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Original Article

The effects of kindling during pregnancy on long-term potentiation (LTP) induction and M_1 muscarinic acetylcholine receptors in male rat offspring

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Abstract

Background and purpose: Neonates of pregnant women with epilepsy may compromise normal neurodevelopment and hippocampal morphology. Memory and learning disorders and a decrease in verbal IQ scores are seen in these children later in life. In the previous study, we suggested that the central muscarinic cholinergic receptors had an important role in learning and memory deficits induced by prenatal pentylenetetrazol-kindling in pups born to kindled mothers. This study aimed to investigate the effects of kindling during pregnancy on long-term potentiation (LTP) induction and the role of M1 muscarinic acetylcholine receptors in the hippocampus of male offspring.

Experimental approach: Twenty female Wistar rats were divided into two groups on the 13th day of their gestation (kindled and control; n = 10). Animals in the first group were kindled by *i.p.* injections of 25 mg/kg body weight pentylenetetrazol every 15 min until seizures occurred and the control group received normal saline. The effect of maternal seizures and perfusion of specific M1 muscarinic receptors antagonist (telenzepine at doses of 0.01, 0.1, and 1 nmol) on the LTP induction of 80 pups were tested at 12 weeks of age by field potential recordings.

Findings/Results: The results of the electrophysiological study revealed that recurrent seizures during pregnancy impaired field excitatory postsynaptic potentials (fEPSP)-LTP induction and normal development of M1 muscarinic receptors in the hippocampus of male offspring. Also, the results demonstrated that maternal seizure did not significantly affect the paired-pulse indexes and population spike-LTP in the hippocampus of male offspring.

Conclusion and implications: Our study showed that recurrent seizures during pregnancy cause impaired fEPSP-LTP induction and abnormal development of the M1 muscarinic receptor in the hippocampus.

***Keywords*:** LTP induction; Maternal seizure; M1 muscarinic acetylcholine receptor; Telenzepine.

INTRODUCTION

Epilepsy is one of many chronic neurological diseases, which is characterized by recurrent episodic seizures (1,2). The World Health Organization estimates that approximately 50 million people worldwide have been suffering from epilepsy. It is also estimated that the prevalence of epilepsy is between 25% and 40% in women of childbearing age (3). Epilepsy in pregnant women can lead to abnormal nerve development, changes in the morphology of the hippocampus, and consequently, impairments in learning and memory and reducing verbal IQ scores in their offspring (4-6).

Long-term potentiation (LTP) is described as long-lasting activity-dependent enhancement in synaptic strength that is commonly regarded as the closest neural model for the molecular and cellular mechanism complicated in learning and memory.

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LTP in synaptic transmission expansively can be evaluated in the hippocampal CA1 region (7,8). A previous study reported that acetylcholine stimulates pyramidal neurons of the CA1 area by stimulating the M1 receptor and can induce LTP (9). Moreover, Le Duigou *et al. in vitro* studies showed that blockage of M1 muscarinic acetylcholine receptors (M1 mAchRs) with telenzepine could prevent the induction of non-NMDA form of LTP (10). Muscarinic cholinergic receptors also play an important role in learning and memory. It is well established that muscarinic receptors (M1-M3) are expressed in the hippocampal formation, among them, M1 muscarinic receptor subtype is widely expressed in the soma and dendrite of pyramidal cells of the CA1 area (11).

It is reported that a single episode of a neonatal seizure can cause disturbance in spatial memory as well as impairment of LTP induction in the CA1 region of the hippocampus in adult rats (12). Our previous studies demonstrated that central muscarinic receptors are involved in spatial memory and passive avoidance learning impairments in adolescent offspring of pentylenetetrazol-kindled pregnant rats (13,14). However, the effect of maternal seizure on offspring synaptic plasticity and its mechanism has not been studied. Therefore, we examined the effects of kindling during pregnancy on LTP induction and the role of M1 muscarinic acetylcholine receptors in the hippocampal of adolescent male offspring by field potential recordings.

MATERIAL AND METHODS

Animals

All procedures were performed according to the Guide for the Care and Use of Laboratory Animals, eighth edition, 2011 and approved by the local Animal Ethics Committee of KUMS (Ethical No. IR.KUMS.REC.1397.022). Ten male and twenty female Wistar rats aged 3-4 months at the beginning of the experiments were purchased from the animal house of Kermanshah University of Medical Sciences (KUMS), Iran. The animals were kept in Plexiglas cages under standard temperature (23 ± 2 °C) and were exposed daily to a 12/12-h light/dark cycle (lights on at 07:00 AM) and free access to standard laboratory rat diet and water.

