Hydromethylthionine enhancement of central cholinergic signalling is blocked by rivastigmine and memantine

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Abstract
The prevention of tau protein aggregations is a therapeutic goal for the treatment of Alzheimer’s disease (AD), and hydromethylthionine (HMT) (also known as leucomethylthioninium-mesylate [LMTM]), is a potent inhibitor of tau aggregation in vitro and in vivo. In two Phase 3 clinical trials in AD, HMT had greater pharmacological activity on clinical endpoints in patients not receiving approved symptomatic treatments for AD (acetylcholinesterase (AChE) inhibitors and/or memantine) despite different mechanisms of action. To investigate this drug interaction in an animal model, we used tau-transgenic L1 and wild-type NMRI mice treated with rivastigmine or memantine prior to adding HMT, and measured changes in hippocampal acetylcholine (ACh) by microdialysis. HMT given alone doubled hippocampal ACh levels in both mouse lines and increased stimulated ACh release induced by exploration of the open field or by infusion of scopolamine. Rivastigmine increased ACh release in both mouse lines, whereas memantine was more active in tau-transgenic L1 mice. Importantly, our study revealed a negative interaction between HMT and symptomatic AD drugs: the HMT effect was completely eliminated in mice that had been pre-treated with either rivastigmine or memantine. Rivastigmine was found to inhibit AChE, whereas HMT and memantine had no effects on AChE or on choline acetyltransferase (ChAT). The interactions observed in this study demonstrate that HMT enhances cholinergic activity in mouse brain by a mechanism of action unrelated to AChE inhibition. Our findings establish that the drug interaction that was first observed clinically has a neuropharmacological basis and is not restricted to animals with tau aggregation pathology. Given the importance of the cholinergic system for memory function, the potential for commonly used AD drugs to interfere with the treatment effects of disease-modifying drugs needs to be taken into account in the design of clinical trials.

KEYWORDS
acetylcholine, Alzheimer’s disease, hippocampus, hydromethylthionine, memantine, microdialysis, rivastigmine
1 | INTRODUCTION

Central cholinergic transmission via its predominantly excitatory neurotransmitter acetylcholine (ACh) is pivotal for memory formation and learning processes (Hasselmo, 2006; Sarter & Lustig, 2019; Thiele, 2013). Cholinergic innervation arising from basal forebrain, namely the medial septum, the diagonal band of Broca and the nucleus basalis of Meynert, projects directly into the neocortex and the limbic system, which includes the hippocampus and the entorhinal cortex (Mesulam & van Hoesen, 1976; Sakanaka et al., 1980). The latter brain areas are part of the Papez neuron circuit, which drives functional changes in hippocampal pyramidal cells and represents the structural basis for consolidation of memory (Vertes et al., 2001). Notably, signalling in the limbic system is not exclusively cholinergic, but is modulated by GABAergic interneurons and glutamatergic transmission (Bonanno et al., 1991; Giovannini et al., 1994).

The indispensable role of cholinergic signalling in memory processing is evident in Alzheimer’s disease (AD), which is characterised by memory loss and cognitive decline and is accompanied by a marked and progressive degeneration of cholinergic neurons (Arendt et al., 1999; Schliebs & Arendt, 2006; Zimmermann, 2020). The cholinergic innervation originating in the basal forebrain and projecting to cortico-limbic areas declines severely in AD (Mesulam, 2013; Vana et al., 2011; Whitehouse et al., 1981). Furthermore, autopsied brains of AD patients show a reduction in presynaptic markers, such as choline acetyltransferase (ChAT) activity, in the limbic system and cerebral cortex (Hampel et al., 2018; Mesulam, 2013; Pepeu & Grazia Giovannini, 2017). These findings implicate a loss of cholinergic terminals in those areas.

The rationale for treatments aiming to counteract the cholinergic deficit seen in AD by acetylcholinesterase (AChE) inhibition appears reasonable. The approved drugs, namely donepezil, galantamine and rivastigmine, are capable of reducing symptoms initially, but they have limited long-term therapeutic value (Kaduszkiewicz et al., 2017). Memantine, an NMDA receptor antagonist developed to restore and preserve cholinergic function in AD, is considered effective but pharmacologically limited (Bonanno et al., 1991; Giovannini et al., 1994).

Methylthioninium chloride (MTC, commonly known as methylene blue) was the first selective tau aggregation inhibitor in vitro (Wischik et al., 1996). MTC treatment was found to slow cognitive decline at a dose of 138 mg/day as monotherapy in a phase 2 trial in AD (Wischik et al., 2015). A reduced form of this compound, hydromethylthioninium (HMT; the new International Non-Proprietary Name for leucomeethylthioninium, LMT), was tested in two phase 3 trials in mild to moderate AD treatment in which doses in the range 150–250 mg/day were compared with a low dose of 8 mg/day intended as an inactive control. There were no differences between high and low doses in either of the two trials (Gauthier et al., 2016; Wilcock et al., 2018). The lack of dose-dependent difference has since been explained by the fact that, in contrast to MTC, 8 mg/day is the minimum effective dose of HMT and there is a response plateau at higher levels of exposure (Schelter et al., 2019). Highly significant differences were seen in both trials between patients receiving HMT as monotherapy and those receiving HMT as an add-on to standard symptomatic treatments (Gauthier et al., 2016; Wilcock et al., 2018). In the present study, we have investigated how prototypic symptomatic drugs (rivastigmine and memantine) modify cholinergic signalling responses to HMT in murine hippocampus. We have used microdialysis measurements of ACh levels and measurements of AChE and choline acetyltransferase (ChAT) activities to compare responses to HMT and symptomatic drugs alone and in combination in wild-type and tau transgenic mice expressing the core tau unit of the PHF (Melis, Zabke, et al., 2015).

