Title: In vitro acetylcholine release is not a straightforward model to study hippocampal 5-HT4 receptors

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In vitro acetylcholine release is not a straightforward model to study hippocampal 5-HT₄ receptors

5-HT₄R activation in hippocampal brain slices

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Abstract

5-HT₄ receptor (5-HT₄R) activation induces pro-cognitive effects. This might be related to stimulation of hippocampal acetylcholine (ACh) release, which has been shown for 5-HT₄R agonists in in vivo models. We investigated the influence of the 5-HT₄R agonists prucalopride and BIMU-8 on ACh release in rat hippocampal brain slices. In contrast to the report by Siniscalchi et al. [1], no facilitating effect of 5-HT₄R agonists on electrically evoked ACh could be shown. The in our hands absence of an effect by 5-HT₄R agonists illustrates that the in vitro evaluation of 5-HT₄R agonists on hippocampal ACh release is not a straightforward model to study the relationship between hippocampal 5-HT₄Rs and hippocampal ACh release.

3-10 keywords or short phrases for cross-indexing

ACh release; Hippocampal brain slices; prucalopride; BIMU-8
Introduction

Pharmacological studies have shown that 5-HT₄ receptor (5-HT₄R) activation induces pro-cognitive effects and increases memory and learning [2-5]. 5-HT₄R have been shown to be present in the hippocampus [6,7]. The pro-cognitive effects of 5-HT₄R stimulation might be the result of the induction of LTP (long-term potentiation) as shown in the amygdala [8] and suggested in the hippocampus [9], where 5-HT₄R activation causes a long-lasting increase in excitability of CA1 pyramidal neurons [4,10,11]. On the other hand, the pro-cognitive effects of 5-HT₄R agonists might also be related to the increased acetylcholine (ACh) release, as shown in vivo in the hippocampus [4] and the frontal cortex [12] of conscious freely moving rats. The loss of hippocampal cholinergic markers is indeed one of the major neurochemical deficits in Alzheimer’s disease (AD), which correlates well with the loss of cognitive abilities [13], and actual treatment of AD patients is mainly with cholinesterase inhibitors [14]. In vitro, the facilitation of ACh release from guinea pig and rat hippocampal brain slices by 5-HT₄R activation has been described by Siniscalchi et al. [1] and this paper is regularly cited to suggest the presence of presynaptic 5-HT₄ heteroreceptors on hippocampal cholinergic nerve terminals [15,16] although no other reports on increased ACh release by 5-HT₄R agonists in hippocampal brain slices have been published. In view of the report of Siniscalchi et al. [1], this study investigated the influence of the highly selective 5-HT₄R agonist prucalopride on ACh release in rat hippocampal brain slices.
Materials and Methods

