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Interplay between 5-HT$_4$ receptors and GABAergic system within CA1 hippocampal synaptic plasticity.

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Running title: 5-HT$_4$R and hippocampal synaptic plasticity

Abstract:

The type 4 serotonin receptor (5-HT_4R) is thought to be highly involved in cognitive process such as learning and memory. Indeed, behavioral studies showed a beneficial effect of its activation on memory performances, and conversely reported memory impairments by its blockade. Further, whether it has been demonstrated that 5-HT_4R can modify hippocampal synaptic plasticity, the mechanisms involved remains elusive. To shed light on mechanisms at work, we investigated through two different electrophysiological protocols, the effects of the 5HT_4R agonist RS67333, on long-term potentiation (LTP) within the hippocampal CA1 area. While high-frequency stimulation (HFS) induced LTP remained unaffected by RS67333, the magnitude of LTP induced by theta-burst stimulation (TBS) was significantly decreased. This effect was blocked by the selective 5-HT_4R antagonist RS39604. Further, the 5-HT_4R-induced decrease in LTP magnitude was fully abolished in the presence of bicuculline, a $GABA_A$R antagonist; hence, demonstrating involvement of GABA neurotransmission. Additionally, we showed that application of a $GABA_B$R antagonist, CGP55845, mimicked the effect of 5-HT_4R activation while concurrent application of CGP55845 and RS67333 did not elicit additive inhibition effect on LTP. Altogether, these data show for the first time a negative regulation of 5-HT_4R on functional plasticity induced in hippocampal CA1 by theta burst afferent stimulation, that involve some interplay between 5-HT_4R and $GABA_B$R.

Introduction

Serotonin type 4 receptor (5-HT_4R) has gained an increasing interest in the field of therapeutic strategy development to treat memory disorders. Indeed, better cognitive performances were recently observed in healthy human subject, after a single intake of prucalopride (a 5-HT_4R agonist), a drug clinically authorized in some countries for treatment of irritable bowel [1]. Besides, numerous preclinical studies have shown beneficial effects of either acute or chronic pharmacological activation of 5-HT_4R on memory and learning functions [2-4]. Conversely, blockade of these receptors leads to learning and memory impairments. More interestingly in the field of Alzheimer Disease (AD), its activation, both *in vivo* and *in vitro [5-7]*, is enable to inhibit the amyloid protein precursor processing (APP), favoring soluble APPα (sAPPα) production rather than amyloid beta peptide (Aβ). Hence, a decrease in amyloid load as well as in neuro-inflammation markers, have recently been described in a transgenic mouse model of AD following a chronic treatment with RS67333, a 5-HT_4R agonist [8]. Additionally, this disease-modifying effect was associated with decreased memory impairments [8,9]. Finally, it is worth mentioning that in response to the early degeneration of the serotoninergic system [10], 5-HT_4R density is upregulated through early and mild stages of the disease [11]. Such phenomenon should strengthen the effect of a pharmacological intervention on this receptor.

In line with its cerebral expression and notably within the hippocampus [12], behavioral studies investigating the effect of 5-HT_4R modulation, have focused on hippocampus-dependent memory tasks. A recent study reported that optogenetic activation of serotoninergic fibers in CA1 area was associated with an increase in spatial memory performances [13]. Quite interestingly, this activation also elicited synaptic potentiation, which was blocked by 5-HT_4R antagonism.