2 | METHODS

2.1 | Animal model

The transgenic line 1 (L1) mouse expresses a truncated core PHF-tau fragment that consists of tau296–390 from the longest human CNS tau isoform htau40 on an NMRI background. Under control
of the Thy1 regulatory element, expression of the transgene leads to the accumulation of oligomeric tau in the absence of intracellular neurofibrillary tangles (Melis, Zabke, et al., 2015). In mice up to 6 months of age the tau protein aggregates were found in hippocampus and entorhinal cortex. By 12–18 months of age, tau aggregates spread to further cortical structures, such as visual and retrosplenial cortex. The neuroanatomical distribution and the progressive spreading of these aggregates occur in a pattern similar to the Braak staging of tau pathology in AD (Braak & Braak, 1991). Cognitive deficits were moderate in younger mice (up to 3 months) and increased with higher age (6 months); significant impairments in sensorimotor function were not observed (Melis, Zabke, et al., 2015). In the present study, we used mice at 6–8 months of age because tau pathology was fully developed at this age and learning deficits were significant (Melis, Magbagbeolu, et al., 2015; Melis, Zabke, et al., 2015).

2.2 | Animals and drug treatment

A total of 120 female mice (60 NMRI (RRID: IMSR_TAC:nmri) and 60 L1, 6–8 months old, delivered by Charles River) were housed in groups of 5 or 6 in stock boxes prior to testing. Animals had access to food and water ad libitum and were kept under standard conditions (temperature 20–22°C, 50–65% relative humidity; 17–20 air changes per hour) and on a 12-h light/dark cycle (07:00 AM to 07:00 PM). After at least 1 week of habituation to the animal facility, mice were randomly assigned to study groups (10 per group) by using block randomisation (Latin square design). Based upon five or six animals per cage, mice were assigned to either five or six groups in the following order: ABCDE, BAECD, CDAEB, DEBAC, ECDBA or ABCDEF, BFDCBA, CDEFBa, DAECBF, ECDABF, and FEDABC. All animal procedures were carried out to minimise animal suffering in accordance with German and European law (EU directive 2010/63/EU). Exclusion criteria were death during anaesthesia, blocked microdialysis probes, probe leaking, or animals under severe pain. Three mice of different genetic background died because of respiratory failure during surgery, another nine had to be excluded because of probe leakage. Three mice were tested on day one but were excluded on day two because of blocked probes. The study was registered with the local authorities (RP Darmstadt; FR1011).

HMT was synthesised in-house; its chemical characterisation has been reported (Baddeley et al., 2015). HMT (TauRx Therapeutics Ltd.) was stored at 8°C. Immediately before administration it was dissolved in nitrogen-sparged water to minimise oxidation. Rivastigmine (Tocris Bioscience; Cat. 129101-54-8) and memantine (Tocris Bioscience; Cat. 41100-52-1) were diluted in aqua ad injectabilia prior to filling minipumps.

Figure 1 shows the different groups and therapy regimes. Rivastigmine (0.5 mg/kg/d) and memantine (1 mg/kg/d) were delivered via an osmotic minipump (ALZET® pump model 1004; DURECT) which was implanted 4 weeks before dialysis. After filling, the pump was placed subcutaneously in the back region and the small incisional wound was sealed with wound closure clips (FST). This procedure was sufficient to deliver the drug constantly for at least 28 days. The doses were selected according to a previous study in our lab (Deiana et al., 2009) or converted from human doses using a dose conversion routine (Nair & Jacob, 2016). Saline (0.2 ml/day) or HMT (5 mg/kg/day) were given daily by oral gavage for 2 weeks prior to the experiment. Drugs and chemicals of general use were supplied by Merck or Sigma at the highest purity available.

| N (NMRI | Line 1) | Week 1 | Week 2 | Week 3 | Week 4 | Excluded animals on
| Implantation day | MD day 1 | MD day 2 |
| 10 | 10 |  |  |  |  |  |
| 10 | 10 | Saline |  |  |  |  |
| 10 | 10 | HMT |  |  |  |  |
| 10 | 10 | Rivastigmine |  |  |  |  |
| 10 | 10 | + Saline |  |  |  |  |
| 10 | 10 | Memantine |  |  |  |  |
| 10 | 10 | + Saline |  |  |  |  |
| 10 | 10 | Memantine |  |  |  |  |
| 10 | 10 | + HMT |  |  |  |  |