Experimental design

Before the experiment, the rats spent two weeks adapting to the environment and laboratory conditions, then 3 female rats with a male rat were placed in each cage for mating. Vaginal smears were performed on the females every morning (07:00-08:00 AM) to check the presence of sperm. The presence of sperm is marked as day 1 of pregnancy (14,15). Each pregnant rat was transported to another cage and stored singly (E0 being the day on which a positive vaginal smear was observed).

The effect of the maternal seizure (on the 13th day of pregnancy) on the hippocampal LTP of the 12-week-old offspring was investigated (5). Twenty pregnant rats were divided into two groups (kindled and control) consisting of 10 animals in each group. Seizures were induced by repetitive intraperitoneal injections of 25-mg/kg body weight pentylenetetrazol (Sigma, St. Louis, MO, USA) every 15 min until seizures occurred. The total dose did not exceed 75 mg/kg (three injections) (16). The control group received an equipollent volume of normal saline on a similar plan. Instantly after injection, seizure activity was observed and scored according to the Racine method (17) and the modified method by Becker *et al*. (18), as follows: stage 0, no response; stage 1, ear and facial twitching; stage 2, myoclonic jerks without rearing; stage 3, myoclonic jerks, rearing; stage 4, turn over into side position, clonic-tonic seizures, and stage 5, turn over into back position, generalized clonic-tonic seizures. Animals that reached stage 4 or 5 seizures were selected for this study. After parturition, pups were counted and weighted. Pups were housed with their mothers until weaning (21st day of postnatal) (19). To reduce possible parturition effects, a maximum of four male offspring from any parturition were used in this experiment. Animals in the kindled group (n = 40) and the control group (n = 40) were divided into four subgroups (n = 10). The pups in subgroups were allowed to grow to 12 weeks of age. All of the subgroups were used for the electrophysiological study in four doses 0, 0.01, 0.1, and 1 nmol of antagonist M1 mACh R (telenzepine).

Electrophysiological study

Slice preparation

The method used in this study to prepare the hippocampal slices was according to the previous protocol performed in our laboratory (20,21). Overall, eighty male rats 12 weeks old were anesthetized with chloroform and decapitated. Then, their brain was kept in the ice-cold artificial cerebrospinal fluid (aCSF) consisting of NaCl 125 mM, KCl 2.5 mM, $NaHPO_4$ 1.25 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1.3 mM, and glucose 10 mM (pH = 7.4 ± 0.5, 290-300 mOsm) and bubbled with a gas mixture of O_2 95%: CO_2 5% (carbogen) for 1 min.

Right hippocampi were slowly removed, and 4 to 5 thick slices (400 μm) of the dorsal hippocampus were cut with a vibratome slicing. The slices were immediately put in a recovery chamber consisting of aCSF at room temperature and then bubbled with carbogen. All slices were maintained in the recovery chamber for at least 1.5 h. Then, slices were transferred to a submerged recording chamber.

Electrophysiological recording

The slices were perfused with carbonated aCSF (warmed to 32 ± 2 °C) at a rate of 2 mL/min in the recording chamber. The glass micropipettes (Sarasota, FL 34240-9258, USA) were pulled using a vertical programmable microelectrode puller (PC-100, Narishige, Japan) and filled with aCSF. Electrode tip resistance was 2-5 MΩ. Extracellular field excitatory postsynaptic potentials (fEPSPs) and population spike (PS) were recorded by placing microelectrode on stratum radiatum and stratum pyramidale of CA1 area, respectively. A twisted pair of Teflon-coated stainless steel wires were placed on the stratum radiatum to stimulate Schaffer collateral afferents. Stimuli consisted of constant current rectangular pulses (200 μs, 0.033 Hz, 10-120 μA) delivered by a stimulator-isolator (A365R, WPI, Sarasota FL, USA). Recording signals were amplified by an amplifier (D3111; Science Beam, Iran), displayed on a computer screen, and saved at a sampling rate of 10 kHz on a personal computer hard disk for offline analysis.