Hippocampal synaptic plasticity, notably long-term potentiation/depression (LTP/LTP), is widely recognized as a cellular mechanism for memory storage [14-17]. To date, literature data argued for a complex regulatory role of 5-HT_4R on the hippocampal synaptic plasticity. Indeed, modulation of this receptor may (or not) affect both LTP and LTD, but also in a different way according notably to the subzone considered [3]. In the dentate gyrus (DG) of freely moving rats, 5-HT_4R activation dose-dependently inhibits LTP [18] and blocks LTD [19]. Conversely, its blockade facilitates LTD, but does not affect LTP [19]. In the CA3 area, 5-HT_4R activation inhibits both LTP and LTD, while its blockade facilitates both form of plasticity *in vivo* [19]. In the subiculum, through *ex vivo* investigations, it has been shown that LTP is unaffected by either blockage or activation of 5-HT_4R, while LTD is inhibited by blockade and conversely enhanced by activation of this receptor [20]. Regarding the CA1 area, things are a bit complex, as two different studies conducted *in vivo* have led to conflicting results. One researchers' group showed that activation of 5-HT_4R enhanced LTP [21], while the other one reported no effect on LTP but an inhibited LTD [22]. However, in any case, neither LTP nor LTD in CA1 area was affected by 5-HT_4R blockade.

To summarize, *i.e.* looking at hippocampal formation as a whole, activation of 5-HT_4R seems to favor the induction of LTP, over LTD. So far still misunderstood, the involved neurobiological processes underlying 5-HT_4R regulation of functional plasticity might imply the GABAergic system. Indeed, activation of 5-HT_4R has been shown to modulate GABA release from hippocampal slices [23,24] and to regulate $GABA_A$ receptors in the cortex [25]. The present study therefore aims to better characterize the interplay between 5-HT_4R and GABAR involved in the modulatory effect of hippocampal Schaeffer's collateral-CA1 synaptic plasticity. To this end, the effects of 5-HT_4R activation on two conditioning protocols for LTP induction that differentially recruit the GABAergic system were analyzed.

Materials and methods:

Animals

Experiments were performed on adult (10-14 weeks old) NMRI male mice (Janvier Labs, France), weighting 25–30g. Mice were group housed of 8 within standard polycarbonate cages, with food and water *ad libitum* and maintained in a regulated environment (22±1°C) under 12h reversed light/dark cycle (light on from 8pm to 8am). All experiments complied with the European Community guidelines and the French law on animal experimentation.

Pharmacology

All drugs used were perfused at least 15min before any recording to ensure full diffusion in the tissues and full expression of their effects. Based on their pharmacological profiles [26] [27], selective 5-HT_4R agonist and antagonist (respectively, 1-(4-Amino-5-chloro-2-methoxyphenyl)-3-[1-butyl-4-piperidinyl]-1-propanone hydrochloride and 1-[4-Amino-5-chloro-2-(3,5-dimethoxyphenyl) methyloxy]-3-[1-[2-methylsulphonylamino]ethyl]piperidin-4-yl]propan-1-one hydrochloride; also called RS67333 and RS39604) were used at 10µM and 1µM (supplementary experiments were performed with agonist – RS67333 – at 1µM). NMDA (N-methyl-d-aspartate) and non-NMDA receptor antagonists (respectively, 2-amino-5-phosphonopentanoic acid and 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo [*f*]quinoxaline-7-sulfonamide, also called APV and NBQX) were used at 50µM and 10µM respectively. $GABA_A$ and ABA_B receptor antagonist ([R-(R*,S*)]-5-(6,8-Dihydro-8-oxofuro[3,4-e]-1,3-benzodioxol-6-yl)-5,6,7,8-tetrahydro-6,6-dimethyl-1,3-dioxolo[4,5-g] isoquinolinium iodide and (2*S*)-3-[[(1*S*)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl] (phenylmethyl) phosphinic acid, also called bicuculline methiodide and CGP55845) were used respectively at

10 and 1µM. Except RS67333 and RS39604 which were obtained from Tocris biosciences®, all others pharmacological compounds were purchased from Sigma-Aldrich®.