FIGURE 1 Administration regime for different drug treatments. Rivastigmine (0.5 mg/kg/d) and memantine (1 mg/kg/d) were administered using an osmotic mini-pump, which was implanted 28 days prior to the experiments. Saline (0.2 ml/kg/d) or HMT (5 mg/kg/d) were given daily by oral gavage in the last 2 weeks before the experiment, starting on day 15. Probe implantation was done on day 26, following two consecutive days of microdialysis experiments. For each group, a number of 10 animals was intended. The exact number of animals for each experiment is given in the respective figure description.
2.3 | Microdialysis

Prior to probe implantation mice were single housed in microdialysis cages in the experimental room. A habituation phase of at least 1 h was left before the surgery started. Self-built probes (Lietsche et al., 2014) were tested for leakage and recovery of ACh, which was found to be 12.2 ± 3.0% (N = 20). The microdialysis membrane (Filtral 12 AN69-HF; Hospal Industrie) had a molecular cut-off of 10 kDa and the exchange area was limited to 2 mm with silicon glue. By means of a stereotaxic apparatus (Stoelting), the probes were implanted into the ventral hippocampus under anaesthesia (isoflurane maintenance dose: 2.0–2.5%, Iso-Vet; Dechra Veterinary Products). The head was fixed, aligned with a tooth bar, and shaved. After sagittal skin incision and mechanical cleaning of the skull bone, a circular hole of 0.8 mm diameter was drilled at the following coordinates from Bregma: AP=2.7 mm; L=3.0 mm; DV=3.8 mm. After implantation, bupivacain 0.5% Jenapharm® (mibe) was applied and the probe was fixed to the skull with glass ionomer eluting cement (Micron® i-Cem; PrevestDenPro). After surgery, the mice were placed back into their home cages in the experimental room to recover having access to food and water ade libitum. A minimum of 18 h was kept between probe implantation and experimental start.

Microdialysis was performed on two consecutive days starting at 09:00 ± 01:00 AM and ending in the afternoon around 2 PM. Artificial cerebrospinal fluid (aCSF) served to perfuse the probes via a microdialysis pump (KD Scientific) and had the following composition: 147 mM NaCl, 2.7 mM KCl, 1.2 mM MgCl2, 1.2 mM CaCl2, 100 mM neostigmine (Acros Organics). Mice were briefly restrained for connecting the probe to the pump. Prior to sampling start, the microdialysate was discarded for 30 min. Then, the perfusion speed was set to 1 µL/min and samples were collected in microwials every 15 min. It should be noted that very low concentrations of neostigmine in the perfusate (as used in this study) cause a stabilisation of ACh release but do not prevent changes in ACh release, for example by therapeutic levels of AChE inhibitors (Chang et al., 2006; Erb et al., 2001).

On the first day, basal samples were collected while the animals stayed in their home cages for 90 min. The first two samples (collected after the start of the experiment) gave variable ACh levels and are not shown in Figures 3–6. Basal levels of ACh were calculated as averages from samples #3–#6 (30–90 min). Subsequently, mice were transferred into an open field box (45 × 32 × 20 cm). The animals were able to freely explore the new environment. A maximum of three animals was recorded in parallel in the open field. Open field boxes were cleaned carefully between animals. After 90 min, mice were placed back into their home cages. Sampling was continued for another 90 min to monitor post-interventional ACh release.

On day two, baseline levels were sampled again for 90 min in the home cage. Then, the perfusion fluid was switched to aCSF supplemented with scopolamine (1 µM) for 90 min. Finally, perfusion fluid was switched back to aCSF and samples were again collected for 90 min. After finishing microdialysis on day two, mice were anaesthetised with 5% isoflurane and decapitated. To confirm the implantation site on a random basis, some probes were perfused with the dye Fast Green (50 mM in aCSF; F7258) prior to sacrifice.

For the determination of cholinergic enzyme activities, the brain was harvested directly after decapitation. While kept on an ice-cooled petri dish, the cerebellum, the olfactory bulb and the right hemisphere were quickly removed and discarded, and the left hemisphere was weighed in a cooled potter vessel. Cold HEPES (10 mM)-sucrose (320 mM) buffer (pH 7.4) was added in a ratio of 1:10 (cortex: buffer), immediately followed by homogenisation (15 hits at 1500 rpm; Potter S. B. Braun). Aliquots of the resulting homogenate were frozen in liquid nitrogen and stored at −80°C until analysis.

2.4 | Measurement of acetylcholine

ACh was analysed using high-performance liquid chromatography (EICOM HTEC-500 HPLC + Shimadzu SIL-20AC autosampler) equipped with an electrochemical detector (ECD) sensitive to fmol amounts of ACh. The composition of the mobile phase was as follows: 50 mM KHCO3, 134.3 mM EDTA-2Na (BDH) and 1.64 mM sodium decane-1-sulfonate (Alfa Aesar) in HPLC grade water, pH 8.4. Flow rate was maintained at 150 µl/min.

