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Effects of memantine and donepezil on cortical and hippocampal acetylcholine levels and object recognition memory in rats

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ABSTRACT

This preclinical study investigated the ability of memantine (MEM) to stimulate brain acetylcholine (ACh) release, potentially acting synergistically with donepezil (DON, an acetylcholinesterase inhibitor). Acute systemic administration of either MEM or DON to anesthetized rats caused dose-dependent increases of ACh levels in neocortex and hippocampus, and the combination of MEM (5 mg/kg) and DON (0.5 mg/kg) produced significantly greater increases than either drug alone. To determine whether ACh release correlated with cognitive improvement, rats with partial fimbria-fornix (FF) lesions were treated with acute or chronic MEM or DON. Acute MEM treatment significantly elevated baseline hippocampal ACh release but did not significantly improve task performance on a delayed non-match-to-sample (DNMS) task, whereas chronic MEM treatment significantly improved DNMS performance but only marginally elevated baseline ACh levels. Acute or chronic treatment with DON (in the presence of neostigmine to allow ACh collection) did not significantly improve DNMS performance or alter ACh release. In order to investigate the effect of adding MEM to ongoing DON therapy, lesioned rats pretreated with DON for 3 weeks were given a single intraperitoneal dose of MEM. MEM significantly elevated baseline hippocampal ACh levels, but did not significantly improve DNMS task scores compared to chronic DON-treated animals. These data indicate that MEM, in addition to acting as an NMDA receptor antagonist, can also augment ACh release; however, in this preclinical model, increased ACh levels did not directly correlate with improved cognitive performance.

1. Introduction

Memantine (MEM), an uncompetitive antagonist (open-channel blocker) of N-methyl-D-aspartate (NMDA) receptors, is used clinically for the treatment of Alzheimer's disease (AD). In several randomized, placebo-controlled clinical trials involving patients with moderate to severe AD, MEM has been shown to provide global, cognitive, functional, and behavioral benefits (Reisberg et al., 2003; Tariot et al., 2004; Winblad and Poritis, 1999). Because MEM acts via a mechanism that does not involve inhibition of acetylcholinesterase (AChE) it is commonly administered in combination with AChE inhibitors (AChEIs) in patients who are in moderate to severe stages of the disease. Support for this practice comes from two large randomized, placebo-controlled studies in patients with moderate to severe AD, which demonstrated that

Abbreviations: Aβ, amyloid-β; ACh, acetylcholine; AChE, acetylcholinesterase; AChEIs, AChE inhibitors; aCSF, artificial cerebrospinal fluid; AD, Alzheimer's disease; ANOVA, analysis of variance; DNMS, delayed non-match to sample; DON, donepezil; FF, fimbria-fornix; i.p., intraperitoneal; LC–MS/MS, liquid chromatography–mass spectrometry; KW, Kruskal–Wallis; MEM, memantine; NMDA, N-methyl-D-aspartate.

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adding MEM to a stable AChEI regimen resulted in significantly better outcomes than AChEIs alone on multiple clinical measures (Grossberg et al., 2008; Tariot et al., 2004).

Despite the favorable clinical outcomes of such studies, relatively a little progress has been made in addressing the mechanism behind this combined effect. *In vitro* measurements suggest that the combined effect does not likely occur on the level of AChE inhibition, since MEM has been shown to have no effect on the activities of the AChEIs donepezil (DON) and galantamine (Wenk et al., 2000). Although MEM and DON have independently demonstrated efficacy in improving memory in animal models, no preclinical study has thus far shown a cooperative effect from the co-administration of both drugs (Wise and Lichtman, 2007; Yamada et al., 2005).

One mechanism by which MEM could clinically augment the effect of DON is through the enhancement of acetylcholine (ACh) release, an effect that would lead to increased synaptic concentrations of ACh and could therefore potentiate the effect of AChEIs. Systemic or intracerebroventricular administration of high-affinity NMDA receptor antagonists, such as MK-801 (Hasegawa et al., 1993) and CPP (Giovannini et al., 1994, 1997; Hutson and Hogg, 1996), has previously been shown to increase ACh release in several brain regions. This possible mechanism is supported by a microdialysis study in freely moving rats, which found increased ACh release in the ventral tegmental area and nucleus accumbens after acute subcutaneous injection of MEM at 20 mg/kg; however, this dose is several-fold higher than the concentration used clinically (Shearman et al., 2006).

In the present study, we tested whether administration of MEM at a clinically relevant dose enhanced ACh release in the hippocampus and cerebral cortex of anesthetized or freely moving rats. To ensure sufficient detection of changes in ACh levels and to equalize degradation of ACh between treatments, we used the AChEI neostigmine in the dialysate. This prevented us from seeing the already well-established increase in extracellular ACh levels by systemically administered AChEI DON, but allowed us to see potential interactions between MEM and DON on ACh release. We also assessed whether clinically relevant doses of MEM and DON, in combination, improved cognition in fimbria-fornix (FF)-lesioned rats in a short-term object recognition task. The model and the task were chosen based on previous evidence that FF-lesion in rats impairs short-term object recognition similarly to a local microinfusion of scopolamine into the hippocampus, whereas local microinfusion of physostigmine enhances object recognition (Hunsaker et al., 2007).

2. Materials and methods

2.1. Animals

A total of 89 male Wistar rats (225–350 g) were anesthetized and used in the initial microdialysis studies, while 61 male Wistar rats (32 weeks old; 400–550 g at the beginning of the experiment) were used for the behavioral study. Rats in the behavioral study were initially food-deprived until they reached 80–85% of their free-feeding weight, but were allowed access to fresh water *ad libitum*. Rats were housed individually in a controlled environment (illuminated from 7:00 AM–7:00 PM, temperature 22 ± 1 °C, humidity 50%–60%). The experiments were conducted according to the Declaration of Helsinki and the guidelines of the Council of Europe (Directive 86/609), the Finnish and the Canadian Councils of Animal Care and the NIH Guide for the Care and Use of Laboratory Animals, and approved by the State Provincial Office of Eastern Finland and by the Dalhousie University Committee on Laboratory Animals.