Extra-cellular recordings

Mice were deeply anesthetized (isoflurane 5%) and decapitated. The brain was rapidly extracted from the skull and submerged half a minute in ice-cold artificial cerebrospinal fluid (aCSF). The aCSF composition was as follows (in mM): NaCl 124; KCl 3.5; $MgSO_4$ 1.5; NaH_2PO_4 1.2; $CaCl_2$ 2.5; $NaHCO_3$ 26; D-glucose 12. The solution was bubbled with O_2/CO_2 carbogen gas mixture (95%/5%) to keep the pH around 7.4. Hippocampi were removed from each hemisphere and cut in 400µm thickness transverse slices with a tissue chopper (McIlwain®). Slices were then deposed in a holding chamber containing aCSF at 28°C at least half an hour to recover from the slicing.

For electrophysiological recordings, slices were placed between two nylon meshes and completely submerged in a recording chamber perfused with a constant flow rate of 2mL/min of a bubbled aCSF at room temperature. All drugs were applied *via* direct bath perfusion. Extracellular synaptic responses in CA1 area were elicited by stimulation of the Schaffer collateral. Stimulation pulses (0.02msec duration) triggered by a computer controlled by the WinLTP® software [28] were delivered by a stimulus isolation unit through a bipolar electrode. Responses were recorded with glass micropipettes filled with 2M NaCl placed in the apical dendritic layer of the CA1 area.

For LTP recording, stimulation pulses were delivered every 10sec and field excitatory post-synaptic potentials (fEPSP) were recorded. The mean slope of 3 successive fEPSPs was considered as a data point and electrical intensity of the pulses was set to obtain a baseline fEPSP slope of 0.1mV/s. After 15min stable recordings for baseline, a conditioning stimulus was applied to induce long-term potentiation (LTP). Conditioning stimulus was realized either through a High Frequency Stimulation protocol (HFS, *i.e.* 100Hz tetanus during 1sec) or a

Theta Burst Stimulation protocol (TBS, *i.e.* 4 repetitions at 0.1Hz of 5 bursts – each constituted of 4 pulses at 100Hz - separated by 200ms at 5Hz). Interestingly, HFS and TBS protocols impact network activity differently since only the second requires specific GABAergic regulation through the $GABA_B$ receptors to induce LTP [29,30]. Whatever the protocol considered, baseline recording has been resumed for 60min after the conditioning stimulus. Therefore, the last 15min recording, reflecting LTP magnitude, was used for statistical analysis. Additionally, for TBS protocol, area under curve (AUC) of the two first bursts responses were calculated. We then evaluated the potentiation of the second burst corresponding to AUC2/AUC1, which reflect the efficacy of the TBS.

For the determination of NMDA receptors (NMDAR) activation, fEPSPs were recorded in a low magnesium aCSF (0.1mM) supplemented with NBQX. Stimulations were delivered as for the LTP baseline (every 10s with a data point recorded based on the average of three successive responses) and fEPSP and fiber volley (FV) slopes were recorded at increasing stimulus intensity (300 to 500µA). To evaluate the level of receptor activation, an index of synaptic efficacy (I_{SE}) corresponding to the fEPSP slope/FV slope ratio was plotted against stimulus intensity. Input/output (I/O) curves were thus constructed in order to assess changes in NMDA receptor activity after 5-HT_4R activation or blockade.

Patch-clamp recordings

Whole-cell patch-clamp recordings of CA1 pyramidal neurons were performed at room temperature from acute hippocampal slices perfused with aCSF. Borosilicate patch pipettes (5 MΩ) were filled with (in mM) $CsCH_4O_3S$ 140, CsCl 6, $MgCl_2$ 2, HEPES 10, EGTA 1,1, QX-314 5, ATP 4, (pH 7.3; 290mosM) for recordings. Membrane currents were acquired and filtered at 2Hz using an AxoPatch 1-D amplifier (Axon Instruments). On-line acquisitions and analysis were performed using WinLTP software. Series resistance was compensated and regularly monitored throughout the experiment and recordings showing unstable (>20%)

series resistance were rejected. GABAR-dependent synaptic current (IPSC) was evoked at 0.07Hz by electrical stimulation of the Schaffer collateral/commissural pathway using a bipolar electrode located in the *stratum radiatum*, in the presence of NBQX and APV to block glutamatergic transmission. RS67333 was added during 30min and then washed out. A continuous recording was performed, from the 10min preceding application of RS67333 (baseline measurement) until the 10min following the wash out.