Evoked field potential recording was started 15 min after putting up electrodes. The recording was continued until a stable baseline for 10 min (less than 10% variation) then, telenzepine was perfused. Telenzepine was dissolved in distilled water and was diluted in aCSF to final concentrations of 0, 0.01, 0.1, or 1 nM for each experimental group. Ten min after stable baseline recording, eight gradual increases of stimulus intensities were used to define the input/output curve. The lowest intensity that evoked a measurable response was considered as the threshold intensity (T), and 2T-8T until the maximum stimulus intensity (120 nμ) were other tested intensities. The slope of the downward deflection of field excitatory postsynaptic potential (fEPSP) was assumed as an index of field synaptic response (fEPSP-I). The saturation of elicited response was obtained as over 120 μA; therefore, it was possible to characterize the stimulation intensity as the percentage of the maximum response. After the development of the input/output curve, the stimulus intensity that elicited 40-50% of the maximum response was adjusted as test pulse 1 (T1) and applied for 5 min. Paired pulse responses were taken at an inter-pulse interval of 10, 20, 80, 100, and 200 ms for 2 min. Fifteen min later high-frequency stimulation (HFS, a train of 100 stimuli at 100 Hz) was induced for induction of LTP, with the recording continuing for 60 min. The second paired pulse was taken, then an additional input/output curve was created and the experiment was terminated.

Statistical analysis

Statistical analyses were accomplished using IBM SPSS Statistics 24 for Windows. The difference between the control and kindle groups was analyzed using a *t*-test. Two-way ANOVA was conducted to analyze the effect of seizure and telenzepine on the LTP induction. Further analysis for multiple comparisons was done using the Bonferroni *posthoc* test. All data were presented as mean ± SEM, with a significant *P*-value at the level of less than 0.05 for all tests. The percentage (%) of changes in the amplitude of PS and slope of fEPSP due to HFS was normalized in the 10-min baseline recording. The averaged response got during the last 10-min baseline recording was used as a reference, and was compared with the averaged response obtained during the 60-min post-HFS recording. A potentialize data

analysis package (Science Beam, Iran) was used to determine the fEPSP or the PS magnitude, as previously described (22). Paired pulse PS index and paired-pulse index of fEPSP were driven from averaged waveforms as the pulse2/pulse1 ratio at inter-pulse intervals of 10, 20, 80, 100, and 200 ms (23,24).

RESULTS

Maternal kindling induces offspring fEPSP impairment

To evaluate the effect of maternal kindling on the 13th day of gestation on the offspring CA1 area synaptic plasticity, we studied the fEPSP-LTP slope and amplitude of PS-LTP in the hippocampal CA1 area of 12-week-old male offspring born from maternal-kindled (kindled group) and non-kindled (control group). Analysis of data revealed a significant difference in the magnitude of fEPSP-LTP between groups ($t_{20} = 3.28$). As can be seen, a significantly shorter mean fEPSP-LTP slope recorded from hippocampal slices of the kindled group was observed, compared with the analogous value in the control group (Fig. 1A and B). However, the analysis of the population spike showed that the PS-LTP amplitude recorded from hippocampus slices of the kindled group had no significant difference from those of the control group ($t_{20} = 1.566$, $P = 0.205$; Fig. 1C and D).

