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Differential inhibition by trifluoperazine of responses of hippocampal CA1 pyramidal cells to NMDA and AMPA *in vivo*

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ABSTRACT   The effects of trifuoperazine (TFP), a phenothiazine neuroleptic drug having potent anticalmodulin activity, were studied on the responses of hippocampal CA1 pyramidal cells to N-methyl-D-aspartic acid (NMDA) and (*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) *in vivo*. Single-unit activity was recorded using multibarrel carbon fiber containing microelectrodes whilst all drugs were delivered by microiontophoresis. NMDA and AMPA were iontophoresed alternately so that they evoked comparable responses in terms of peak heights as peristimulus time histograms were recorded. We observed that changing the stimulation intensity of one classes of receptors (*e.g.* NMDA) greatly influenced the responsiveness of the other (*e.g.* AMPA) and *vice versa*. In the presence of iontophoretically applied TFP responses to both NMDA and AMPA were significantly decreased. More interestingly, NMDA-evoked responses were significantly more inhibited by TFP than responses to AMPA under the same experimental conditions. In our conclusions, these results are due to the inhibition by TFP of the second messenger cascade events leading from NMDA receptors *via* Ca^{2+}/calmodulin to AMPA receptors and, in consequence, for the blocking of phosphorylation of AMPA receptors and their sensitization. It is also likely that the function of NMDA receptors by itself is, at least in part, dependent on the Ca^{2+}/calmodulin-mediated events.
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Trifluoperazine (TFP), a phenothiazine antipsychotic drug, is primarily used in the treatment of schizophrenia. This compound has been shown to exert a variety of effects in the hippocampus. It antagonizes dopamine D_1 and D_2 receptors (Seeman et al. 1976; Creese et al. 1996) or α_1 adrenergic effects (Huerta-Bahena et al., 1983). Binding studies showed that neuroleptics such as TFP can also compete for serotonin (5-HT), α-adrenergic, and histamine receptors (Peroutka and Snyder 1980). In low concentration (0.1-1 µM), TFP antagonizes nicotinic actions of acetylcholine (ACh) by increasing the rate of desensitization (Clapham and Neher 1984). At concentrations of 1-100 µM, TFP blocks noncompetitively γ-aminobutyric acid (GABA)-gated chloride currents in hippocampal and spinal cord neurons (Zorumski and Yang 1988)

At micromolar concentrations, TFP is also a potent calmodulin inhibitor (Levin and Weiss 1977). Interference with calmodulin-dependent processes may in turn give rise to a wide spectrum of effects. For instance, the release of 5-HT or noredrenaline from hippocampal slices was completely abolished or significantly decreased by TFP (Satoh et al.

1996; Jaffe 1998). The evoked release of acetylcholine (ACh) from slices of hippocampus was decreased in a concentration-dependent manner by preincubation with TFP (Budai and Kasa 1987). In pyramidal neurons freshly dissociated from the rat hippocampal CA1 region, ACh-induced two types of muscarinic current was reversibly and concentration dependently inhibited by TFP (Wakamori et al. 1993). Neuroleptics decrease calcium-activated potassium conductance in hippocampal pyramidal cells. Bath application of a wide variety of neuroleptics was found to depress the slow afterhyperpolarization, which is mediated in these neurons by a calcium-dependent potassium conductance occurring following a burst of spikes (Dinan et al. 1987). Tetanic stimulation or brief exposure to Ca^{2+} produced a long-lasting augmentation of the extracellular excitatory postsynaptic potentials (EPSP) and of the responses of the population spikes in the CA1 region of hippocampal slices. Both forms of potentiation were inhibited by perfusion of TFP, an effect which is unlikely to involve interactions with dopamine or norepinephrine receptors, but rather a potent blockade of calmodulin-mediated events (Mody et al. 1984). Similarly, both somatic population spikes and dendritic EPSP fields were depressed reversibly by TFP when applied by microiontophoresis in the CA1 region of hippocampal slices (Agopyan and Krnjevic 1993).
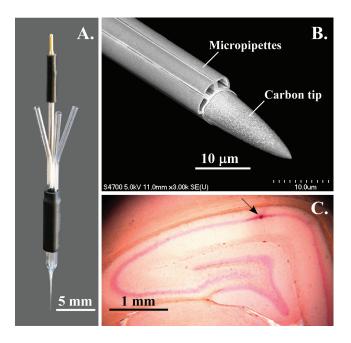
Figure 1. Macroscopic view of a multibarrel carbon fiber microelectrode used in this study (panel A) and the scanning electron micrograph of its tip (panel B). Carbon fiber was used as lead element for recording electrical signals whereas the attached micropipettes allowed iontophoretic application of electrically charged compounds in the near vicinity of the recorded neuron. The site of extracellular single-unit recording (arrow in panel C) was marked by ejection of pontamine sky blue after completing the experiment. Spikes recorded from this site are shown in Figure 2.

