receptor agonists and antagonists demonstrate the involvement of the M1 receptors in the induction of cLTP of the lateral amygdala. These findings suggest that activation of M1 receptors plays a role in conditioned fear by modulating synaptic plasticity in the lateral amygdala.

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Acknowledgements: This study was supported by a grant from the Korea Health 21 R&D Project (02-PJ1-PG1-CH06-0001), Ministry of Health & Welfare, Republic of Korea.

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