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7-Methoxyderivative of tacrine is a 'foot-in-the-door' open-channel blocker of GluN1/GluN2 and GluN1/GluN3 NMDA receptors with neuroprotective activity *in vivo*

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HIGHLIGHTS

- 7-MEOTA is a potent "foot-in-the-door" open-channel blocker of NMDA receptors.
- 7-MEOTA potently inhibits GluN1/GluN2A-M817V receptors with a pathogenic mutation.
- 7-MEOTA exhibits neuroprotective activity in rats with NMDA-induced lesions.
- 7-MEOTA attenuates MK-801-induced pre-pulse inhibition deficit in rats.

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ABSTRACT

N-methyl-D-aspartate receptors (NMDARs) are ionotropic glutamate receptors that mediate excitatory neurotransmission in the mammalian central nervous system (CNS), and their dysregulation results in the aetiology of many CNS syndromes. Several NMDAR modulators have been used successfully in clinical trials (including memantine) and NMDARs remain a promising pharmacological target for the treatment of CNS syndromes. 1,2,3,4-Tetrahydro-9-aminoacridine (tacrine; THA) was the first approved drug for Alzheimer's disease (AD) treatment. 7-methoxyderivative of THA (7-MEOTA) is less toxic and showed promising results in patients with tardive dyskinesia. We employed electrophysiological recordings in HEK293 cells and rat neurones to examine the mechanism of action of THA and 7-MEOTA at the NMDAR. We showed that both THA and 7-MEOTA are "foot-in-the-door" open-channel blockers of GluN1/GluN2 receptors and that 7-MEOTA is a more potent but slower blocker than THA. We found that the IC_{50} values for THA and 7-MEOTA exhibited the GluN1/GluN2A < GluN1/GluN2B < GluN1/GluN2C = GluN1/GluN2D relationship and that 7-MEOTA effectively inhibits human GluN1/GluN2A-M817V receptors that carry a pathogenic mutation. We also showed that 7-MEOTA is a "foot-in-the-door" open-channel blocker of GluN1/GluN3 receptors, although these receptors were not inhibited by memantine. In addition, the inhibitory potency of 7-MEOTA at synaptic and extrasynaptic hippocampal NMDARs was similar, and 7-MEOTA exhibited better neuroprotective activity when compared with THA and memantine in rats with NMDA-induced lesions of the hippocampus. Finally, intraperitoneal

Abbreviations used: AD, Alzheimer's disease; AMPA, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid; ANOVA, Analysis of Variance; CAS, Czech Academy of Science; CGC, cerebellar granule cell; CNS, central nervous system; CTZ, cyclothiazide; D-AP5, D(−)-2-amino-5-phosphonopentanoic acid; DCKA, 5,7-dichlorokynurenic acid; DGh, granular cell layer of dentate gyrus higher part; DGl, granular cell layer of dentate gyrus lower part; EPSC, excitatory postsynaptic current; FBS, foetal bovine serum; FDA, US Food and Drug Administration; FJB, Fluoro-jade B; HEK293, human embryonic kidney 293 cells; IC_{50}, the half maximal inhibitory concentration; M, membrane domain; NMDA, *N*-methyl-D-aspartate; NMDARs, *N*-methyl-D-aspartate receptors; P, postnatal; PPI, pre-pulse inhibition; SEM, standard error of the mean; THA, 1,2,3,4-tetrahydro-9-aminoacridine (tacrine); TTX, tetrodotoxin; 7-MEOTA, 7-methoxytacrine

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administration of 7-MEOTA attenuated MK-801-induced hyperlocomotion and pre-pulse inhibition deficit in rats. We conclude that 7-MEOTA may be considered for the treatment of diseases associated with the dysfunction of NMDARs.

1. Introduction

N-methyl-D-aspartate receptors (NMDARs) are a subclass of glutamate receptors that play an essential role in mediating excitatory neurotransmission and synaptic plasticity in the mammalian central nervous system (CNS) (Horak et al., 2014; Traynelis et al., 2010). NMDARs have also been implicated in the pathology of many neuropsychiatric disorders and conditions, often associated with their excessive activation, known as "excitotoxicity". For example, excitotoxicity has been associated with the pathophysiology of focal brain ischemia (Lai et al., 2014), traumatic brain injury (Mehta et al., 2013), Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, multiple sclerosis, glaucoma, amyotrophic lateral sclerosis, epilepsy, anxiety, depression, bipolar disorder, schizophrenia, and lupus erythematosus (Lau and Zukin, 2007; Paoletti et al., 2013). Thus, NMDARs are an important target for the treatment of many CNS syndromes and the development of novel compounds with known molecular mechanisms of action is essential for designing effective therapies.