Induction of long-term potentiation (LTP) in the CA1 region of hippocampal slices is associated with increased activity of Ca^{2+}/calmodulin-dependent protein kinase II (CaM K II) (Fukunaga et al. 1995, 1996). Neuroleptic drugs such as TFP were able to block LTP almost completely. The ability of neuroleptics to antagonize LTP was more closely related to their ability to block calmodulin than to their relative potencies as dopamine antagonists. It would appear that neither norepinephrine nor adrenergic antagonists influence the amount of LTP elicited by repetitive stimulation; however, drugs which have been shown to interfere with calmodulin-mediated cellular processes do antagonize this phenomenon (Dunwiddie et al. 1982).

The ionotropic glutamate receptors include the N-methyl-D-aspartate (NMDA) receptors, 2-amino-3-hydroxy-5-methyl-oxazole-4-propionic acid (AMPA) receptors and kainic acid (KA) receptors (for recent review, see (Peng et al. 2011). The NMDA receptor is dual voltage and ligand-gated channel which combines with the Mg^{2+} channels to maintain a resting potential state, with this activity being voltage-dependent. Channel activation depends on the depolarization of the postsynaptic membrane and the neurotransmitter release from the presynaptic membrane. When NMDA receptors are open Ca^{2+} streams into the intracellular space, serving as a second messenger to activate a series of biochemical reactions, which may result in the manifestation of LTP. NMDA receptor activation requires the participation of the non-NMDA glutamate receptors, including the AMPA receptors and KA receptors. The non-NMDA glutamate receptors mediate low-frequency synaptic transmission in the resting state, and serve as the main receptor of Na^+, K^+ permeability. The inflow of Ca^{2+} into the postsynaptic membrane can activate a wide range of calcium-dependent enzymes including the CaM K II. After CaM K II activation, the AMPA receptor subtypes of GluR1 are phosphorylated, the AMPA receptors from non-synaptic sites are redistributed to the synaptic site. At the same time, the function of the AMPA receptor also significantly increased, as shown by the increase of the single-channel AMPA receptor synaptic transmission as well as the development of a phosphorylation site.

In the present study, our aim was to test the effects of the potent calmodulin inhibitor TFP on the activity of hippocampal CA1 pyramidal cells evoked by alternate NMDA and AMPA iontophoretic applications *in vivo*. This approach allowed us, at least in principle, to activate the two classes of ionotropic glutamate receptors independently from one another. TFP was also delivered by microiontophoresis having the advantage of affecting only a small sphere around the recorded site instead of affecting all regions of the hippocampus.

Materials and Methods

Animals and surgery

Twelve male Wistar rats (*Rattus norvegicus*) weighing 350–450 g were used in this study. Animals were purchased from the local University owned breeder and were allowed to acclimatize for at least one day before use. There was automatic control of light cycle and temperature. Light hours were from 06 to 18 h and temperature was kept within the target range of $22°C \pm 3°C$.

After an initial dose (400 mg/kg) of intraperitoneally applied chloral hydrate solution (40 mg/ml in physiological saline) (Sigma, St. Louis, MO), the jugular vein on one side was cannulated with a 60 cm-long PE-50 plastic tubing. A continuous anesthetic inflow was provided through this cannula at a rate of 1-2 ml/hour using diluted (8 mg/ml) chloral hydrate solution as needed during the whole experiment.