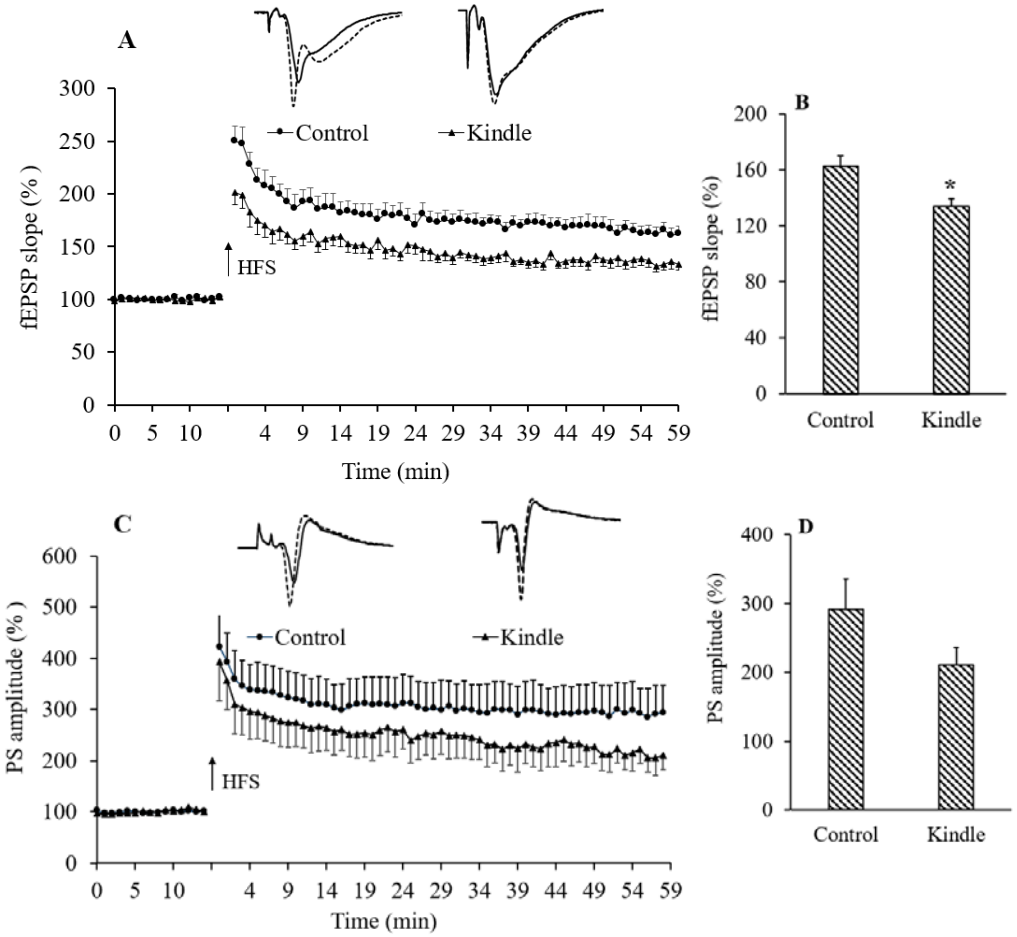

Fig. 1. The effect of maternal kindling on the fEPSP-LTP and PS-LTP. The magnitude of (A) fEPSP-LTP and (C) PS-LTP changes (%) versus time for hippocampal slices perfused with pure artificial cerebrospinal fluid in the control and kindled groups before and after tetanic stimulation; the upward arrows show the moment of high-frequency stimulation; solid line represents superimposed fEPSPs and PSs trace at baseline and the dashed line represents 60 min after tetanic stimulation, on top of parts A and C, respectively, in the hippocampal CA1 area of control and kindled groups. (B) and (D) show the mean values (%) of the fEPSP-LTP and PS-LTP, respectivelyThe data are presented as the mean ± SEM. **P* < 0.05 Indicates significant difference with the control group. fEPSP, Field excitatory postsynaptic potentials; LTP, long-term potentiation; PS, population spike; LTP, long-term potentiation.

The effects of kindling during pregnancy and telenzepine on the magnitudes of the fEPSP and population spike

Kindling during pregnancy and telenzepine have the same effect on the amount of CA1 LTP recorded from slices of male offspring. To reveal the role of the M1 muscarinic receptor on the LTP induction and the LTP impairment effect of maternal seizure, we perfused slices taken from offspring of the control and kindled groups with 0.01, 0.1, and 1 nM telenzepine. Statistical analysis showed that there was a significant difference in the slope of fEPSP-LTP among groups ($F_{[3,72]} = 8.425$, $P = 0.0001$; Fig. 2). However, there was also an interaction between seizure and telenzepine effects, further analysis using Bonferroni post hoc showed that telenzepine dose-dependently decreased the slope of fEPSP-LTP of the control group (Fig. 2A and C) but had no significant effect on the fEPSP-LTP slope of the kindled group (Fig. 2B and C). Although, the fEPSP-LTP slope of 0 and 0.01 nM of telenzepine-treated slices in the control group were significantly higher than the slope of fEPSP-LTP of 0 nM and 0.01 nM treated slices in the kindled group (Fig. 2C) there were no significant differences between 0.1 and 1 nM telenzepine-treated slices in the control and kindled groups (Fig. 2C). The analysis of PS-LTP did not show any significant difference in the amplitude of the PS-LTP in none of these experimental groups ($P > 0.05$, Fig. 3).

