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NO EVIDENCE FOR ROLE OF EXTRACELLULAR CHOLINE-ACETYLTRANSFERASE IN GENERATION OF GAMMA OSCILLATIONS IN RAT HIPPOCAMPAL SLICES *IN VITRO*

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Abstract—Acetylcholine (ACh) is well known to induce persistent γ-oscillations in the hippocampus when applied together with physostigmine, an inhibitor of the ACh degrading enzyme acetylcholinesterase (AChE). Here we report that physostigmine alone can also dose-dependently induce γ-oscillations in rat hippocampal slices. We hypothesized that this effect was due to the presence of choline in the extracellular space and that this choline is taken up into cholinergic fibers where it is converted to ACh by the enzyme choline-acetyltransferase (ChAT). Release of ACh from cholinergic fibers in turn may then induce γ-oscillations. We therefore tested the effects of the choline uptake inhibitor hemicholinium-3 (HC-3) on persistent γ-oscillations either induced by physostigmine alone or by co-application of ACh and physostigmine. We found that HC-3 itself did not induce γ-oscillations and also did not prevent physostigmine-induced γ-oscillation while washout of physostigmine and ACh-induced γ-oscillations was accelerated. It was recently reported that ChAT might also be present in the extracellular space (Vijayaraghavan et al., 2013). Here we show that the effect of physostigmine was prevented by the ChAT inhibitor (2-benzoylethyl)-trimethylammonium iodide (BETA) which could indicate extracellular synthesis of ACh. However, when we tested for effects of extracellularly applied acetyl-CoA, a substrate of ChAT for synthesis of ACh, physostigmine-induced γ-oscillations were attenuated. Together, these findings do not support the idea that ACh can be synthesized by an extracellularly located ChAT.

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Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; aCSF, artificial cerebrospinal fluid; BETA, (2-benzoylethyl)-trimethylammonium iodide; ChAT, choline-acetyltransferase; DMSO, dimethyl sulfoxide; HC-3, hemicholinium-3.

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INTRODUCTION

In the central nervous system acetylcholine (ACh) is critically involved in regulation of arousal, attention and rapid eye movement (REM) sleep (Metherate et al., 1987; Steriade et al., 1993). Elevated ACh levels during explorative behavior are likely to induce network oscillations in the theta (θ)- and γ-bands (Klinkenberg et al., 2011; Picciotto et al., 2012). These oscillations can be reproduced *in vitro* in hippocampal slices by application of cholinergic agonists such as carbachol (Fisahn et al., 1998; Weiss et al., 2003). Carbachol readily induces γ-oscillations in area CA3 in horizontal and parasagittal slices (Wójtowicz et al., 2009). While in longitudinal slices θ-oscillations emerge as reported also for kainate-induced oscillations (Gloveli et al., 2005a). In contrast to the basal ganglia and septum, cortex and hippocampus contain only few cholinergic neurons (Frotscher et al., 2000) whose function is presently unclear but which could contribute to cholinergic oscillations as suggested by the findings that both types of oscillations can coexist in hippocampal slice cultures (Fischer et al., 2002) without septal input. In the cortex, the main sources of ACh are terminals originating from the nucleus basalis (Johnston et al., 1979; Struble et al., 1986), and in the hippocampus, fibers which originate from the septum (Hasselmo and Bower, 1993). Stimulation of the nucleus basalis not only induces θ- and superimposed γ-oscillations (Lamour et al., 1986; McLin et al., 2003), but also leads to a rapid neurovascular response (Moro et al., 1995). Activation of the septum has similar effects in the hippocampus (Vandecasteele et al., 2014). ACh binds to both ionotropic nicotinic receptors, and metabotropic G protein-dependent muscarinic receptors. Nicotinic receptors containing α4 subunits found on hippocampal pyramidal cells show rapid desensitization (Liotta et al., 2011), while nicotinic receptors containing α7 subunits are typically expressed on parvalbumin-negative basket cells (Freund and Gulyás, 1997). Muscarinic receptors, on the other hand, are present in different types of neurons and can be blocked by atropine, and, in case of M1 receptors, by pirenzepine (Müller and Misgeld, 1989). ACh also exerts effects on non-neuronal non-excitable cells abundant in the brain, including microglia, astrocytes, oligodendrocytes,

endothelia, and vascular smooth muscle cells (Wessler and Kirkpatrick, 2008; Kawashima and Fujii, 2008).

