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SHORT COMMUNICATION
Bioactivity of a peptide derived from acetylcholinesterase: electrophysiological characterization in guinea-pig hippocampus

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Abstract

Acetylcholinesterase is well known to have noncholinergic functions. Only recently, however, has the salient part been identified of the molecule responsible for these nonclassical actions, a peptide of 14 amino acids towards the C-terminus of acetylcholinesterase. The aim of this study was to test the bioactivity of this 'acetylcholinesterase-peptide' using intracellular recordings in guinea-pig hippocampal slices. In the presence of tetrodotoxin, acetylcholinesterase-peptide alone affected neither the membrane potential nor the input resistance of CA1 neurons; however, a modulatory action was observed, as a concentration-dependent decrease of *N*-methyl-D-aspartic acid-induced depolarization. When calcium potentials were elicited by a depolarizing current pulse, application of acetylcholinesterase-peptide increased or reduced the degree of calcium spike firing in, respectively, the presence or absence of the *N*-methyl-D-aspartic acid antagonist D(–)-2-amino-5-phosphonopentanoic acid. In contrast, a peptide derived from the equivalent region of butyrylcholinesterase, which also hydrolyses acetylcholine, had no effect. In conclusion, acetylcholinesterase-peptide has a selective bioactivity in the hippocampus; it could thus offer new ways of targeting mechanisms of calcium-induced neurotoxicity.

Introduction

In various brain regions, acetylcholinesterase (AChE) may have functions unrelated to cholinergic neurotransmission (Greenfield, 1991; Soreq & Seidman, 2001). For example, in the substantia nigra, AChE increases calcium entry through *N*-methyl-D-aspartic acid (NMDA) receptors (Webb *et al*., 1996), and thereby activates adenosine triphosphate (ATP)-sensitive potassium channels by compromising oxidative phosphorylation, due to mitochondrial uptake (Webb & Greenfield, 1992), and thereby leads to a net hyperpolarization of the cell membrane (Greenfield *et al*., 1988; Greenfield *et al*.,1989). This nonclassical calcium action may play a key role in development, as certain neurons (Robertson & Yu, 1993), perhaps even a majority (Soreq & Seidman, 2001), transiently express AChE in development; even commitment of stem cells to a neuronal differentiation pathway is characterized by the appearance of AChE messenger ribonucleic acid (mRNA) (Coleman & Taylor, 1996). Moreover, the appearance of the protein, in a developmental context, can be dissociated from its familiar hydrolytic action (Ling *et al*., 1995), as can its seemingly vital role in the process of LTP (Appleyard, 1995) and trophic action in the hippocampus (Day & Greenfield, 2002).

Only recently, however, a peptide sequence within AChE has been identified as a candidate for its noncholinergic functions (Greenfield & Vaux, 2002): AEFHRWSSYMVH. The aim of this study was therefore to test whether this 'AChE-peptide' was indeed bioactive. Accordingly, we have studied the effects of exogenous AChE-peptide upon the passive and active membrane properties of guinea-pig CA1 hippocampal neurons *in vitro*. Because the nonclassical action of AChE itself is modulatory (Greenfield, 1991; Greenfield 1998; Webb & Greenfield, 1992; Soreq & Seidman, 2001), we have also tested for a possible action of AChE-peptide occurring only in conjunction with another signalling event, such as the activation of NMDA or other calcium potentials induced by a depolarizing current pulse. These particular paradigms were chosen as an appropriate initial scenario, as AChE has already been shown to have a noncholinergic action in plasticity of CA1 cells dependent on glutamate receptors (Appleyard, 1995), and, more recently, activation of calcium channels during development of the hippocampus (Day & Greenfield, 2002). All effects were compared with an analogous 14-residues peptide, AGFHRWNNYMMDWK (Greenfield & Vaux, 2002), from a comparable region of the C-terminus of butyrylcholinesterase (BuChE), which also hydrolyses acetylcholine, but does not display that same novel action of AChE, in the adult brain.