Statistical analyses

Data are expressed as mean±SEM. R® software was used for statistical analysis and p-value lesser than 0.05 was considered significant. Analysis of variance (ANOVA) was performed; followed, when necessary, by post-hoc multiple comparison test with a Bonferroni-Dunn correction factor to investigate inter-group differences.

Results:

RS67333 (5-HT$_4$R agonist) did not affect HFS-induced LTP, but inhibited TBS-induced LTP.

When using HFS protocol, ANOVA with repeated measurements of mean fEPSP slopes during the last 15min showed no group effect ($F_{(1,14)}$=0.397, p=0.5388), no time effect ($F_{(30,420)}$=1.101, p=0.3293) and no group x time interaction ($F_{(30,420)}$=0.823, p=0.7350) (figure 1A). Thus, RS67333 did not change the stable and robust tetanus-induced LTP normally expressed in control conditions (respectively 137±8% *versus* 131±7% of baseline, n=8 slices for each conditions, figure 2A).

Conversely, when using the TBS protocol, ANOVA revealed a group effect ($F_{(1,14)}$=14.907, p=0.0017), a time effect ($F_{(30,420)}$=1.488, p=0.0494), but no group x time interaction ($F_{(30,420)}$=1.129, p=0.2948) (figure 1B and 2B). In control condition, LTP magnitude was of 150±9% of baseline value, whereas its value dropped down to 116±3% in RS67333-treated slices (n=8 slices for both conditions). Besides, while stable over time in control condition (one-way ANOVA, $F_{(30,210)}$=0.695, p=0.8822), a decrease of LTP magnitude was significantly observed in RS67333-treated slices (one-way ANOVA, $F_{(30,210)}$=1.8828, p=0.0078) (figure 2C).

Further, having a look at AUCs' ratio values for the two first bursts of conditioning stimulus (AUC for 2nd burst compared to the 1st one, *i.e.* AUC2/AUC1), one-way ANOVA revealed a group effect, with RS67333 treated slices having an AUC ratio significantly lower than control group ($F_{(1,14)}$=4.654, p=0.0488, figure 2C). Besides, only control group displayed a ratio significantly higher than 100% (univariate t-test, p=0.0490 and 0.8370 for respectively control and RS67333 treated groups).

RS67333 (5-HT$_4$R agonist) did not affect NMDAR activation.

Because LTP expression is closely related to activation of the NMDA subtype of glutamate receptors, we looked at the effects of RS67333 on isolated NMDAR synaptic potentials. Two-way ANOVA with repeated measurements showed no group effect ($F_{(1,20)}$=1.143, p=0.2978), and neither stimulation intensity effect ($F_{(2,40)}$=0.481, p=0.6215), nor group x stimulation intensity interaction ($F_{(2,40)}$=0.207, p=0.8142) (n=12 slices for both condition, see supplementary data).

Blockade of $GABA_A$ receptors suppressed the inhibitory effect of RS67333 (5-HT_4R agonist) on TBS-induced LTP.

We then assessed whether inhibitory system is involved in the effect of RS67333 on TBS-induced LTP. Thus, RS67333-induced modulation of synaptic plasticity was measured in the presence of bicuculline ($GABA_A$ receptors antagonist, 10µM), and compared to both control conditions (*i.e.* aCSF with or without bicuculline). ANOVA with repeated measurements showed neither group effect ($F_{(2,22)}$=0.080, p=0.9231), nor time effect ($F_{(30,660)}$=1.394, p=0.0806), and no group x time interaction ($F_{(60,660)}$=0.811, p=0.8438) (figure 3A). Accordingly, TBS-induced LTP was similar with or without bicuculline (148±10% and 148±5% of baseline, respectively, figure 2D) and not different from LTP observed in RS67333 plus bicuculline-treated slices (145±6% of baseline). This last result suggested that the decreased magnitude of TBS-induced LTP observed with 5-HT_4R activation involves the contribution of the $GABA_A$ receptors.