After the compounds of the dialysate were separated on the column, ACh was cleaved to acetate and choline by AChE in an enzyme reactor, and choline was oxidized by choline oxidase. Resulting hydrogen peroxide was detected by the ECD. External standards were used to quantify the ACh concentration of the collected samples. Peaks were integrated with the software eDAQ PowerChrom (version 2.7.12).

2.5 | Cholinergic enzyme activities

AChE enzyme activity was determined following Ellman’s procedure (Ellman et al., 1961). Samples of the left hemisphere were thawed on ice and mixed with 5% Triton X-100 in PBS. Tetraisopropylpyrophosphoramide (iso-OMP) diluted in Ellman buffer (NaH2PO4/NaH2PO4, pH 8.0) was used at a final concentration of 100 µM to inhibit butyrylcholinesterase. After centrifugation (12 000 g, 4°C, 10 min), the supernatant was used for enzyme activity and protein determination. A standard was prepared using AChE from Electrophorus electricus.

The activity of ChAT was determined by formation of [3H]ACh from [3H]acetyl-Coenzyme A (specific activity: 200 mCi/mmol; Biotrend Chemikalien) and choline chloride (Fonnun, 1969). Supernatant, as described above, was mixed with the following components (final concentrations): NaCl (0.3 M), EDTA-Na (0.02 M), Na2PO4 (0.05 M; pH 7.4), Triton X-100 (0.5%), neostigmine (1 mM) and choline chloride (2 mM). The mixture was incubated at 37°C for 5 min, then [3H]acetyl-CoA (0.4 µCi) was added and incubated for 10 min. In ice-cooled PBS buffer, [3H]ACh was extracted with 0.5% Na-tetraphenylborate in toluene/acetonitrile (85%/15%).
Protein concentrations were determined according to Bradford (Bradford, 1976), using albumin fraction V 96% as standard.

2.6 | Data analysis and statistics

This is an exploratory study using absolute ACh values and cholinergic enzyme activities as outcomes. Both, ACh analysis and measurements of cholinergic enzyme activities, were performed by an investigator who was blinded to the study groups. Apart from that, no blinding was performed. Normal distribution was tested using the Kolmogorov–Smirnov test. No outliers were identified using the Grubbs test. Since time courses of ACh release analysis with Bonferroni’s post-test. Since time courses of ACh release analysis with Bonferroni’s post-test.

The sample size was calculated by the formula \( N = 2 SD^2 \times \text{power index}/\text{delta}^2 \). Based on many years of experience, an SD of 20% was expected and a treatment effect of 25% was defined as goal of the study. The value for the power index (\( \alpha = 0.05 \), two-sided; \( \beta = 0.2 \); 80%) was taken from the book Intuitive Statistics by Harvey Motulsky (Oxford University Press, 1995).

Basal levels of ACh (Figure 2) were analysed by one-way ANOVA with Bonferroni’s post-test. Since time courses of ACh release analysis with three-way ANOVA (genotype, treatment, time) did not yield any effects with the factor genotype, we compared data for each genotype by two-way ANOVA (genotype by treatment) followed by Bonferroni’s post-test. ACh values are shown as absolute values and given as mean ± SEM. Cholinergic enzyme activities were normalised to protein and given as mean ± SEM.

3 | RESULTS

The experiment was designed so that treatments were randomised for recording days leading to single cohorts for each drug (saline/HMT/rivastigmine alone, rivastigmine + HMT; memantine, memantine + HMT). A global statistical three-way analysis of variance (treatment/genotype/time) revealed no difference between L1 and WT controls, neither at baseline nor during open field or scopolamine exposure. Also, there was no genotype by treatment interaction indicating similar levels of ACh release in response to drug administration in WT and L1 mice. We, therefore, reasoned that a more meaningful analysis should address reactions to drug exposure within each genotype separately (with the caveat that saline and HMT groups are included repeatedly in the analyses).

3.1 | Basal levels of ACh

Recorded in their home cages over 90 min, the first six samples on day one were averaged to obtain basal ACh levels (Figure 2). The control group of NMRI mice had basal ACh values in the hippocampus of \( 8.8 \pm 1.1 \) nM (\( N = 10 \)). HMT treatment more than doubled mean basal ACh levels to \( 22.2 \pm 1.9 \) nM (\( N = 9 \); 252% of controls; \( p < 0.001 \)). Rivastigmine also led to a significant increase in basal ACh levels to \( 16.0 \pm 1.2 \) nM (\( N = 8 \); 182% of controls; \( p < 0.05 \)). Notably, the combination therapy—4 weeks of rivastigmine plus 2 weeks of HMT administration—produced only a non-significant increase in