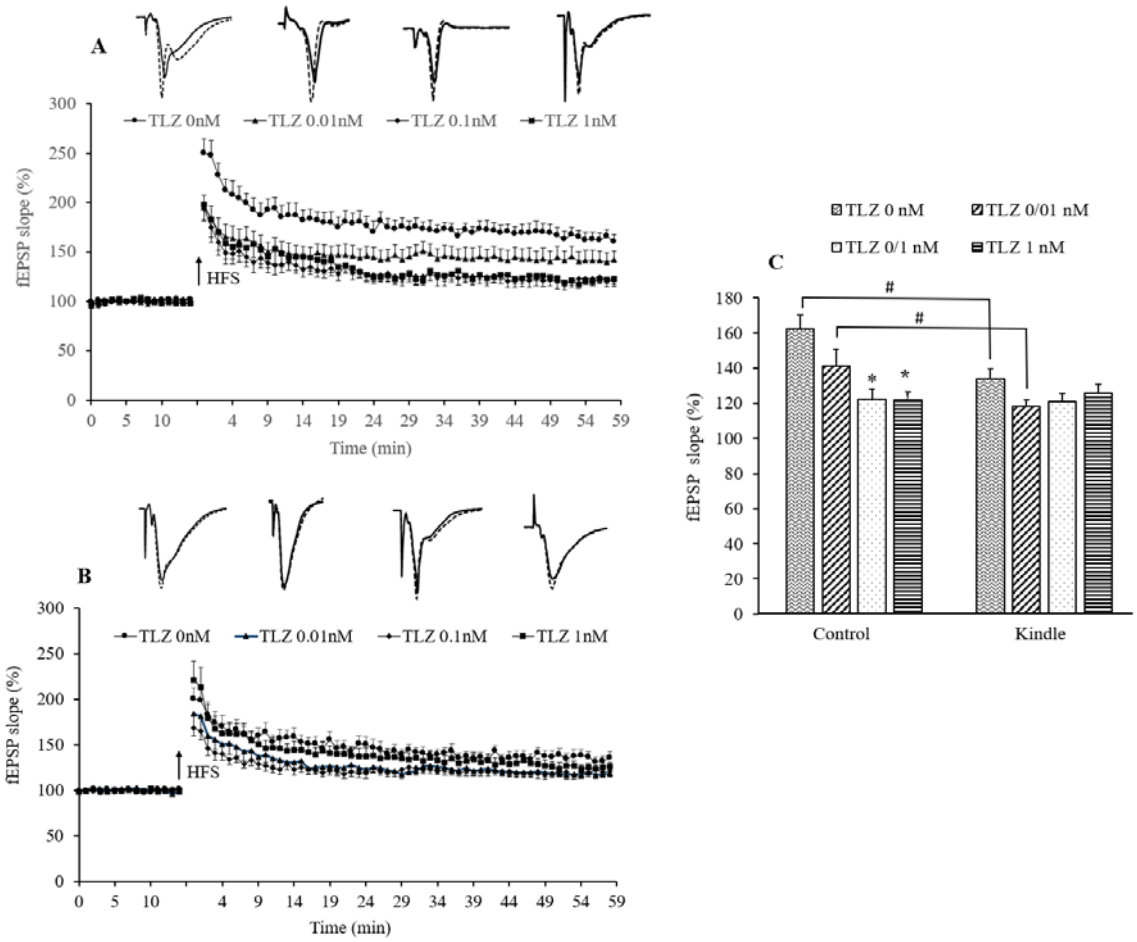

Fig. 2. The effect of M1 receptor inhibition on the fEPSP-LTP induction. The amount of fEPSP-LTP slope change (%) versus time for slices taken from (A) the control group and (B) kindled group that perfused with pure artificial cerebrospinal fluid and difference doses of TLZ (0.01, 0.1, and 1 nM); the upward arrows indicate episodes of high-frequency stimulation; solid line represents fEPSPs trace in control and kindled groups at baseline and dashed line for 60 min after tetanic stimulation which are on top of parts A and B, respectively. (C) The diagram reveals the mean values (%) of the fEPSP-LTP magnitude between different groups. The data are presented as the mean ± SEM. $^{*}P < 0.05$ indicates significant differences in comparison with the control group; $^{\#}P < 0.05$ between the specified groups. fEPSP, Field excitatory postsynaptic potentials; LTP, long-term potentiation; PS, population spike; LTP, long-term potentiation; TLZ, telenzepine.