Activation of 5-HT_4R had no effect on $GABA_A$ receptor activities of CA1 pyramidal cells.

Evoked IPSCs were recorded in CA1 pyramidal cells (n=4) in the three conditions, i.e. when RS67333 was added to the bath of aCSF, but also before (which enabled baseline level

calculation), as well as afterwards, during washout. ANOVA of the time course revealed neither condition effect ($F_{(2,9)}$=0.631, p=0.5539), nor time effect ($F_{(39,351)}$=1.201, p=0.1984), or condition x time interaction ($F_{(78,351)}$=0.806, p=0.8747). Here, we showed no effect of 5-HT_4R activation on evoked IPSCs since their amplitude remained constant after addition of RS67333 in the recording medium, as well as after washout (figure 4). These results therefore demonstrate that 5-HT_4R activation did not directly enhance inhibitory transmission toward CA1 pyramidal neurons.

$GABA_B$ receptors blockade mimicked and occluded the inhibitory effects of 5-HT_4R activation on LTP.

We then tested the effects of an antagonist of the second type of GABAergic receptors, $GABA_B$ receptors. ANOVA with repeated measurements showed a group effect ($F_{(3,39)}$=8.573, p=0.0002), a time effect ($F_{(30,1170)}$=1.583, p=0.0243), and a group x time interaction ($F_{(90,1170)}$=1.475, p=0.0035) (figure 3B). Compared to control slices group (142±4% of baseline, n=15), magnitude of TBS-induced LTP was significantly decreased in slices infused with either RS67333 or CPG55645 (selective $GABA_B$ receptor antagonist) alone (respectively, p=0.0006 and 0.0002; 116±3% and 115±7% of baseline, n=8 and 9), or in combination (p=0.0009; 119±6% of baseline, n=10) (figure 2E). Furthermore, all three treated groups were not different from each other (p>0.5). Thus, the concurrent 5-HT_4R activation and $GABA_B$R blockade did not produce a stronger impairment of TBS-induced LTP than modulation of each of these receptors taken apart.

Discussion:

We demonstrated here for the first time that 5-HT_4R activation on the expression of LTP in CA1 field of hippocampus lead to highly contrasting effects according to the conditioning protocol used. Indeed, through *ex vivo* experiments, we observed either an unaffected LTP or a conversely highly decreased potentiation (HFS *versus* TBS). Furthermore, we showed that the in-between key difference of the two conditioning stimulation protocols, standing in the recruitment of $GABA_BR$, is central in the effects of 5-HT_4R.

The classic HFS conditioning protocol (100Hz tetanus during 1sec) is one of the most frequently used in the literature. While using such protocol, we observed no effect of 5-HT_4R activation on the magnitude of CA1 hippocampal LTP. Interestingly, this result is consistent with an *in vivo* study conducted in the CA1 area of freely moving rats [22]. In this study, Manahan-Vaughan's team used a similar (100Hz tetanus during 1sec) induction protocol, but repeated it 4 times. However, as evoked earlier, among the only two *in vivo* studies published so far, the other revealed contrasting results. Led by the group of Mastumoto, they showed an enhanced LTP after 5-HT_4R activation [21]. One would have been tempted to rely on the methodological difference in the origin of the signal recorded to explain the discrepancy. Indeed, whereas Manahan-Vaughan group and us recorded fEPSPs slopes (dendritic response), the population spike amplitude (PS) (somatic response) were recorded in the work of Matsumoto. However, previous works on effects of 5-HT_4R activation collected in other hippocampal formation area (*i.e.* dentate gyrus [18,19] and CA3 area [19]) indicated that changes in fEPSP slopes are similar to those in PS amplitudes. Hence, it is more likely that the entirely different LTP induction protocol (5 trains at 1Hz, each composed of 8 pulses at 400Hz) used by the group of Matsumoto would account for such discrepancy.