![FIGURE 2](image-url) Basal ACh concentrations in the ventral hippocampus of wild-type (a + b) and tau-transgenic mice (c + d) on day one. Data are presented as means ± SEM and given as absolute values. Basal values are averages of the first six samples collected in the home cage prior to intervention on day one. Number of experiments (NMRI/L1): Saline (\( N = 10 \)|\( N = 10 \)), HMT (\( N = 9 \)|\( N = 8 \)), rivastigmine (\( N = 8 \)|\( N = 7 \)), rivastigmine + HMT (\( N = 10 \)|\( N = 10 \)), memantine (\( N = 10 \)|\( N = 10 \)) and memantine + HMT (\( N = 8 \)|\( N = 8 \)). Statistics (one-way ANOVA): (a) \( F_{3,36} = 10.79, p < 0.001 \). (b) \( F_{3,34} = 8.53, p < 0.001 \). (c) \( F_{3,34} = 8.80, p < 0.001 \). (d) \( F_{3,35} = 11.95, p < 0.001 \). Bonferroni’s post-test; asterisks over the error bar consider saline group as reference: *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \); \( N \) = number of animals
ACh to 14.4 ± 2.1 nM (N = 10; 164% of controls) (Figure 2a). In other words, pre-treatment with rivastigmine prevented the increase in basal ACh induced by HMT, but HMT also reduced the effect of rivastigmine on basal levels of ACh.

Long-term treatment with memantine had less effect on basal ACh levels than rivastigmine (Figure 2b). While HMT alone significantly increased basal ACh levels (see above), memantine did not (13.5 ± 2.6 nM; N = 10; 153% of controls; p > 0.05). Memantine pre-treatment blocked the ACh-enhancing effect of HMT, with ACh levels after memantine plus HMT significantly less than HMT alone (13.4 ± 1.5 nM; N = 8; p < 0.05).

The results in L1 mice, the tau-transgenic AD model, were comparable to those seen in wild-type mice (Figure 2c,d). The L1 control group had basal levels similar to wild-type mice (10.8 ± 1.3 nM; N = 10; t-test vs. wild-type: p > 0.2). HMT alone produced a highly significant increase in basal ACh values (24.8 ± 2.3 nM; N = 8; 230% of controls; p < 0.001). Treatment with rivastigmine alone also increased ACh levels significantly (19.7 ± 1.9 nM; N = 8; 182% of controls; p < 0.05), whereas the combination of rivastigmine and HMT significantly reduced ACh levels compared to HMT alone (15.3 ± 2.3 nM; N = 10; 142% of controls; p < 0.05). This again suggests an interference of rivastigmine on the response to HMT, but also an interference by HMT on the response to rivastigmine. Memantine did not significantly change extracellular ACh (13.6 ± 1.4 nM; N = 10; 126%; p > 0.05). When HMT was given to memantine-treated mice, ACh levels were only 14.2 ± 2.0 nM; N = 9; 131% of controls), a concentration that was significantly lower than HMT alone (p < 0.01) and not significantly different from saline-treated controls.

3.2 | ACh levels in the open field

Exposure to the open field triggered exploratory behaviour in mice and led to a clearly visible ACh release in the hippocampus as shown in Figures 3 and 4. In all treatment groups, exploration of the novel environment produced a 2- to 3-fold increase in ACh levels which returned to baseline following return to the home cage. We did not observe differences in the exploratory behaviour of either wild-type or L1 mice, and this is consistent with our previous observation that L1 mice have minimal motor deficits (Melis, Zabke, et al., 2015).

In saline-treated NMRI mice, ACh levels reached a maximum extracellular concentration of 27.3 ± 5.2 nM (equivalent to 310% of basal values) during exploration of the open field (Figure 3a). HMT treatment led to a significantly higher stimulated ACh output (F1,221: 13.14, p < 0.01); a maximum value of 53.7 ± 7.6 nM was recorded (Figure 3a). However, since the basal ACh levels were higher in HMT-treated mice the relative increase was equivalent to only 252% of basal levels. ACh levels in the rivastigmine group reached a maximum 39.2 ± 6.1 nM (245% of basal levels), exceeding the absolute values for the control treatment, but remaining lower than HMT alone. Combination of rivastigmine with HMT reached a maximum ACh level of 32.7 ± 4.6 nM in the open field (227% of basal values). Total ACh release remained significantly lower than in the HMT monotherapy group (F1,221: 8.90, p < 0.01) (Figure 3a).

Memantine alone did not significantly increase ACh levels (33.0 ± 5.5 nM; 244% of basal levels) (Figure 4a) when compared with saline controls. The combination of memantine plus HMT resulted in ACh values which were significantly lower than HMT alone (F1,195: 11.50, p < 0.01; maximum of 33.2 ± 3.7 nM) (Figure 4a).