Materials and methods

Slice preparation

Male albino guinea-pigs (300–500 g; University Park Farm, Oxford, UK) were decapitated after cervical dislocation in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines, the European Community Council Directive of 24 November 1986 (86/609/EEC). The whole brain was quickly removed and cooled

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with ice-cold oxygenated artificial cerebrospinal fluid solution (aCSF) containing (in mM): NaCl, 120; KCl, 5; KH_2PO_4, 2; $MgSO_4$ $7H_2O$, 2; $NaHCO_3$, 20; $CaCl_2$ $2H_2O$, 1.25; D-glucose, 10, HEPES acid, 6.7; HEPES sodium salt, 3.3; gassed with 95% O_2 and 5% CO_2. Transverse hippocampal slices were cut at a thickness of 450 μm using a vibratome (Lancer Series 1000). At the beginning of the recording session, a single slice was transferred to a Perspex submersion-type recording chamber (volume of 5 mL), where it was sandwiched between two nylon nets and constantly perfused with preheated, oxygenated modified Ringer's solution at a rate of 3 mL/min at 32 °C. The composition of the superfusate was as follows (in mM): NaCl, 124; KCl, 2; KH_2PO_4, 1.25; $MgSO_4$ $7H_2O$, 1.3; $NaHCO_3$, 26; $CaCl_2$ $2H_2O$, 2.4; D-glucose, 5. The remaining slices were stored until required in small vials filled with oxygenated aCSF solution at room temperature. Drugs were added via the superfusion medium at fixed concentrations, and there was a delay of approximately 50 s before the new solution arrived in the recording chamber.

Electrophysiological recordings

Intracellular recordings were obtained in the CA1 stratum radiatum of a hippocampal slice using standard intracellular recording techniques, as described previously (Bon *et al.*, 1998). The range of electrode resistance was 80–120 MΩ. Neurons accepted for inclusion had a stable resting membrane potential greater than −60 mV and a membrane resistance superior to 40 MΩ.

All experiments were performed in the presence of tetrodotoxin (TTX) added to the superfusion medium to block synaptic transmission and Na^+-dependent action potentials. Electrotonic potentials were elicited by constant current pulses (−0.2 nA; 200 ms; 0.5 Hz). The membrane potential was determined by measuring the change in potential seen when the electrode was pulled out of the cell.

Concentration–response curves

In the presence of 1 μM TTX, increasing concentrations of NMDA were applied to hippocampal neurons for 5 min with wash periods between each application. Slices were exposed to ascending concentrations of AChE-peptide at least 10 min prior and 5 min during NMDA perfusion. In this case, each drug application was separated by a 15 min washout.

Calcium potentials induced by a depolarizing current pulse

Slices were pre-incubated for 10 min with a solution containing 1 μM TTX and 5 mM tetraethyl-ammonium chloride (TEA), a blocker of potassium channels, before calcium potentials were elicited by injection of direct current to the cells. Once the response was stable, AChE-peptide was added to the medium for 15 min. The number of Ca^{2+} spikes was measured at 5 min intervals before and during drug application, and also after 30 min of washout.

Statistics

In all cases, the maximal membrane potential change (mV) and the number of calcium spikes were measured from the chart recorder. All numerical data are expressed as the mean ± SEM. Statistical comparisons between groups were performed using a two-tailed, paired *t*-test. *P*-values < 0.05 were considered statistically significant.

Special chemicals

Drug sources were as follows: TTX and TEA (Sigma, France), NMDA and D(–)-2-amino-5-phosphonopentanoic acid (D-AP5; Research Biochemicals International, MA, USA), AChE-peptide and BuChE-peptide (synthesized by Oxford Centre for Molecular Studies, UK).

Results

Stable intracellular recordings included in this study were obtained from a total of 55 guinea-pig hippocampal neurons with a resting membrane potential and membrane resistance of -64 ± 6 mV and 57 ± 12 MΩ, respectively (means ± SD). No significant differences were observed in the passive membrane properties of the groups of cells used to test the action of the NMDA and AChE-peptide (ANOVA, $P = 0.36$ membrane potential, $P = 0.27$ membrane resistance).