Most NMDARs are heterotetramers composed of the GluN1 and GluN2A-D subunits (GluN1/GluN2 type) which are activated by agonists of the glutamate binding site within the GluN2 subunit, and by co-agonists of the glycine-binding site within the GluN1 subunit (Traynelis et al., 2010). The minor NMDAR type composed of the GluN1 and GluN3A-B subunits (GluN1/GluN3 type) binds only agonists of glycine binding sites within both the GluN1 and GluN3 subunits. All GluN subunits share the same membrane topology, with four membrane domains (M1 - M4), an extracellular *N*-terminus and loops between M3 and M4, an ion channel pore formed by M2 and M3, and an intracellular C-terminus. Different regions of the GluN subunits are targets of a plethora of pharmacological compounds; in clinical studies, some of these compounds have been used successfully for the alleviation of the symptoms of various neuropsychiatric disorders. For example, an open-channel blocker of the GluN1/GluN2 receptor type, memantine, is an approved drug for AD treatment (Spilovska et al., 2016). Another open-channel blocker, ketamine, is used for the induction of dissociative analgesia and might be misused for its hallucinogenic properties (Wolff and Winstock, 2006) and has attracted recent interest as a fast-acting antidepressant (Serafini et al., 2014). Dizocilpine (MK-801) acts by a similar mechanism to memantine and ketamine and has been used as a pharmacological model of schizophrenia (Andine et al., 1999).

1,2,3,4-Tetrahydro-9-aminoacridine (tacrine; THA) was the first drug approved (by the US Food and Drug Administration (FDA) in 1993) for AD treatment, and was expected to act mostly *via* the cholinergic system. However, the mechanism seems to be more complex and probably involves more than the interaction with cholinesterases (Adem, 1992). THA was withdrawn from the market because of its hepatotoxicity and gastrointestinal side effects (Watkins et al., 1994). Interestingly, THA was also previously identified as a voltage-dependent inhibitor of native NMDARs, but with a relatively low affinity (Hershkowitz and Rogawski, 1991). However, the published IC_{50} values for THA inhibition at native NMDARs differ substantially between studies and THA effects at different types of the NMDAR have not been examined previously (Horak et al., 2017). 7-methoxyderivative of THA (7-MEOTA) was synthesised as a pharmacologically similar compound (Soukup et al., 2013). Interestingly, according to recently declassified reports, 7-MEOTA has succesfully passed the clinical trial stage I and stage II, with recommendation for the stage III. However, the stage II task focusing on the efficacy against the AD has never been accomplished due to change of the regime in the 1989 in former Czechoslovakia and the development of 7-MEOTA was discontinued. In the stage I, the compound has been found to be safe at repetitive dosing of 2 mg/kg p.o. and 1 mg/kg i.m. with oral bioavailability around 10% and reaching maximal plasmatic concentration around 1 μM after dose 8 mg/kg p.o. (Filip, 1988). The clinical stage II preliminarily proposed the efficacy in the treatment of tarditive dyskinesia and against organic psychosyndrome showing a potential benefit in mild impairment and mild improvement in recent memory, psychic condition (Sram, 1990). Notably, no hepatotoxicity was observed in those trials. likely due to a different metabolic pathway that avoids the production of toxic quinon methide (Patocka et al., 2008); therefore it may be considered for future pharmacological interventions against human neuropsychiatric disorders.

In this study, we aimed to examine the inhibitory effect of THA and 7-MEOTA at different NMDAR types expressed in HEK293 cells and in rat autaptic hippocampal neurones and rat cerebellar granule cells (CGCs), using electrophysiology. Furthermore, we examined neuroprotective activity of 7-MEOTA in a model of NMDA-induced lesion in the rat dorsal hippocampus as well as the effect of 7-MEOTA on the hyperlocomotion or pre-pulse inhibition (PPI) deficit, both induced by the administration of MK-801 in rats. We conclude that 7-MEOTA is a promising compound for future treatment of human diseases associated with the dysfunction of NMDARs.

2. Materials and methods

2.1. Molecular biology

The cDNA vectors expressing rat versions of GluN1-1a, GluN1-4a, GluN2A-D and GluN3A-B subunits have been described previously (Horak et al., 2006; Smothers and Woodward, 2009; Vyklicky et al., 2015). Here, the GluN1-1a (GluN1) subunit was used in all experiments with the GluN2 subunits, the GluN1-4a-F484A (GluN1-F484A) and GluN1-4a-F484A-T518A (GluN1-F484A-T518A) subunits were co-expressed with the GluN3 subunits. Human versions of the GluN1-1a and GluN2A subunits were generously provided by Prof. Stephen F. Traynelis (Hedegaard et al., 2012). PCR-based site-directed mutagenesis was performed using an established approach and the DNA constructs were verified by DNA sequencing (Kaniakova et al., 2012).

2.2. Mammalian cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were maintained in Opti-MEM I media containing 5% foetal bovine serum (FBS; v/v) and transfected with cDNA constructs carrying the GluN subunits and green fluorescent protein using Lipofectamine 2000 (Thermo Fisher Scientific) as described previously (Kaniakova et al., 2016). Cells were then trypsinised, re-suspended in Opti-MEM I containing 1% FBS, 20 mM $MgCl_2$, 1 mM D,L-2-amino-5-phosphonopentanoic acid, and 3 mM kynurenic acid (to inhibit excessive activation of NMDARs during cell culturing) and plated on poly-L-lysine-coated glass coverslips. Experiments were performed within 24–48 h of transfection.

2.3. Preparation of primary autaptic hippocampal neurones and cerebellar granule cells

Autaptic cultures of hippocampal neurones were prepared as described previously (Burgalossi et al., 2012). In brief, glass coverslips were rinsed in 1 M HCl and 70% ethanol, transferred to 6-well plates, coated with 0.15% agarose solution (w/v), and sterilised under UV