Comparing wild-type NMRI to L1 mice, a two-way ANOVA did not reveal any differences on day one (open field) (F1,195: 0.19, p > 0.5; data from Figure 3a,b). Under open-field stimulation, 28.8 ± 4.8 nM was the peak concentration in L1 mice (267% of basal level) (Figure 3b). L1 mice showed robust responses to HMT given alone (maximum: 48.9 ± 5.1 nM; 197% of basal values; two-way ANOVA vs. saline-treated L1 mice: F1,195: 10.35, p < 0.01). The effect of rivastigmine in L1 mice was similar to HMT (maximum: 47.6 ± 7.5 nM; 242% of basal values; two-way ANOVA vs. saline-treated L1 mice: F1,195: 6.13, p < 0.05). However, the ACh increase under rivastigmine plus HMT was reduced to near-saline levels (maximum 34.5 ± 5.6 nM; 225% of basal values), and the time course of ACh release was not significantly different from saline-treated L1 mice (Figure 3b). L1 mice showed enhanced ACh levels under memantine monotherapy with a maximum of 39.3 ± 4.2 nM; 289% of basal levels (Figure 4b); this response was nominally higher than that seen in wild-type mice (Figure 4a), but not significantly different (see above for lack of overall genotype effect). Combined administration of memantine and HMT produced a similar time course in ACh response as in saline-treated L1 mice: ACh levels were significantly reduced relative to HMT alone (F1,195: 11.51, p < 0.01; maximum 31.7 ± 2.5 nM; 223% of basal values) (Figure 4b).

3.3 | ACh levels under scopolamine

On day 2 of the experiment, perfusion of the probe with scopolamine stimulated release of ACh (Figures 5 and 6). This response is because of a block of presynaptic M2-type muscarinic auto-receptors in hippocampus that confer negative feedback regulation of ACh release (Köppen et al., 1997), an effect which is enhanced when AChE is partially inhibited (Liu & Kato, 1994). Consequently, the infusion of scopolamine produced a five- to seven-fold stimulation of ACh release (Figures 5 and 6). As scopolamine is a highly lipophilic molecule, ACh levels return to baseline in a delayed fashion after the end of scopolamine infusion (Hartmann et al., 2010; Kopf et al., 2001). All groups of mice responded to scopolamine with strong increases of ACh release.

In saline-treated NMRI mice, ACh levels rose from 13.0 ± 2.7 nM to a maximum of 87.3 ± 24.7 nM during scopolamine perfusion (672% of basal values) (Figure 5a). NMRI mice receiving HMT or rivastigmine alone had higher basal levels than controls (27.0 ± 2.5 nM and 23.6 ± 3.6 nM, respectively) and significantly enhanced ACh levels during scopolamine perfusion (F1,122: 10.25, p < 0.01 and F1,208: 6.13, p < 0.05 vs. saline-treated NMRI mice, respectively). Maximum peaks of ACh were observed at 196.5 ± 33.5 nM for HMT (728%
of basal levels) and 167.9 ± 35.3 nM for rivastigmine (711% of basal values) (Figure 5a). Again, the combination of both drugs produced lower basal ACh levels (15.8 ± 2.3 nM), with a lower maximum release (70.6 ± 20.5 nM; 447% of basal values), and a significant decrease compared to either treatment alone (Figure 5a). Memantine-treated NMRI mice had a maximum of 119.6 ± 19.0 nM (595% of basal values) (Figure 6a). The combination of memantine and HMT produced basal levels of 16.8 ± 1.8 nM and a maximum ACh release of 95.5 ± 13.8 nM (568% of basal values); these values were not significantly different from those of saline-treated NMRI mice (Figure 6a).

Tau-transgenic L1 mice also responded with strong increases of ACh levels following scopolamine infusion, albeit at a somewhat lower level than NMRI mice. Although the maximum ACh level in saline-treated L1 mice was lower by 20% than in wild-type mice, statistical analysis of the ACh time courses showed no significance (two-way ANOVA: F_{1,224} < 0.001, p > 0.9). Scopolamine treatment in L1 mice raised basal ACh levels from 15.0 ± 1.8 nM to a maximum of 69.3 ± 15.9 nM (462% of basal values) (Figure 5b). Following HMT treatment alone, extracellular ACh was enhanced significantly versus saline-treated L1 mice (F_{1,195} = 16.51, p < 0.01); it increased from 30.5 ± 2.7 nM to 139.2 ± 24.8 nM (456% of basal values). A significant increase versus saline-treated L1 mice was also observed in the rivastigmine group (F_{1,195} = 7.12, p < 0.05); ACh basal levels started at 23.5 ± 3.9 nM and reached a maximum of 129.5 ± 19.2 nM (551% of basal values). Rivastigmine in combination with HMT produced basal ACh levels of 20.1 ± 2.2 nM, which increased to 108.1 ± 20.6 nM (538% of basal values) following scopolamine (Figure 5b). Interestingly, memantine alone was also able to
increase ACh release significantly in L1 mice ($F_{1,221} = 12.45, p < 0.01$),
starting from 21.9 ± 2.2 nM at baseline to a maximum ACh value of
132.6 ± 22.0 nM (605% of basal values) following scopolamine.
This increase is comparable to the increase produced by rivastigmine
(Figure 5b) or HMT (Figure 6b). By contrast, combination of
memantine with HMT produced a scopolamine response which was
significantly lower than HMT alone ($F_{1,156} = 10.42, p < 0.01$),
starting at 20.1 ± 2.5 nM and reaching a maximum of 87.6 ± 9.6 nM (436% of
basal values) (Figure 6b). It follows that treatment-dependent effects
on basal ACh levels are largely identical in L1 mice and NMRI mice,
whereas the relative increase induced by scopolamine is somewhat
lower in transgenic L1 mice.