ACh is synthesized from choline by choline-acetyltransferase (ChAT), an enzyme which transfers acetate from acetyl-CoA onto choline. This enzyme is considered to be localized inside cholinergic terminals and serves as a marker of cholinergic neurons (Léránth and Frotscher, 1987). After synthesis, ACh is transported into vesicles and eventually released. Recently it has been suggested that ChAT is also present in the extracellular space thus permitting synthesis of ACh outside neurons (Vijayaraghavan et al., 2013). In presence of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), ACh is very short-lived (Kaufer et al., 1999). AChE comes in different forms. The soluble read-through form is not anchored close to release sites, permitting wide-spread diffusion of ACh (Meshorer et al., 2002). Animals lacking AChE show a strong increase in extracellular ACh in the dorsal hippocampus which can be detected by microdialysis (Hartmann et al., 2008). These increased ACh levels are accompanied by decreased levels of extracellular choline. Addition of choline (10 μM) to the perfusion fluid, while ineffective in wild-type animals, more than doubles extracellular ACh levels in AChE-deficient mice suggesting that ACh synthesis is probably not saturated (Hartmann et al., 2008).

Additionally, it has been suggested that in brain slices choline originates also from the degradation of lipid membranes. Using Corning type tri- and tetra alkylated ammonia and K^+-sensitive electrodes in comparison to measurements with valinomycine-based K^+-selective microelectrodes suggested concentrations of about 10 μM choline in the extracellular space (Müller et al., 1988). We hypothesized that this source of choline is used for ACh synthesis after cellular uptake and thus may induce network oscillations, provided ACh degradation is inhibited.

In this study we tested for effects of physostigmine alone and physostigmine in combination with ACh on γ-oscillations in hippocampal slices. We found that physostigmine alone could dose-dependently induce γ-oscillations which were strongly augmented by ACh. Surprisingly, the choline uptake inhibitor hemicholinium-3 (HC-3) did not prevent generation of γ-oscillations by physostigmine but rather led to a slight augmentation suggesting that ACh synthesis can occur under conditions when choline transport is blocked.

To clarify whether additional ACh can be synthesized by extracellular ChAT we applied (2-benzoylethyl)-trimethylammonium iodide (BETA) which antagonizes the synthesis of ACh by blocking ChAT (Galea and Estrada, 1991; Chen et al., 1993). In another set of experiments we applied acetyl-CoA, a substrate for ChAT to form ACh. While we observed a (slight) reduction of physostigmine-induced γ-oscillations by BETA, there was no evidence for a facilitation of γ-oscillations in the presence of acetyl-CoA. Together these findings suggest that there is no evidence for an extracellularly located ChAT.

EXPERIMENTAL PROCEDURES

Slice preparation and solutions

Animal procedures were performed in accordance with the guidelines of the European Communities Council and approved by the State Office of Health and Social Affairs Berlin (Landesamt für Gesundheit und Soziales, LaGeSO, Berlin: T0096/02). Six to eight-week-old male Wistar rats were decapitated under deep isoflurane/laughing gas anesthesia. The brain was rapidly removed and then washed in ice-cold carbogenated (95% O_2–5% CO_2) artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 129, $NaHCO_3$ 21, KCl 3, $CaCl_2$ 1.6, $MgSO_4$ 1.8, NaH_2PO_4 1.25, and glucose 10. Horizontal hippocampal-entorhinal cortex slices, 400-μm thick, were prepared on a vibrating blade microtome (Leica Microsystems, Wetzlar, Germany) and immediately transferred to an interface chamber and perfused with carbogenated aCSF at 36 ± 0.5 °C (flow rate: ~1.8 ml/min, pH 7.4, osmolarity: 300 ± 3 mosmol/kg). Slices were left to recover for at least 2 h before recording was started.

Recordings

Extracellular field potentials (FP) were recorded under interface conditions with carbon-fiber electrodes in AC mode. Electrodes were placed in the pyramidal layer of area CA3. Potentials were amplified 200 times using a custom-made amplifier, low-pass filtered at 3 kHz, digitized at 10 kHz using Spike2 software, and stored on a computer disk for offline analysis (1401 interface, CED, Cambridge, UK).

Data analysis

Signals were analyzed off-line using custom programs written in Matlab (The MathWorks, Natick, MA, USA). We performed power spectrum analyses on 1-min data epochs collected during the last 5 min in a given series of measurements. We determined peak power, peak frequency and power between 30 and 90 Hz. In addition we analyzed the 40–10-Hz quotient in a given treatment termed γ–θ ratio. Finally, we did autocorrelation analysis of 5 min of data and used the distance of nearest peak to corroborate dominant frequency. By fitting an exponential to the different peaks in the autocorrelation function we determined inner coherence following Stenkamp et al. in their analysis on carbachol-induced γ-oscillations (Stenkamp et al., 2001). All numerical data are expressed as mean ± standard error. If data were normally distributed, statistical evaluation was performed by a one-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons to identify significant differences between conditions. Non-parametric tests (Kruskal–Wallis as well as Friedman) were used and followed by Dunn's multiple comparisons, when data were not normally distributed. *p*-Values less than 0.05 were considered to indicate a significant difference between means (indicated by asterisks and/or plusses).