Effect of NMDA on CA1 pyramidal neurons

We first characterized the response elicited by application of different concentrations of NMDA on CA1 neurons (Fig. 1). In the presence of 1 μM TTX, superfusion of NMDA (1–30 μM) for 5 min produced a concentration-dependent depolarization, which was associated with an

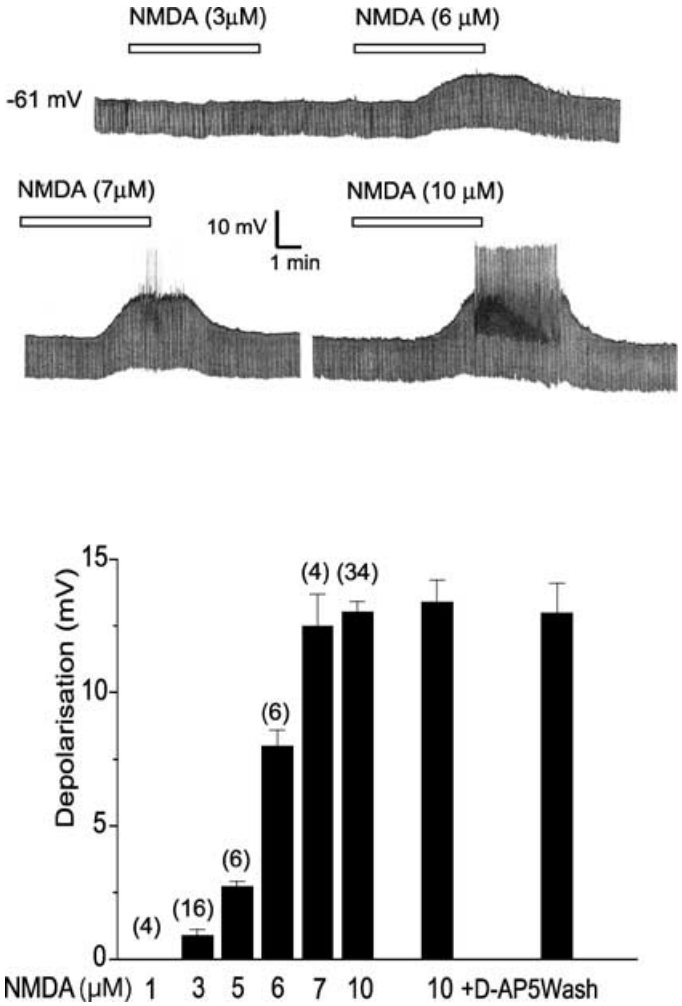
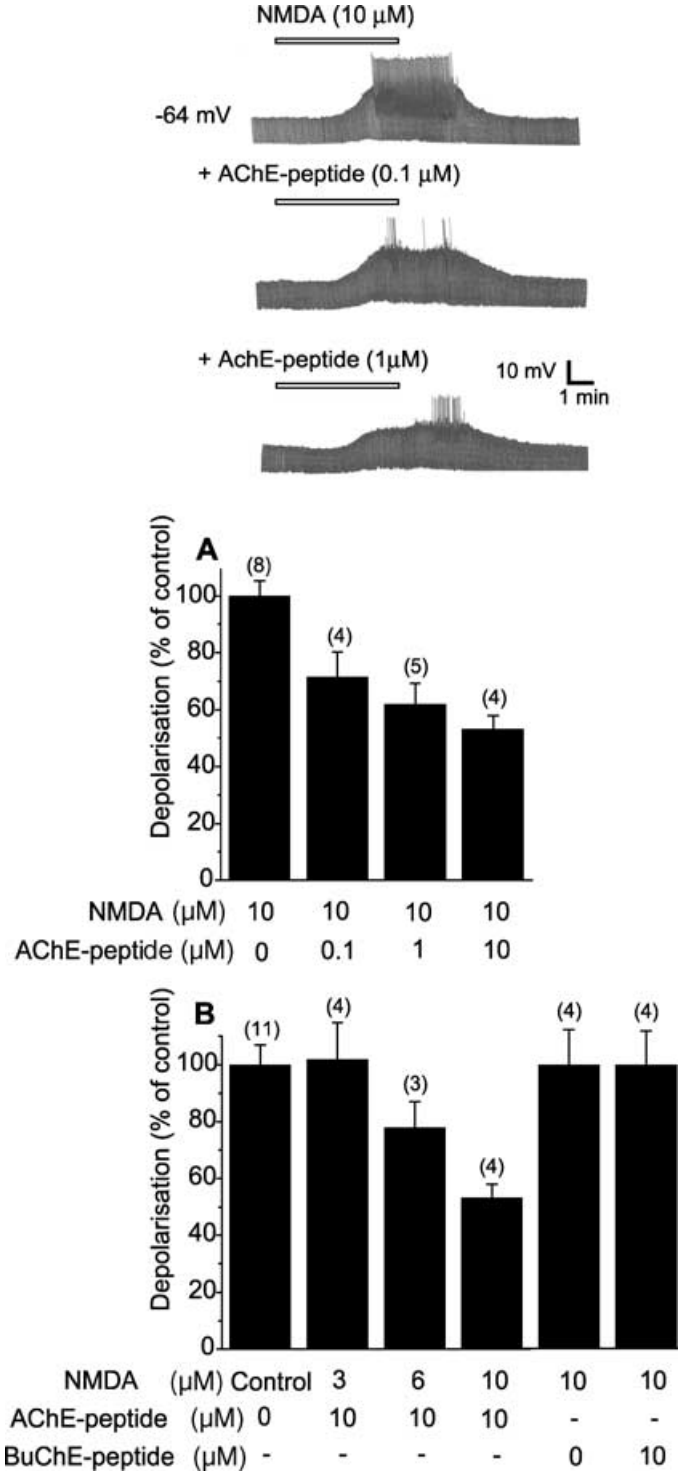