### 3.4 Cholinergic enzyme activities

AChE activities and responses to drug treatments were similar in
both strains (Figure 7a). While AChE activity was slightly lower in
NMRI mice (408.1 ± 14.2 mU/mg) than in L1 mice (456.5 ± 18.6 mU/
mg; t-test: $p > 0.4$), significant changes in either strain were seen
only in animals receiving rivastigmine. In wild-type animals, riv-
astigmine significantly decreased AChE activity when given alone
(to 302.1 ± 14.5 mU/mg; 74% of controls) or in combination with
HMT (to 330.0 ± 20.4 mU/mg; 81% of controls). L1 mice responded
similarly, showing decreased AChE activity after rivastigmine
alone (326.6 ± 20.6 mU/mg; 72% of controls) and after the riv-
astigmine/HMT combination (373.6 ± 15; 82% of controls). AChE
ACh in hippocampus. As shown previously (Hartmann et al., 2010; Kopf et al., 2001), exploration of a novel environment causes an increase in hippocampal ACh levels by 2- to 3-fold, whereas pharmacological stimulation (by infusion of scopolamine) causes a strong, 5- to 7-fold increase in ACh (Hartmann et al., 2010; Kopf et al., 2001). L1 mice that express a truncated tau protein and develop tau pathology had similar hippocampal ACh levels as controls and were also able to enhance cholinergic tone during behavioural activation in the open field. However, their ACh levels remained somewhat lower during pharmacological stimulation which may indicate a slightly reduced cholinergic capacity during global neuronal stimulation. This reduction in central cholinergic tone is probably age-dependent and is reminiscent of a more pronounced central cholinergic dysfunction in aged P301L tau-transgenic mice that we investigated previously (Stein et al., 2019).

The focus of the present study was to investigate cholinergic actions of HMT, a novel anti-dementia drug that yielded beneficial results in drug-naïve patients in a clinical study of dementia (Gauthier et al., 2016; Schelter et al., 2019; Wilcock et al., 2018). Notably, HMT had less benefit when patients had been pre-treated with cholinesterase inhibitors and/or memantine. The main objective of the present study was, therefore, to determine whether a similar negative interaction occurs in wild-type mice and in a mouse model of Alzheimer’s disease, the tau-transgenic L1 mouse, using cholinergic activity as the outcome parameter. We used a drug treatment schedule in which we treated either with rivastigmine (0.5 mg/kg/day) or memantine (1 mg/kg/day) given by chronic systemic infusion, before HMT (5 mg/kg/d) was added for a further 2 weeks by oral gavage. The study was carried out in 6- to 8-month-old mice because cognitive deficits were already visible in L1 mice at this age (Melis, Zabke, et al., 2015).

In our hands, HMT had robust pro-cholinergic actions because it increased hippocampal ACh levels more strongly than the AChE inhibitor rivastigmine. The most interesting finding of the present study is that a negative interaction between HMT and rivastigmine or memantine, respectively, was observed in the tau-transgenic mouse model of AD. Administration of HMT in combination with either rivastigmine or memantine had less effect on ACh levels than HMT alone, and this surprising interaction was seen both in wild-type NMRI and in tau-transgenic L1 mice. Our finding in murine models exactly matches the picture seen in the completed clinical trials, that is pre-treatment with two established anti-dementia drugs reduced the beneficial clinical effects of LMT. In mice, HMT given alone caused a highly significant rise of ACh levels and rivastigmine (at the chosen dose) a more limited increase, while memantine only affected the ACh response in L1 mice. Intriguingly, the pronounced effect of HMT alone on ACh levels during exploration in the open field was completely prevented by rivastigmine or memantine co-administration in both strains (Figures 3 and 4). At the same time, the lesser increase in ACh levels induced by rivastigmine was also blocked by the combination with HMT. This...
is surprising and remains unexplained mechanistically. What appears certain is that while rivastigmine inhibited AChE, the strong increases in ACh levels after long-term HMT treatment are not the result of the same mechanism of action. Our preliminary evidence seems to suggest an indirect action via glutamatergic or other neurotransmitter (Cranston et al., in preparation), or more globally through actions on mitochondrial functions (see below) or synaptic protein levels and release mechanisms (Riedel et al., 2020). These alternative mechanisms would also be consistent with enhanced basal levels of ACh in hippocampus under HMT treatment in both genotypes despite a reduction in ChAT-positive neuronal labelling in the basal forebrain and AChE levels in cortex and hippocampus of L1 mice (Cranston et al., 2020).

Upon scopolamine infusion, disinhibition because of the blockade of hippocampal M2-autoreceptors produced ACh increases of up to 5- to 7-fold (Figures 5 and 6). It is notable that NMRI and L1 mice had comparable ACh values in the control condition. L1 mice appeared to have a lower cholinergic capacity under maximum stimulation (during perfusion with scopolamine), but again, the lack of overall genotype and genotype/treatment differences should be born in mind. During scopolamine perfusion, extracellular ACh concentrations reached 160–200 nM while maximum levels in L1 mice ranged lower (between 130 and 140 nM). This reduced ACh release was not accompanied by an overall reduction in ChAT activity in L1 mice. However, a recent study reported a reduction in ChAT-positive neuronal labelling in the basal forebrain of L1 mice (Cranston et al., 2020). While ChAT is not rate-limiting for ACh synthesis under basal conditions (Brandon et al., 2004), the high demand for ACh synthesis induced by scopolamine perfusion is consistent with an impairment in cholinergic function resulting from tau aggregation pathology.