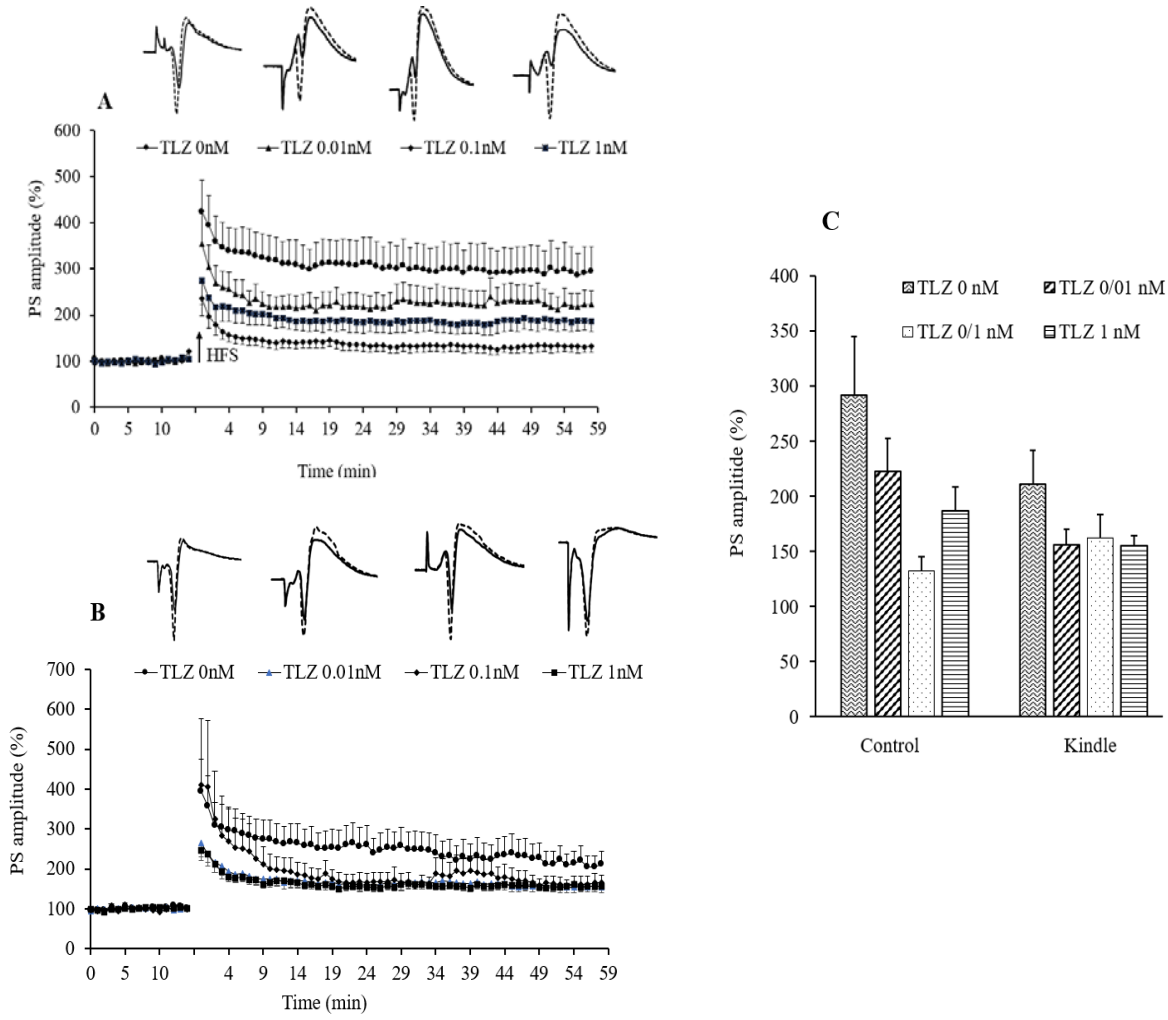

Fig. 3. The effect of M1 receptor inhibition on the PS-LTP induction. The amount of PS-LTP amplitude change (%) versus time for slices taken from (A) the control group and (B) kindled group that perfused with pure artificial cerebrospinal fluid and different doses of TLZ (0.01, 0.1, and 1 nM). The upward arrows indicate episodes of high-frequency stimulation; the solid line represents superimposed PSs trace in control and kindled groups at baseline and the dashed line represents 60 min after tetanic stimulation; (C) revealing the mean values (%) of the PS-LTP magnitude between groups. The data are presented as the mean ± SEM. PS, Population spike; LTP, long term potentiation; TLZ, telenzepine.

The effects of kindling during pregnancy and telenzepine on the paired-pulse

Kindling during pregnancy and telenzepine did not affect the paired-pulse index recorded from slices of male offspring. To determine the effect of maternal seizures and telenzepine on neurotransmitter release from presynaptic terminals at CA1 synapses, we also examined the paired-pulse population spike index and the paired-pulse index of fEPSP. There was no significant difference in none of these experimental groups ($F_{[3,71]} = 0.973$, $P = 0.411$; Table 1) ($F_{[3,69]} = 1.228$, $P = 0.306$; Table 2).

Table 1. The average paired-pulse index of fEPSP was recorded for baseline synaptic response and 60 min after induction of HFS in the CA1 region of the hippocampus.