The head of the animal was mounted in a stereotaxic frame, the skull was opened above the hippocampus by drilling the bone (antero-posterior: 2.8 to 3.8 mm from bregma; lateral: 2 mm on either side from the midline) (Paxinos and Watson 1998), and the dura mater was carefully removed. The brain surface was always kept moist using physiological saline. All efforts were made to minimize animal suffering. The principles of laboratory animal care (NIH publication No. 85-23) and the protocol for animal care approved by the Hungarian Health Committee (1998) and the European Com-
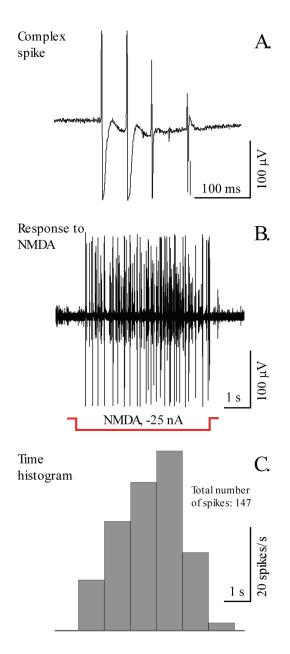
Complex spike

A.

100 µV

100 ms

Response to NMDA

B.

100 µV

1 s

NMDA, -25 nA

Time histogram

C.

Total number of spikes: 147

20 spikes/s

1 s

Figure 2. Representative single-unit recording from a hippocampal pyramidal cell located in the CA1 region as shown in Fig. 1, panel C. A complex spike typical to pyramidal cells in this region is shown in panel A. Neuronal spiking in response to iontophoresed NMDA and its computed time histogram, using 1s bin width, of this activity are shown in panels B and C, respectively.

munities Council Directive of 24 November 1986 (86/609/EEC) were followed. The animals were killed by exposure to intravenously applied lethal amount of chloral hydrate.

The level of anesthesia was monitored by tail-flick test (applying noxious pressure to the tail by thumb) and by pupilla reflex (using a drop of physiological saline) throughout the experiment. The venous inflow of anesthetic (2-3 ml/h using 8 mg/ml chloral hydrate in physiological saline) was adjusted correspondingly so that the tail-flick test was just observable.

Extracellular recording and microiontophoresis

Single-unit activity was recorded extracellularly from the hippocampal CA1 neurons by means of a low-impedance (<1 MΩ) 7 µm carbon fiber containing combined recording and iontophoresis microelectrodes (Figs. 1 and 2) (Budai et al. 2007; Budai 2010). Recordings were commenced at least 1 h after surgery from a depth of about 2.1 mm from brain surface. The action potentials (spikes) were amplified using an ExAmp-20K amplifier (Kation Scientific, Minneapolis, MN), filtered, (Budai, 2004) and then monitored using an oscilloscope. A window discriminator (WD-2, Dagan, Minneapolis, MN) was used for spike discrimination. The amplified signals were sampled and digitalized at 80 kHz. The number of action potentials per second was counted by the computer and peristimulus time histograms were calculated using 1 s bin width (for example, see Fig. 2, panel C), displayed in line and digitally stored for off-line analysis.

Iontophoretic drug delivery and experimental data collection were controlled by a PCI-6221 multifunction instrument control and data acquisition board (National Instruments, Austin, TX) placed in a desktop computer and programmed in a LabView environment. Retention and ejection currents were generated using Union-40 iontophoresis pumps (Kation Scientific, Minneapolis, MN).

N-Methyl-D-aspartic acid (NMDA) and (*RS*)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid hydrobromide (AMPA) solutions was made at respective concentrations of 25 mM and 10 mM in 100 mM NaCl solution. Their pHs were adjusted to 8.5 using NaOH. Both compounds were purchased from Tocris (Bristol, UK). Trifuoperazine (TFP) was purchased from Sigma (St. Louis, MO) and was dissolved in 100 mM NaCl in a final concentration of 50 mM. Pontamine sky blue dye was from Sigma and was used at 2% in 100 mM NaCl. Microampere iontophoresis was performed using a BAB-501 iontophoresis pump (Kation Scientific, Minneapolis, MN).

NMDA and AMPA were ejected alternately from the multi-barrel electrodes for 5 s in every 2 min using negative currents ranging from 10 to 40 nA. TFP was ejected using positive current of 100 nA. Currents of about 20 nA of opposite polarity were used for all drugs to prevent leakage in between ejection periods. Only cells showing no or a very low basal activity (a few spikes/s at the most) were selected for recording. The control NMDA or AMPA receptor responses were set between 30 and 60 spikes/s.