In most experiments male Wistar rats (200-300g) were used. To rule out possible species differences due to variations in 5-HT$_4$ receptor density, male Dunkin-Hartley guinea pigs (350-500g) were used in a series of experiments. Animals were housed under controlled conditions of temperature, humidity and light (12-h light/dark), and received food and water *ad libitum*. On the day of the experiment the animals were sacrificed by decapitation, under light CO$_2$ anesthesia, and the brain was quickly removed to ice-cold modified Krebs’ solution of the following composition (mM): NaCl 124.0, KCl 3.0, KH$_2$PO$_4$ 1.25, MgSO$_4$ 1.0, CaCl$_2$ 2.0, NaHCO$_3$ 26.0, ascorbic acid 0.3, Na$_2$EDTA 0.03, glucose 10.0 (pH 7.4) oxygenated with 95% O$_2$ and 5% CO$_2$. In experiments with guinea pig, the composition of the Krebs’ solution was as described by Siniscalchi [1]. The hippocampi were dissected from the cooled brain and coronal sections (rat: 350 µm; guinea pig 400 µm) were cut with a McIlwain tissue chopper to yield 16 dorsal hippocampal slices per animal. In a limited number of experiments, slices from rat were cut with a Microm HM 650V (Thermo Fisher Scientific, Waltham, MA) vibratome. To prepare the brain for cutting, the cerebellum and the frontal lobe were removed by a coronal section; the brain was then tilted on the frontal end to remove ventral-lateral areas (20°-30° off the horizontal axis). Finally, a medial sagittal cut was performed on the remaining tissue and the ventral lateral areas were glued onto the specimen holder of the vibratome. About eight horizontal 350 µm hippocampal slices were obtained per animal. Slices were loaded with [³H]-choline chloride (10 µCi/ml, 0.1 µM) in 2 ml Krebs’ solution for 45 min at 37°C. The slices were rinsed three times and transferred to 200 µl chambers (4 per chamber) in an automated perfusion system (Brandel SF-2518B, Brandel Inc., Gaithersburg, MD). Perfusion was
done with carbogen-saturated Krebs’ solution containing 2µM hemicholinium-3 at a flow rate of 1.2 ml/min. After 1 hour of perfusion, 3 min samples of the effluent were collected and assayed for tritium content as a measure for \[^3\text{H}\]-ACh. Electrical field stimulation (EFS) through platinum ring electrodes was applied twice at 15 and 45 min after the beginning of the collection period (S\(_1\), S\(_2\)): biphasic square wave pulses were delivered by an ES-218 (Brandel) stimulator at 50 mA, 1 ms, at a frequency of 2 Hz for 2 min (240 pulses). In guinea pig experiments, square wave pulses of alternating polarity were used. Drugs were added 15 min before S\(_2\). At the end of the experiments, tissues were homogenized by sonication in 0.5 ml of 10% trichloroacetic acid for 30 min and a 100 µl aliquot was used to determine the amount of tritium remaining in the tissue. The tritium content of the sample was assayed by adding a 0.5 ml aliquot of perfusate samples to 2 ml Ultima Gold scintillation fluid (Perkin Elmer, Waltham, MA) and measured by liquid scintillation counting (Tri-Carb 2100 TR, Perkin Elmer) for 5 min. Tritium outflow was expressed as fractional release, i.e. as a percentage of the total amount of tritium in the tissue at the time of sample collection. The uptake of tritium was determined as the sum of release plus tissue content of radioactivity at the end of the experiment. The release of tritium by EFS (stimulation-evoked release S\(_1\), S\(_2\)) was calculated by subtracting basal release, measured for 9 min during the immediate prestimulation period, from the release measured for 9 min from the start of the stimulation. The effect of the drugs on field stimulation-evoked tritium outflow was evaluated as the S\(_2\)/S\(_1\) ratio compared to the S\(_2\)/S\(_1\) ratio in control slices without drug addition.
Drugs: The following drugs were used: $[^3]$H-methyl-choline chloride (Perkin Elmer; specific activity 75.7 Ci/mmol); hemicholinium-3 bromide and atropine sulfate (Sigma-Aldrich, St-Louis, MO); 2-chloroadenosine (CADO; Fluka, Buchs, Switzerland); BIMU-8 (Tocris Cookson, Bristol, UK); prucalopride (Shire-Movetis, Turnhout, Belgium); tetrodotoxin (Serva, Heidelberg, Germany). All compounds were dissolved and diluted in distilled water.

Statistics: For statistical analysis, GraphPad Prism version 5.0 (GraphPad, San Diego, CA) was used to perform one-way ANOVA followed by Bonferroni’s post-hoc test. The data were expressed as means ± s.e.m. p<0.05 was considered significant in all tests.
Results

Fig. 1 illustrates the average tritium outflow from rat hippocampal slices obtained in control perfusion experiments. It can be seen that the basal release was stable and that the amount of electrically evoked tritium during the two episodes of electrical stimulation (S1 and S2) was similar yielding an average S2/S1 ratio of 1.09 ± 0.02 (n=14). The electrically evoked tritium outflow was completely abolished by 3 µM tetrodotoxin (n=4; data not shown) indicating its dependence on action potential propagation through sodium channels. The assay was validated by perfusion of atropine (1 µM) or CADO (10 µM) 15 min prior to S2. As shown in Fig 2., atropine produced a consistent increase (+37%) of the evoked tritium outflow, with the average S2/S1 ratio being 1.48 ± 0.03 (n=8) while perfusion of CADO produced a consistent decrease (-55%) of the evoked tritium outflow, with the average S2/S1 ratio being 0.49 ± 0.04 (n=6) versus an average S2/S1 ratio of 1.08 ± 0.03 (n=10) in control.

However, neither BIMU-8 (2 µM), the 5-HT4R agonist that increased the tritium outflow in the study of Siniscalchi [1] by 43% nor the selective 5-HT4R agonist prucalopride (1 µM) facilitated the ACh release from hippocampal brain slices (Figure 3). None of the drugs modified the basal tritium outflow.