Pre HFS	TLZ (nM)	Inter-pulse interval (ms)				
		10	20	80	100	200
Control	0	2.02 ± 0.16	2.17 ± 0.13	2.07 ± 0.11	2 ± 0.10	1.55 ± 0.08
	0.01	1.6 ± 0.15	1.77 ± 0.09	1.63 ± 0.06	1.55 ± 0.05	1.19 ± 0.04
	0.1	1.83 ± 0.12	1.98 ± 0.07	1.84 ± 0.09	1.67 ± 0.08	1.23 ± 0.08
	1	1.82 ± 0.24	2 ± 0.20	1.95 ± 0.13	1.84 ± 0.11	1.35 ± 0.08
Kindle	0	1.75 ± 0.17	1.87 ± 0.12	1.81 ± 0.08	1.73 ± 0.05	1.36 ± 0.05
	0.01	1.69 ± 0.25	1.97 ± 0.20	1.84 ± 0.13	1.67 ± 0.11	1.23 ± 0.05
	0.1	2.2 ± 0.59	1.89 ± 0.27	1.82 ± 0.17	1.72 ± 0.18	1.24 ± 0.11
	1	2.22 ± 0.18	2.42 ± 0.19	2.24 ± 0.15	2.02 ± 0.14	1.45 ± 0.07
Post HFS						
Control	0	1.31 ± 0.08	1.69 ± 0.10	1.65 ± 0.09	1.55 ± 0.09	1.3 ± 0.09
	0.01	1.34 ± 0.14	1.58 ± 0.10	1.5 ± 0.06	1.44 ± 0.05	1.14 ± 0.03
	0.1	1.87 ± 0.22	2.11 ± 0.19	1.84 ± 0.10	1.74 ± 0.06	1.23 ± 0.04
	1	1.87 ± 0.26	2.06 ± 0.23	1.82 ± 0.17	1.71 ± 0.13	1.25 ± 0.06
Kindle	0	1.48 ± 0.15	1.65 ± 0.12	1.55 ± 0.10	1.58 ± 0.10	1.31 ± 0.05
	0.01	1.61 ± 0.25	1.92 ± 0.18	1.76 ± 0.11	1.68 ± 0.11	1.33 ± 0.07
	0.1	1.71 ± 0.27	1.85 ± 0.17	1.66 ± 0.11	1.64 ± 0.10	1.29 ± 0.10
	1	1.92 ± 0.10	2.03 ± 0.09	1.88 ± 0.08	1.77 ± 0.09	1.38 ± 0.06

fEPSP, Field excitatory postsynaptic potentials; HFS, high-frequency stimulation; TLZ, telenzepine.

Table 2. The average paired-pulse population spike index was recorded for baseline synaptic response and 60 min after induction of HFS in the CA1 region of the hippocampus.

Pre HFS	TLZ (nM)	Inter-pulse interval (ms)				
		10	20	80	100	200
Control	0	3.66 ± 0.56	4.27 ± 0.68	4.09 ± 0.57	3.7 ± 0.50	2.5 ± 0.31
	0.01	3.64 ± 0.80	3.27 ± 0.51	2.86 ± 0.34	2.88 ± 0.47	1.75 ± 0.19
	0.1	2 ± 0.39	2.26 ± 0.34	2.33 ± 0.36	2.16 ± 0.35	1.47 ± 0.15
	1	2.86 ± 0.57	3.11 ± 0.55	2.84 ± 0.35	2.64 ± 0.32	1.82 ± 0.13
Kindle	0	7.14 ± 2.36	7.16 ± 2.28	6.94 ± 2.38	7.03 ± 2.66	3.14 ± 0.86
	0.01	3.90 ± 0.97	3.07 ± 0.43	2.81 ± 0.34	2.58 ± 0.30	1.8 ± 0.22
	0.1	3.11 ± 0.56	4.06 ± 1.06	3.31 ± 0.66	3.3 ± 0.76	2.17 ± 0.47
	1	2.74 ± 0.24	2.94 ± 0.14	2.59 ± 0.12	2.32 ± 0.11	1.59 ± 0.05
Post HFS						
Control	0	1.42 ± 0.12	1.54 ± 0.10	1.59 ± 0.10	1.5 2± 0.08	1.38 ± 0.08
	0.01	2.08 ± 0.33	2.09 ± 0.30	1.9 ± 0.24	1.73 ± 0.19	1.47 ± 0.12
	0.1	1.96 ± 0.27	1.97 ± 0.25	1.87 ± 0.21	1.75 ± 0.21	1.41 ± 0.13
	1	2.77 ± 0.73	3.37 ± 1.19	2.66 ± 0.69	2.41 ± 0.59	1.72 ± 0.28
Kindle	0	2.55 ± 0.50	2.6 ± 0.54	2.36 ± 0.49	2.21 ± 0.40	1.71 ± 0.28
	0.01	3.48 ± 1.13	2.95 ± 0.65	2.58 ± 0.45	2.52 ± 0.47	1.72 ± 0.22
	0.1	3.37 ± 0.99	5.09 ± 2.57	3.42 ± 0.92	4.1 ± 1.88	4.07 ± 2.48
	1	2.17 ± 0.17	2.43 ± 0.20	2.13 ± 0.16	1.9 ± 0.13	1.46 ± 0.05

HFS, high-frequency stimulation; TLZ, telenzepine.