FIG. 1. Effect of NMDA on CA1 neurons. Ascending concentrations of NMDA were applied to the cells for 5 min. The numbers in brackets give the numbers of cells studied. The insets show representative intracellular recordings obtained after the application of different concentrations of NMDA as indicated (horizontal bars). The highest concentrations of NMDA (7 and 10 μM) were associated with calcium spike firing. Downward deflections represent hyperpolarizing electrotonic potentials in response to constant current pulses (−0.2 nA; 200 ms; 0.5 Hz). D-AP5 (50 μM) was added to the bath 10 min before being in contact with 10 μM NMDA for 5 min ($n = 4$). The depolarization was measured 30 min after washout of the drugs. The 50-s delay in the superfusion system (see Materials and methods) has not been corrected for in this or in subsequent figures.

apparent increase in membrane input resistance. Calcium spike firing was also observed for the higher concentrations (from 7 μM) and triggered when the membrane potential reached −48 ± 1 mV. The depolarizing effect was maximal after at least 4 min of NMDA application, and the repolarization of the membrane occurred within 3–5 min after reintroduction of drug-free modified Ringer's solution. The depolarization elicited by 10 μM NMDA was completely blocked by 50 μM D-AP5, a NMDA receptor antagonist, when applied 10 min prior and 5 min during NMDA perfusion (Fig. 1). This inhibition was fully reversible after 30 min of washout. There was no significant difference between the response elicited by NMDA before adding D-AP5 (13.4 ± 0.8 mV) and that recorded after the washout (13 ± 1.1 mV; $P > 0.77$, paired t-test, $n = 4$). We also checked that slices did not desensitize after being exposed to four successive solutions of 10 μM NMDA for 5 min separated by a washout period of 20 min (data not shown); application 1 resulted in a depolarization of 15 ± 1 mV for 8.5 ± 1 min, which was not significantly different from application 4 (14.5 ± 1 mV for 9 ± 1 min; $n = 3$).



Effect of AChE-peptide on NMDA-induced depolarization

Slices incubated with the AChE-peptide alone (0.1−10 μM) for 5 to even 30 min did not show any significant changes in the resting membrane potential or membrane resistance. In contrast, superfusion with AChE-peptide (0.1–10 μM) resulted in a decrease in the amplitude of depolarization induced by 10 μM NMDA (13.8 ± 1.2 mV) in a concentration-dependent manner, with already a significant effect at 0.1 μM (9.9 ± 1.4 mV; $P < 0.04$, paired t-test, $n = 4$; Fig. 2A). In addition, when the effect of 10 μM AChE-peptide on the depolarization was induced by different concentrations of NMDA (3–10 μM; Fig. 2B), it was found that AChE-peptide significantly decreased the amplitude of the depolarization induced by 10 μM NMDA (8 ± 1.1 mV) relative to control (14.9 ± 1.1 mV; $P < 0.001$, paired t-test, $n = 4$). The specificity of the effect was examined by using BuChE-peptide, a peptide which has a sequence different to AChE-peptide, yet which also hydrolyses acetylcholine. Application of 10 μM BuChE-peptide had no significant effect on the amplitude of the depolarization induced by 10 μM NMDA nor on the threshold of calcium spikes (12.5 ± 1.49 mV) relative to controls (12.5 ± 1.54 mV; Fig. 2B).