Prucalopride did not affect the release of ACh at various perfusion times before S2 (24, 18, 6, 3 min) or at different concentrations (0.01 µM, 0.1 µM) (n=1-2 for each condition). The data set presented above was obtained using a total K+ concentration of 4.25 mM and a Ca2+ concentration of 2 mM in the Krebs solution; the effect of prucalopride has also been assessed in Krebs’ solution with high (5.9 mM) concentration of total K+ in combination with low (1.4 mM), intermediate (2 mM), or
high (2.5 mM) Ca\(^{2+}\) concentration (n = 1 for each condition). In addition, an experiment has been performed in the absence of EDTA and ascorbic acid. None of these conditions induced a facilitating effect of prucalopride on ACh release; neither did a different slicing method as similar S\(_2\)/S\(_1\) ratios were obtained with vibratome-made slices (control S\(_2\)/S\(_1\) ratio of 1.11 versus 1.12 with prucalopride; n=1).

The absence of an effect by prucalopride was confirmed in hippocampal slices from guinea pig for two stimulation intensities: standard stimulation intensity as used for rat slices (2 Hz, 50 mA, 1 ms) yielded an S\(_2\)/S\(_1\) ratio of 1.07 in control tissues versus 0.99 in tissues treated with prucalopride (n=1); lower stimulation intensity (0.5 Hz, 50 mA, 1 ms) led to an S\(_2\)/S\(_1\) ratio of 0.93 in controls versus 0.92 with prucalopride present (n=1).
Discussion

Our study confirmed that reproducible stimulation-induced release of ACh can be obtained from rat hippocampal slices [17-19]. In accordance with the literature the muscarinic receptor antagonist atropine increased and the mixed A1/A2a adenosine receptor agonist CADO decreased the evoked tritium outflow resulting from the blockade and stimulation of presynaptic inhibitory control on ACh release through muscarinic and A1 adenosine receptors, respectively [18,19]. In contrast with the report by Siniscalchi et al. [1], however, 5-HT4R activation did not modify the electrically-evoked tritium outflow from rat or guinea pig hippocampal brain slices in the present study. To rule out the possibility that this discrepancy was a result of the slightly different experimental conditions used, the buffer composition, stimulus intensity, agonist and agonist perfusion time of the study by Siniscalchi [1] were additionally tested but no condition was obtained where 5-HT4R agonists showed a facilitating effect on ACh release from the hippocampal brain slices.

Multiple variations in Krebs’ solution composition exist in the literature on rat hippocampal [3H]-ACh release [1,17-20]. Essentially total K+ concentration ranges from 3 to 6 mM and/or Ca2+ concentration ranges from 1.2 to 2.5 mM. Adaptation of the K+ and Ca2+ concentration of the Krebs solution did not induce a facilitating effect of prucalopride. The absence of an effect of the 5-HT4R agonists in the present study was thus not due to the buffer composition.

Multiple variations in stimulation parameters exist in studies on rat hippocampal [3H]-ACh release [1,17,19,20]. Square wave pulses (monophasic, biphasic or with alternating polarity) are used with typical pulse durations of 1 or 2 ms. Both voltage and current stimulation has been described with frequencies ranging from 0.5 Hz to 3 Hz and
stimulation durations from 2 to 4 min. As the intensity of nerve activation could indeed play an important role in the effectiveness of presynaptic receptors modulating transmitter release [21], the lowest stimulation intensity in terms of current and frequency yielding reproducible $S_2/S_1$ was used in the present study. The low stimulation intensity described by Siniscalchi et al. (30 mA/cm² at 0.5 Hz) reduced the ratio of evoked release to basal release in the experiments with rat tissue to such an extent that it impeded obtaining reproducible $S_2/S_1$; as in our experiments too, current stimulation was used, the delivered current should be identical in both setups. From the Siniscalchi data, however, the stability of the basal release and the ratio of evoked release to basal release cannot be appreciated as no graphical representation of the course of the release experiments was given. In experiments with guinea pig tissue, however, the ratio evoked release to basal release was higher than in experiments with rat tissue, which allowed stimulation at 0.5 Hz while preserving reproducible $S_2/S_1$. But still, this did not induce a facilitating effect of 5-HT₄R agonists.

In the study by Siniscalchi, the 5-HT₄R agonist BIMU-8 was added to the perfusion medium 3 min before $S_2$ to minimize the possibility of receptor desensitization [1]. As reducing the perfusion time or concentration of prucalopride before $S_2$ did not increase the $S_2/S_1$ ratio obtained, receptor desensitization can be excluded as possible cause of the absence of an effect of the 5-HT₄R agonists in the present study.