DISCUSSION

The current study demonstrated that maternal seizure at thirteen days of gestation reduced the magnitude of fEPSP-LTP recorded from the hippocampus CA1 area of the male offspring. These findings are important because they suggest that seizure during gestation could lead to disruption of synaptic function and LTP induction in the offspring. It has been indicated that the thirteen days of gestation is a critical and important time point for the development of the hippocampus (25,26). Clinical and experimental studies have revealed that seizures during gestation can result in persistent functional abnormalities in offspring (6,13). Therefore, maternal seizure at this time point has a long-lasting effect on the hippocampal synaptic plasticity which will affect offspring behavioral response patterns.

It is known that the activation of M1 mAchRs in humans with cognitive impairment (27) can improve memory formation. According to the important role of synaptic plasticity in memory processes, it is suggested that acetylcholine release improves memory by modulating the induction of synaptic plasticity (28). Indeed, the induction of hippocampal synaptic plasticity requires muscarinic receptor activation (29). It has been documented that acetylcholine stimulates pyramidal neurons of the CA1 area by stimulating the M1 receptor and can induce LTP (9). Based on the conducted studies, we investigated the effect of seizure at thirteen days of pregnancy on the function of M1 mAchRs in the hippocampus of male offspring. The results of our study revealed that the offspring in the control group exposed to 0.1 and 1 nM doses of antagonist M1 mACh R (telenzepine) showed lower fEPSP-LTP than those that received pure aCSF. This result is consistent with a previous study that showed the role of the cholinergic system in CA1 LTP induction (9,10). However, this reduction effect of the telenzepine did not observe in slices taken from the offspring of the kindled group. The present study suggested that maternal kindling causes abnormal development of the M1 muscarinic receptor which leads to defects in M1 receptor function in adulthood. Abnormal function of the M1 receptor resulted in lower fEPSP-LTP of the kindled group and no further effect on the M1 antagonist. A previous study reported that maternal seizure has a severe effect on the neurobiological development of the offspring hippocampus (30), here we showed the M1 muscarinic receptor is a susceptible candidate that could be affected by maternal seizure and the functional deficiency of the muscarinic system could be traced until adulthood. However, these preliminary results should be confirmed by a further study that will evaluate the amount of M1 receptor proteins or/and mRNA and M1 receptor structure of the offspring.

This study did not show significant changes in PS-LTP magnitude, neither seizure nor telenzepine had a significant effect on the PS-LTP magnitude. These results may propose a site-dependent effect of telenzepine and maternal seizure of CA1 of the hippocampus (31,32).

Paired-pulse facilitation is a very short-term form of dependent synaptic plasticity observed at synapses in different phylogenetic groups (33). In paired-pulse facilitation, transmitter release evoked by a second presynaptic action potential is enhanced when it follows a previous action potential within tens or hundreds of milliseconds (34-36). This phenomenon is generally evaluated as an index of presynaptic alterations during more persistent and complex forms of synaptic plasticity. The results of our study showed that paired-pulse indexes (short-term plasticity) had no significant difference among experimental groups. These results are consistent with a previous -study that observed no change in paired-pulse indexes between the epileptic group and the control group (37). In our study, the lack of change in the paired-pulse indexes in the experimental groups could indicate that the maternal seizures and telenzepine did not affect the permeability of presynaptic neurons and the release of neurotransmitters from presynaptic terminals in offspring.

CONCLUSION

Taken together, these findings suggest that recurrent seizures during pregnancy cause impaired fEPSP-LTP induction and abnormal development of the M1 muscarinic receptor in the hippocampus. While maternal seizure had no effect on the paired-pulse indexes (short-term plasticity) recorded from the CA1 area of the male offspring.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

A. Pourmotabbed conducted the study, and contributed to data collection and analysis; R. Naghizadeh contributed to data collection, data

analysis, and manuscript preparation; F. Moradpour helped design the study and manuscript preparation; M. Veisi and S.E. Nedaei contributed to the conduction of the study, data collection, and data analysis; F. Zarei and Z. Salimi assisted with manuscript drafting and revision. The finalized manuscript was approved by all the authors.

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