The scopolamine response was strong in rivastigmine-treated wild-type mice, an observation that is likely because of the higher feedback inhibition during rivastigmine-induced AChE inhibition which enhanced the efficiency of scopolamine (Liu & Kato, 1994). L1 animals showed this effect to a lesser extent which might point to an altered presynaptic M2-receptor expression, as described before in post-mortem studies of AD patients (Mash et al., 1985), or to a diminished receptor response (Mohr et al., 2015). The fact that both strains respond to rivastigmine with enhanced ACh release, but only transgenic mice responded to the glutamate receptor antagonist memantine, suggests altered glutamatergic transmission in L1 animals, as reported previously (Riedel et al., 2020). Of note, interactions between tau pathology and cholinergic and glutamatergic signalling are highly complex, limiting the extent to which these can be elucidated at a cellular level using microdialysis.

The explanation for the negative interaction between HMT and symptomatic AD drugs remains to be established. It should be noted that MTC, which delivers the oxidised form of the methylthionium moiety, needs to be converted to HMT to permit efficient absorption and distribution to the brain (Baddeley et al., 2015). MTC inhibits AChE in micromolar concentrations in vitro (Augustinsson, 1950; Pfaffendorf et al., 1997). The present data clearly demonstrate that HMT (at the dose given) does not act as an AChE inhibitor in vivo since its action on ACh was counteracted by rivastigmine (Figures 3 and 5). This conclusion was corroborated by measurement of AChE activity in brain homogenates where HMT had no influence (Figure 7a). We conclude that the enhanced ACh levels measured after HMT alone cannot be explained by changes in the rate of enzymatic hydrolysis or of ACh synthesis (Figure 7b). Rather, enhanced cholinergic tone must be because of an effect of the drug on other neuronal systems.

Although the mechanism of the HMT-ACh interaction remains elusive, our data suggest that it is independent of cortical tau load since both HMT-mediated effects and their attenuation by pre-treatment with symptomatic AD drugs were similar in wild-type and in L1 mice.

As MTC is converted to HMT in vivo (Baddeley et al., 2015), several of the effects reported for MTC (Oz et al., 2011; Schirmer et al., 2011) may be relevant for HMT. Both MTC and HMT are effective inhibitors of tau aggregation in vitro (Akoury et al., 2013; Harrington et al., 2015; Wischik et al., 1996). Both MTC and HMT reduced the limbic load of tau in L1 mice (Melis, Magbagbeolu, et al., 2015), and HMT was found to be active in a mouse model of synucleinopathy (Schwab et al., 2017). The methylthionium moiety has a redox potential close to zero and is able to act as a supplementary electron carrier in the mitochondrial electron transport chain (Tretter et al., 2014) and has been shown to improve respiration in isolated mitochondria (Atamna et al., 2008; Harpey et al., 1986; Visarius et al., 1997). Therefore, one of the possible pathways whereby HMT may influence cholinergic neurons is by enhancing metabolism in synaptic mitochondria, a hypothesis that is a focus of our ongoing studies.

An alternative explanation may be an effect on neuronal systems that influence ACh release in hippocampus (Dazzi et al., 1995). Both GABA and glutamate are prominent regulators of hippocampal ACh release, either via direct input from the basal forebrain or through interneurons (Freund & Antal, 1988; Giovannini et al., 1994), and an effect of HMT on GABAergic or glutamatergic mechanisms has been described recently (Riedel et al., 2020). It follows that the action of HMT on the cholinergic systems may well involve interactions with a variety of targets.

In summary, HMT enhances cholinergic activity independently of AChE inhibition. Our findings demonstrate, in both a mouse model of AD and in wild-type mice, that the effects of HMT on cholinergic function are inhibited by pre-treatment with both a prototypic AChE inhibitor and a glutamate receptor modulator. Therefore, the interference by commonly used AD symptomatic drugs in the treatment effects of HMT observed in the AD clinical trials can be reproduced in mouse models. The results we report provide strong support for underlying neuropharmacological mechanisms as the explanation of the interaction. Therefore, clinically relevant interactions can occur in clinical trials even though HMT and symptomatic AD drugs have different mechanisms of action. This has broad-reaching implications for the design of clinical trials of novel disease-modifying drugs in AD.

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**CONFLICT OF INTEREST**

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**AUTHOR CONTRIBUTION**

C.K. performed the experimental work. G.R. and J.K. planned the study and supervised the work. C.R.H and C.M.W. provided input at the planning stage, secured funding contributed to the interpretation of the data and to the final manuscript. C.K., G.R. and J.K. wrote the manuscript. All authors reviewed the final submission.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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