We have no clearcut explanation for the discrepancy between our study and this of Siniscalchi et al.. It can still be mentioned that we used Wistar rats and Dunkin-Hartley guinea-pigs while Siniscalchi et al. used Sprague-Dawley rats and a not specified guinea pig strain. As the effects observed by Siniscalchi et al. were observed in both species, strain-dependency seems an unlikely explanation. More important, however,
could be the procedure used to obtain hippocampal slices. Moreover, it is still unknown whether 5-HT₄ receptors that regulate ACh release within the hippocampus are located on cholinergic nerve terminals, cholinergic cell bodies or interneurons [16]. The absence of an effect of 5-HT₄R agonists in our study could be the result of the disruption of essential cholinergic projections. The present data and the failure of 5-HT₄R agonists to enhance K⁺-evoked ACh release from hippocampal synaptosomes [1] suggest that 5-HT₄ receptors are not located on the hippocampal cholinergic nerve terminals. A preterminal location of the 5-HT₄R modulating ACh release could indeed make its functionality prone to the exact slicing procedure used. Unfortunately, Siniscalchi did not specify which specific part of the hippocampus was sliced and which slicing plane was used, which impeded exact mimicking of their slicing conditions.
Conclusion

In rat and guinea pig hippocampal brain slices, no facilitating effect of the 5-HT₄R agonists BIMU-8 and prucalopride on electrically evoked ACh release could be shown, in contrast to earlier data. The *in vitro* evaluation of 5-HT₄R agonists on hippocampal ACh release is thus not a straightforward model to study the relationship between hippocampal 5-HT₄Rs and hippocampal ACh release.
Acknowledgements. We thank dr. B. Sperlágh and Mrs. C. Csölle for their expert technical advice in setting up ACh release experiments with brain slices, and Miss Choi Sze Men for technical assistance during the experiments. The study was supported by IWT and Movetis via IWT grant 070311.
Reference List


Figure 1

Tritium outflow in control slices. Mean ± s.e.m. (n=14).

Tritium outflow is expressed as a percentage of the tritium retained in the preparations at the time of the sample collection. The bars at $S_1$ and $S_2$ indicate the periods of EFS. In control experiments the evoked release of tritium remained constant during the consecutive stimulation periods, giving $S_2/S_1$ ratios close to unity.
Effect of atropine (1 µM) and 2-chloroadenosine (CADO; 10 µM) on the stimulation evoked release of $[^3\text{H}]-\text{ACh}$ from rat hippocampal slices. Drugs were added to the perfusion solution from 15 min before the second stimulation period ($S_2$) until one minute after $S_2$ was ended. The ratio between the fractional release of $[^3\text{H}]-\text{ACh}$ in response to the first ($S_1$) and second stimulation ($S_2$) was calculated and compared in the absence and the presence of drug. Mean $S_2/S_1$ ratio ± s.e.m. (n=6-10). Asterisks show significant differences from control. *** P<0.001 (One way ANOVA; Bonferroni)
Figure 3

Absence of an effect by prucalopride (1 µM) and BIMU-8 (2 µM) on the stimulation evoked release of [³H]-ACh from rat hippocampal slices. Drugs were added to the perfusion solution from 15 min before the second stimulation period (S₂) until one minute after S₂ was ended. The ratio between the fractional release of [³H]-ACh in response to the first (S₁) and second stimulation (S₂) was calculated and compared in the absence and the presence of drug. Mean S₂/S₁ ratio ± s.e.m. (n=6-8).
Figure 3

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Abstract

5-HT₄ receptor (5-HT₄R) activation induces pro-cognitive effects. This might be related to stimulation of hippocampal acetylcholine (ACh) release, which has been shown for 5-HT₄R agonists in in vivo models. We investigated the influence of the 5-HT₄R agonists prucalopride and BIMU-8 on ACh release in rat hippocampal brain slices. In contrast to the report by Siniscalchi et al. [1], no facilitating effect of 5-HT₄R agonists on electrically evoked ACh could be shown. The absence of an effect by 5-HT₄R agonists illustrates that the in vitro evaluation of 5-HT₄R agonists on hippocampal ACh release is not a straightforward model to study the relationship between hippocampal 5-HT₄Rs and hippocampal ACh release.