Recording sites were marked by ejection of pontamine sky blue using 3 µA negative current for 20 min. At the end of the experiment, animals were euthanized with an overdose of chloral hydrate; the brain was removed and immersed in
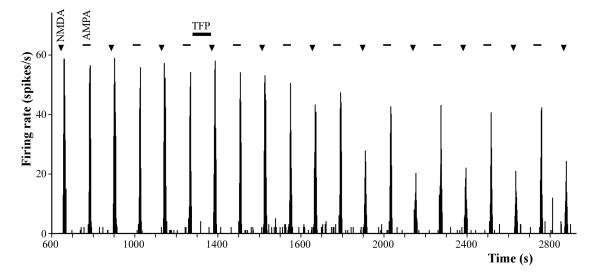
Figure 3. Trifluoperazine (TFP) differentially inhibits NMDA-, and AMPA-induced responses of hippocampal CA1 pyramidal cells. NMDA and AMPA were iontophoresed, alternately, 2 min apart using -45 nA and -20 nA, respectively. TFP was ejected as shown using 120 nA which markedly inhibited responses to either excitatory amino acid analogues. Note the differential effects of TFP on NMDA- versus AMPA-evoked responses. A representative time histogram recording is shown.

10% formalin. Recording locations were histologically verified in 60-μm thin sections counterstained with neutral red. Positions of the Pontamine sky blue marks were established with the stereotaxic atlas of Paxinos and Watson (1998). For example, see Figure 1.

Statistical analysis

Statistical evaluations were performed by using the total number of spikes evoked during each excitation epoch by iontophoretic application of NMDA or AMPA. The background neuronal discharge (or spontaneous activity) was calculated by averaging a 15 s period of ongoing activity preceding and following each excitation epoch, and this value was subtracted from all evoked responses. The total spike number during each excitation epoch was calculated and (Fig. 2, panel C) expressed as a percentage of mean (± S.D.) of the control and compared statistically with the data obtained after iontophoretic TFP application using one-way analysis of variance (ANOVA, with the Bonferroni test for *post hoc* analysis). A *P* value of <0.05 was considered as significant change. Statistical calculations were performed using a SigmaStat software program.

Results

Pyramidal cells in the hippocampal CA1 region were identified on basis of stereotactic coordinates and typical complex spiking patterns (Fig. 2, panel A) (Kandel and Spencer 1961; Mancillas et al. 1986; Henze et al. 2000; Huang et al. 2010). After finding an adequate neuron, NMDA or AMPA were alternately ejected 2 min apart from one another to evoke

cellular firing using ejections currents sufficient to produce a peak firing rate of 30 to 60 spikes/s. Following the establishment of 3 or 4 pairs stable control responses, TFP was iontophoretically delivered using 100 nA for 30 s in between two excitation periods. Responses to NMDA or AMPA were expressed in peristimulus time histograms (Fig. 3) and were quantified by counting the total number of spikes per stimulation period after subtracting the background averaged activity (if any) preceding and following each excitation epoch. Changes in the total number of spikes in NMDA or AMPA responses after TFP application were expressed as percent of their respective controls and pooled over all experiments. At the end of the experiment, recording sites were marked by pontamine sky blue ejection and were verified by histological means (Fig. 1, panel C).

A total of 12 successful experiments were performed in the same number of animals. Adjusting ejection currents for NMDA or AMPA excitation to reach comparable peak heights for both (as seen in Fig. 3) proved to be rather difficult as changing the stimulation intensity of one classes of receptors greatly influenced the responsiveness of the other (data not shown). Iontophoretic application of TFP significantly decreased responses to both NMDA and AMPA (Fig. 3) in 8 of the total 12 pyramidal cells. Maximal inhibition of NMDA- or AMPA-evoked responses were reached 10–16 min after TFP application and remained so during the rest of the approximately 1-h long experiment. Responses to NMDA were decreased to $32 \pm 15\%$ of control whereas AMPA responses were decreased to $74 \pm 19\%$ (mean ± SD, n=8 for both) of control. Both decreases were significant at $p<0.05$ and $p<0.01$ level, respectively. The ANOVA procedure with the Bonfer-
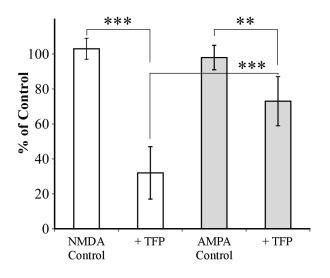
Figure 4. Summary of the effects of trifluoperazine (TFP) on the responses to alternately iontophoresed NMDA and AMPA of hippocampal CA1 pyramidal cells. Note the decrease of the responses in the presence of TFP. Data represent the mean ± SD of 8 experiments. Asterisks denote significant differences by ANOVA (**p< 0.05, ***p< 0.01) as compared to their respective controls or the significant difference between the decrease of NMDA- and AMPA- evoked responses in the presence of TFP. See details in the text.