2.2. In vivo microdialysis in anesthetized rats

To test the effects of MEM and DON on ACh levels in the neocortex and hippocampus, we first administered the drugs in anesthetized rats using a single intraperitoneal (i.p.) injection, and monitored ACh levels using microdialysis probes implanted in each brain region. We then tested whether the drug effects occur at or near the cholinergic terminals by administering the drugs locally within the hippocampus, using reverse dialysis through the hippocampal microdialysis probe.

2.2.1. Surgery

Rats were anesthetized with urethane (Sigma–Aldrich, St. Louis, MO; 1.6 mg/kg, i.p.). This constant level of anesthesia over several hours provided a "behavioral clamp", which enabled a direct assessment of the drugs' effects on ACh levels. After placing the animal in a stereotaxic frame, a microdialysis probe (2 mm membrane length, 0.5 mm outer diameter, 20,000 MW cutoff; CMA, Sweden) was implanted into the left dorsal hippocampus (4.1 mm posterior to bregma, 2.5 mm lateral to midline, and 3.5 mm ventral to the cortical surface). For studies that used peripheral injections of MEM and DON, a second, identical probe was implanted into the left somatosensory cortex (1.3 posterior, 2.0 lateral, and 2.0 ventral).

2.2.2. Treatment

A total of 53 rats (6 or 7 animals/group) received i.p. drug injections of MEM (Forest Research Institute, Jersey City, NJ) at 2.5, 5, or 10 mg/kg, DON (Sigma–Aldrich, Saint Louis, MO) at 0.25, 0.5 or 1.0 mg/kg, or a combination of both drugs (MEM: 5 mg/kg; DON: 0.5 mg/kg). An additional 36 animals received direct hippocampal administration of the drugs dissolved in the perfusate (see below) via reverse microdialysis. Seven of these rats received a low concentration (1 μM) of one of the two drugs, which produced no change in ACh release. Consequently, the remaining 29 animals received a higher concentration (100 μM) of MEM alone, DON alone, or MEM plus DON.

2.2.3. In vivo microdialysis

For both systemic and local administration experiments, the microdialysis probes were perfused continuously at a flow rate of 2 μl/min with artificial cerebrospinal fluid (aCSF; 3 mM KCl, 125 mM NaCl, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$), to which 5 μM neostigmine (Sigma–Aldrich, Saint Louis, MO), an AChEI, was added to prevent ACh hydrolysis. After 1 h of stabilization, a series of ten 15-min microdialysis samples were collected. For peripheral administration, drugs were injected i.p. at the beginning of sample 6; control animals were given an injection of the vehicle (saline). For the reverse dialysis experiments, the dialysis solution was replaced throughout sample 6 with one containing MEM alone, DON alone, or MEM plus DON. The efficiency of the microdialysis probe was tested after each experiment by placing it in a standard solution of ACh (10 pM) for 15 min and measuring the ACh content appearing in the perfusate, to ensure that the *in vitro* recovery of the probe was greater than 5%. The dialysis samples were analyzed for ACh content using high performance liquid chromatography with electrochemical detection (Waters; Mississauga, Ontario, Canada), according to the procedure used by Materi and Semba (2001).

2.2.4. Data analysis and statistics

The mean ACh concentration measured in the first 2 samples was considered the baseline ACh level for each animal. The effect of each drug on spontaneous ACh release was determined using sample 6, immediately after peripheral drug injection, or during reverse dialysis. Statistical evaluation used the 5% level of significance. Student's *t*-test was used when appropriate, but when the assumption of equal variance between groups was not met, a nonparametric Kruskal–Wallis (KW) test was used.

2.3. In vivo dialysis in freely moving rats with fimbria-fornix lesions

2.3.1. Overview

The time course of experimental procedures is summarized in Fig. 1A. To investigate the cognitive effects associated with changes in ACh levels after drug treatment, a DNMS short-term object recognition memory task was developed (Fig. 1B; see below). The training phase continued for each animal until it reached a criterion of 80% or more correct choices on three consecutive testing days (pre-lesion performance), then sham or FF-lesioning was performed to impair afferent cholinergic innervation of the septal (dorsal) hippocampus. Chronic microdialysis probes were implanted to allow *in vivo* assessment of extracellular ACh levels during subsequent DNMS testing. The rats were divided into 5 treatment groups (Table 1), treated for three weeks with MEM, DON, or placebo, and DNMS task performance was again observed (DNMS Test 1; Fig. 1). Finally, rats that had been treated chronically with MEM were given an acute dose of DON, while DON-treated rats were given an acute dose of MEM, to test the effects of drug combinations on task performance (DNMS Test 2). After a 5-day washout of the acute treatment (while continuing chronic treatment), the microdialysis probes were connected to a collection device, and DNMS Tests 1 and 2 were repeated under the same conditions, to monitor ACh release during DNMS task performance.

2.3.2. Fimbria-fornix lesioning

After DNMS training (see below), each rat was randomly assigned to sustain either a sham lesion or a partial fimbria-fornix (FF) lesion (Table 1). The rats were anaesthetized with a 3:1 mixture (2 mL/kg, i.p.) of ketamine (Ketaminol®/Ketalar®, 50 mg/mL, Intervet International B.V, Boxmeer, the Netherlands) and medetomidine (Domitor®, 1 mg/mL, Orion Pharma, Turku, Finland). Each animal was then placed in