More sensitive to variations of GABAergic neurotransmission than HFS protocol, TBS protocol is viewed as a more physiological pattern of stimulation [31]. Indeed, TBS

mimics two particularities of hippocampal physiology: the complex spike discharges of pyramidal neurons [32] and the rhythmic modulation of their excitability during theta rhythm [33].

Hippocampal theta rhythm was originally described as the arousal rhythm [34]. Although at first discussed in line with motor behavior [35], it is now rather associated to the updating of the cognitive spatial map (within hippocampal place cells) [36], as well as with memory and learning processes [37,38]. Numerous studies have shown that TBS-induced LTP is more susceptible than HFS-induced LTP [31] to various experimental manipulations, many of which also lead to memory deficits. While using TBS protocol, we reported herein for the first time that activation of 5-HT_4R led to a significant decrease of the magnitude of LTP. Nonetheless, in 1992, before the discovery of 5-HT_4R, one *ex vivo* experiment had investigated the effect of serotonin application on rat hippocampal slices [39]. In this study, Corradetti and collaborators reported no change of HFS-induced LTP (consistent with our previously discussed result), and a decrease of primed burst-induced LTP. Of note, primed burst protocol in this study shares close properties with the TBS protocol we used, in such a way that a similar time interval was used between priming pulse and the following burst (4 pulses at 100Hz). Hence, in line with our result, one might hypothesize that impairments of LTP observed with serotonin application rely on activation of 5-HT_4R.

At that time, effects on the primed burst induced LTP was attributed to activation of 5-HT_{1a} and 5-HT_3 receptors; the former hyperpolarizing pyramidal cells [40], while the latter enhancing GABA release [41]. Herein, to ascertain that the TBS-induced decreased magnitude of LTP was specific to 5-HT_4R activation, two supplementary experiments were performed. First, considering its pharmacological profile, a low dose of RS67333 (1µM) was tested on TBS-induced LTP and has resulted in a similar effect (decrease magnitude) (see supplementary figure 1 and 2). Therefore, if 5-HT_{1a}R and/or 5-HT_3R (pKi of RS67333=6.4

for both receptors) would be involved in the decrease of LTP magnitude observed at 10µM (~90% receptor occupancy), different results should have been observed at 1µM (~50% receptor occupancy). Second, we used a highly selective antagonist of this receptor, RS39604. This antagonist affords a 1000 time higher affinity for type 4 receptor of serotonin rather than both 1A and 3 [27]. Thus, whereas it has no effect alone, RS39604 blocked effect of RS67333 on TBS-induced LTP (see supplementary figure 2).

Next, we further explored the mechanism at work that could account for differential effect of RS67333 (or of serotonin application) on LTP according to the stimulation protocol used. A quarter century ago, the power of the afferent stimulation was argued to likely overcome the inhibitory effect of serotonin, thus explaining the absence of effect of serotonin application during a HFS protocol. Here, we first investigated whether a direct effect of 5-HT_4R activation on either AMPAR or NMDAR activity was involved. On basal synaptic transmission (relying on AMPAR activity), we reported a small increase at 10µM and no effect at 1µM (see supplementary figure 3). However, any modifications of basal transmission are unlikely to be involved on the reported effect of 5-HT_4R activation on TBS-induced LTP. Indeed, only the highest dose of RS67333 affected basal transmission, while conversely the two doses of RS67333 decrease the magnitude of TBS-induced LTP. Besides, RS39604 failed to block RS67333-induced increase in basal transmission, suggesting that this effect may not involve 5-HT_4R. As regards to NMDAR, while a decrease of their activity would have explained impairments of LTP, RS67333 had no effect on NMDAR activity (recorded in low Mg^{2+} medium with a blockade of non-NMDA receptors). Furthermore, HFS-induced LTP also requires NMDAR and was unaffected by RS67333. Hence, RS67333 effect on TBS-induced LTP cannot be explained by modulation of NMDAR activity.