Actions of AChE-peptide on calcium spikes

In order to characterize the modulatory effect of AChE-peptide on active membrane properties independent of NMDA, the priming action of the endogenous ligand was substituted by a current pulse. Once again, in the presence of TTX and TEA, application of 10 μM AChE-peptide for 15 min significantly reduced the number of calcium spikes fired per min compared with controls (27 ± 2), with a maximal effect after 10 min perfusion (3 ± 1). This effect was partially reversible after 30 min of washout (7 ± 2 spikes/min). In contrast, if D-AP5 was added to the previous medium, AChE-peptide increased the number of calcium spikes per min (Fig. 3B). This effect was maximum after 10 min perfusion with the drugs (33 ± 1 spikes/min), and significantly different from the control value (16 ± 2 spikes/min), but remained partially irreversible after 30 min of washout (24 ± 4 spikes/min).

Discussion

Passive membrane properties

There was no effect of either AChE-peptide or BuChE-peptide on passive membrane properties; AChE-peptide alone does not appear

FIG. 2. Effect of AChE-peptide on the NMDA-induced depolarization. (A) Cells were pre-incubated with different concentrations of AChE-peptide (from 0.1 μM to 10 μM) for 10 min before being in contact with 10 μM NMDA for 5 min. Each drug application was separated by 15 min of washout. The numbers in brackets represent the numbers of cells studied. The control values are pooled. The insets show representative intracellular recordings obtained after the application of 10 μM NMDA (top panel), then in the presence of 0.1 μM (middle panel) and 1 μM of AChE-peptide (bottom panel). The electrotonic potentials were elicited by constant current pulses (−0.2 nA; 200 ms; 0.5 Hz). (B) Cells were pre-incubated with 10 μM of AChE-peptide or BuChE-peptide for 10 min before being in contact with different concentrations of NMDA (from 3 to 10 μM) for 5 min. Each drug application was separated by 15 min of washout. The control values are pooled. The numbers in brackets represent the numbers of cells studied.

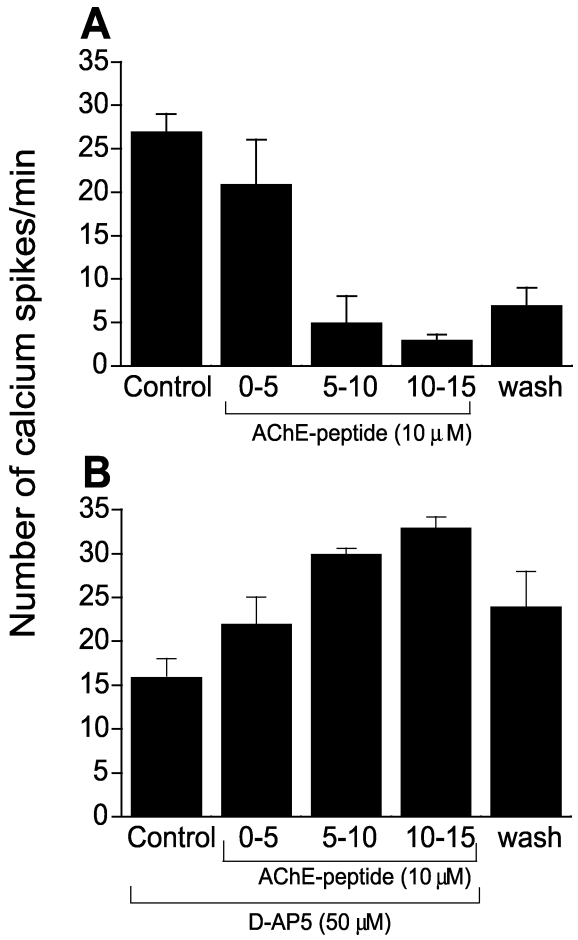


FIG. 3. Effect of AChE-peptide on calcium spike firing. (A) Calcium spikes were elicited by injection of a holding current in the presence of 5 mM TEA and 1 μM TTX. AChE-peptide (10 μM) was in contact with CA1 neurons for 15 min ($n = 6$), and the number of Ca^{2+} spikes was measured at 5-min intervals before and during drug application, and also after 30 min of washout. (B) Same as previously described, except that experiments were performed in the presence of 50 μM D-AP5 ($n = 5$).

to exert any nonspecific or toxic effects that may have occurred as a result of bulk peptide interacting with the bilayer of the plasma membrane.