roni test for *post hoc* analysis revealed a significant difference between the inhibition by TFP of NMDA and AMPA responses. The levels of significance was $p<0.01$ (Fig. 4). The present findings indicate that these two classes of ionotropic glutamate receptors may function in some form of cooperation through a Ca^{2+}/calmodulin-dependent way.

Discussion

We have shown that TFP could significantly decrease responses of hippocampal CA1 pyramidal cells *in vivo* to alternately iontophoresed NMDA and AMPA as compared to their respective controls. Also, inhibition of NMDA responses was significantly greater in the presence of TFP than that of the AMPA responses. Possible explanations for these effects include: (1) TFP prevents the action of tonically released dopamine, noradrenaline, serotonin or ACh (for reviews see (Vizi and Kiss 1998; Lendvai and Vizi 2008)) and/or (2) by binding to calmodulin and protein kinase C (PKC), TFP interferes with the phosphorylation or dephosphorylation of the corresponding ion channels. The former case of a major TFP action mediated through cell surface receptors specialized for a variety of endogenous neurotransmitters is unlikely in the views of most investigators, including ours.

Our observation that selective stimulation of the NMDA-class of the ionotropic glutamate receptors can in fact influence the responsiveness of the AMPA-class of receptors (and *vice versa*) strongly suggests *per se* that there must be an interaction between the function of the two classes of recep-

tors in the hippocampal CA1 pyramidal cells which express calmodulin in large quantities (Palfi et al. 1999, 2002). Most feasibly, this interaction is mediated through second messengers such as CaM K II and PKC. As for the effects of TFP, experimental data show that arginine vasopressin potentiation was blocked by 50 μM TFP, which is consistent with a Ca^{2+}/calmodulin involvement but which might also implicate PKC (Brinton and McEwen 1989). It has also been hypothesized that NMDA receptor activation is positively coupled to adenylyl cyclase *via* Ca^{2+}/calmodulin and are consistent with a role for cyclic AMP metabolism in the induction of NMDA receptor-dependent LTP in area CA1 of the hippocampus. In intact hippocampal slices, TFP blocked the increase in cyclic AMP levels caused by both NMDA application and stimulation of Schaffer collateral fibers. Exposure of hippocampal slices to elevated extracellular potassium to induce calcium influx also caused increased cyclic AMP levels; the increase in cyclic AMP caused by high potassium was also blocked by TFP. (Chetkovich and Sweatt 1993). Inhibition of calmodulin by calmidazolium also produced a differential inhibition of NMDA and KA stimulation of dorsal horn neurons of the spinal cord where NMDA-evoked responses were significantly more inhibited than KA ones (Olah et al. 2007).

Our present experiments may provide evidence *in vivo* for the inhibition by TFP of the second messenger cascade events leading from NMDA receptors *via* Ca^{2+}/calmodulin to AMPA receptors and, in consequence, for the blocking of phosphorylation of AMPA receptors and their sensitization. This blocking, in turn, prevents he AMPA receptors to enable NMDA receptors to become more sensitive to presynaptic glutamate release (or to iontophoretic NMDA ejection in our case) through phosphorylation enzyme activity. In fact, NMDA receptors became less sensitive to ejected NMDA in our experiments after application of TFP and they became more inhibited than AMPA receptors under the same conditions. Based on our experimental data, it is also likely that the function of NMDA receptors itself is, at least in part, dependent on the Ca^{2+}/calmodulin-mediated events.

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