As stated earlier, several studies have advanced converging arguments for a role of GABAergic transmission in the modulatory role of 5-HT_4R on synaptic plasticity [23-25].

Here, we reported that 5-HT_4R activation did not change the amplitude of evoked IPSC at low frequency. Consistent with the absence of modification after application of a 5-HT_4R agonist of endogenous GABA release from hippocampal slices at rest [23], this result suggest that 5-HT_4R activation did not modify basal GABA neurons excitability. Contrarily, effects of 5-HT_4R activation on TBS-induced LTP appeared to mainly rely on modifications of GABAergic functioning. Indeed, we did not observe burst potentiation across conditioning stimuli during TBS protocol when RS67333 was applied. However, one of the key property of TBS protocol relies on the increase of action potential firing across bursts repetition, which results from cumulative loss of synaptic inhibition [31]. Post-burst hyperpolarization – caused by synaptic activation of both $GABA_AR$ and $GABA_BR$ – is more or less suppressed across bursts according to the interval used. Of note, the loss of inhibition has been described to most rapidly occur with a 200msec burst interval (as used here) [42]. In our experiments, this loss of inhibition was confirmed since no change of TBS-induced LTP was observed in the presence or the absence of the postsynaptic $GABA_AR$ antagonist, bicuculline. This phenomenon, that lasts for about a second [43], relies on the activation of presynaptic $GABA_B$ auto-receptors to disinhibit the postsynaptic element [44,45] (through a decrease GABA release). This allows sufficient depolarization of postsynaptic element and hence the activation of NMDA receptor leading to LTP.

Quite interestingly, it has been reported that the concurrent blockade of both pre- and post-synaptic $GABA_BR$ exert different effects on HFS- or TBS-induced LTP [46], leaving the former unaffected and impairing the latter. Here, we reported that blockade of $GABA_BR$ (through CGP55845) induced similar impairments of TBS-induced LTP than 5-HT_4R activation. Besides, concomitant $GABA_BR$ blockade with 5-HT_4R activation did not produce additive effect. Then, when investigating $GABA_AR$ neurotransmission, we demonstrated that

co-application of RS67333 with a selective $GABA_AR$ antagonist, fully blocked effect of 5-HT_4R activation on TBS-induced LTP.

Taken altogether, our results argue for a major interplay of 5-HT_4R activation with GABAergic functioning. Indeed, we demonstrated for the first time that activation of 5-HT_4R induces a preserved inhibitory neurotransmission during a TBS protocol induced LTP. Consecutively, the preservation of GABA release (or absence of loss of inhibition) enable an activation of postsynaptic $GABA_AR$ neurotransmission, which in turn, has decreased the excitability of Schaffer collateral-CA1 glutamatergic synapse eliciting impairments of TBS-induced LTP. Still remains open the question of how 5-HT_4R interact with GABAergic functioning. A direct effect through 5-HT_4R localized on GABAergic interneurons seems unlikely. Indeed, in situ hybridization study has reported that expression of 5-HT_4R mRNA does not seem to co-localize with the Gad-65 mRNA (a marker of GABAergic neurons) in the hippocampus [47]. An indirect pathway would then require the release of other neurotransmitters, such as the acetylcholine, whose receptors are presents on interneurons in the CA1 [23].

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Acknowledgments:

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Caption to figures:

Figure 1: Time-course of fEPSP slopes in CA1 hippocampal slices during HFS- (A) and TBS-induced LTP (B). Data are expressed as mean±SEM. Control corresponds to a bath of artificial cerebrospinal fluid and RS67333 (5-HT_4R agonist) was used at 10µM. Arrow marks the time when conditioning stimulation was applied (respectively HFS- and TBS, **A** and **B**). Insets show representative traces of fEPSP during both baseline (plain line) and the last 15min of recording (dashed line) (** $p<0.01$ *versus* control).