Active membrane properties: NMDA

In CA1 pyramidal neurons, depolarization obtained during bath application of NMDA was concentration dependent and associated unexpectedly with an increase in the membrane resistance; as previously described (Mayer & Westbrook, 1987), this effect can be explained by the current voltage relationship that results from amino acids acting at NMDA receptors, which is highly nonlinear.

The total reversible blockade of the response obtained with D-AP5, a selective and competitive NMDA receptor antagonist (Stone, 1986; Davis *et al*., 1992), demonstrates that the depolarization evoked by NMDA is mediated exclusively, as predicted, by the NMDA receptor. Although NMDA receptors could desensitize (Ascher & Johnson, 1989), in our conditions, there was no change in the amplitude of the depolarization during prolonged application of NMDA, probably due to the slow time course of agonist application which will obscure the relatively rapid (seconds) NMDA receptor desensitization.

Active membrane properties: NMDA and AChE-peptide

In the presence of a high concentration of NMDA, AChE-peptide decreased the amplitude of the depolarization, leading to a reduction of calcium spikes in a selective, dose-dependent manner. Were this reduction due to an inhibition of calcium channels, it would most likely be due to phosphorylation resulting from an excessive calcium entry (Standen, 1981). The notion that AChE-peptide could modulate calcium channels in this way is not surprising. In the cerebellar cortex, exogenous AChE can increase synaptic responses mediated by excitatory amino acids in Purkinje cells by a mechanism which is insensitive to inhibition of AChE catalysis (Appleyard & Jahnsen, 1992). Indeed, the effects of AChE, acting in a nonenzymatic capacity during neuronal growth, have been directly linked to activation of the L-type calcium channel (Luo *et al*., 1996; Day & Greenfield, 2002).

Active membrane properties: direct current stimulation and AChE-peptide

When the contribution of the NMDA receptor/ion channel complex activated by endogenous ambient glutamate (Sah *et al*., 1989) is blocked by APV, then AChE-peptide has a reversible, enhancing effect on depolarization induced by direct current injection. This depolarization would, of course, activate a range of voltage-dependent calcium channels, including the L-channel. This time, however, the net effect of AChE-peptide on calcium spikes is one of enhancement. The most likely explanation for this observation is that in the absence of APV, the net influx of calcium caused by NMDA receptor activation, voltage-dependent channels and, finally, AChE-peptide, would be sufficient to cause channel inactivation. Any decrease in ambient endogenous glutamate would be offset by a similar decrease in efficacy of glutamate transporters in the *in vitro* preparation, resulting in a residual availability of glutamate. However, once the contribution of NMDA receptors is removed, then the enhancing effects of AChE-peptide are unmasked on the calcium channels that remain operational.

Physiological and pathological significance of AChE-peptide

This study has shown that AChE-peptide has a selective bioactivity in the hippocampus, in a fashion similar to that seen for the noncholinergic actions of AChE itself. This bioactivity is to enhance calcium, perhaps through voltage-sensitive calcium channels (Day & Greenfield, 2002), which could be activated by electrical stimulation, thereby resulting in a net enhancement in calcium potentials, or in addition to a priming NMDA receptor activation, resulting in calcium channel inactivation, and hence a net inhibition. In any event, it is possible that the peptide tested here could indeed underlie the familiar noncatalytic action of AChE. This action could be an important factor, not only in development, but in other calcium-dependent phenomena, such as the aberrant activation of developmental mechanisms resulting in neurodegeneration (Greenfield & Vaux, 2002) as well as in excitotoxicity (Olney, 1978).

Abbreviations

AChE, acetylcholinesterase; aCSF, artificial cerebrospinal fluid; ATP, adenosine triphosphate; BuChE, butyrylcholinesterase; D-AP5, D(–)-2-amino-5-phosphonopentanoic acid; mRNA, messenger ribonucleic acid; NMDA, *N*-methyl-D-aspartic-acid; TEA, tetraethyl-ammonium chloride; TTX, tetrodotoxin.

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