Figure 2: Mean of fEPSPs slopes during the last 15min of LTP recordings, using either HFS (A) or TBS (B, D and E) condition protocol. (C) AUCs ratio during TBS conditioning stimulus. Data are expressed as mean±SEM. Control corresponds to a bath of artificial cerebrospinal fluid. RS67333, bicuculine ($GABA_A$R antagonist) and CGP55845 ($GABA_B$R antagonist) were respectively used at 10, 10 and 1µM. Insets in figure 1C show representative traces of first (grey line) and second burst (black line) during TBS conditioning protocol (* and ** for $p<0.05$ and 0.01 *versus* control; # for $p<0.05$ *versus* 100%)

Figure 3: Time-course of fEPSP slope in CA1 hippocampal slices during TBS-induced LTP. Data are expressed as mean±SEM. Arrow marks the time when conditioning stimulation was applied. Control corresponds to a bath of artificial cerebrospinal fluid. RS67333, bicuculine ($GABA_A$R antagonist) and CGP55845 ($GABA_B$R antagonist) were respectively used at 10, 10 and 1µM. Insets show representative traces of fEPSP during both baseline (plain line) and the last 15min of recording (dashed line) (** $p<0.01$ *versus* control).

Figure 4: Time-course of evoked IPSCs normalized amplitude in CA1 pyramidal cells (N=6 cells from 6 different mice). After 10min baseline recordings, RS67333 (10µM) was added to the bath for 20min period. Recordings was follow-up during 10min after washout. Insets show representative traces of evoked IPSCs in a pyramidal CA1 neuron during baseline, after 15min of RS67333 infusion, and during washout (* $p<0.05$).

Supplementary figure 1: Time-course of fEPSP slopes in CA1 hippocampal slices during TBS-induced LTP. Effect of combination of RS67333 (10µM) with RS39604 (5-HT_4R antagonist, 1µM) and of a low dose (1µM) of RS67333 (5-HT_4R agonist). Data are expressed as mean±SEM. Control corresponds to a bath of artificial cerebrospinal fluid. Arrow marks the time when conditioning stimulation was applied. Insets show representative traces of fEPSP for each group, during both baseline (plain line) and the last 15min of recording (dashed line) (* $p<0.05$ *versus* control).

Supplementary figure 2: Index of either AMPA (A) or isolated NMDA receptors (B) mediated Synaptic Efficacy (I_{SE} defined as the fEPSP/PFV ratio) according to intensity stimulation. Control corresponds to a bath of artificial cerebrospinal fluid and RS67333 (5-HT_4R agonist) was used at 1 and 10µM (* for $p < 0.05$). Insets show representative AMPA or NMDA field recordings in control condition (plain line) and in the presence of RS67333 at 10µM (dashed line).

Supplementary results:

Selectivity of action of RS67333 on for 5-HT4 receptor was tested in two ways during this supplementary experiment. Not only, effect of RS67333 (10µM) on magnitude of TBS-induced LTP was assessed in the presence of RS39604 (a highly selective antagonist of 5-HT_4 receptor), but a very low dose of RS67333 was also tested (1µM). Thus, ANOVA with repeated measurements of mean fEPSP slopes during the last 15min showed a group effect ($F_{(2,24)}$=4.223, p=0.0268), due to a statistical difference between low dose RS67333 and control group (p=0.0124) (suppl figure 1). Set at 145±10% of baseline value in control group (n=7 slices) and 138±12% in slices infused with the cocktail of agonist plus antagonist (respectively, 10 and 1µM of RS67333 and RS39604, n=8 slices), LTP magnitude was dropped down to 118±4% in slices treated with lowest dose RS67333 (n=12 slices). Altogether, these results demonstrated the specificity of action of RS67333, through 5-HT_4 receptors.

Considering either basal (AMPA-mediated) neurotransmission or isolated NMDA-mediated current, ANOVA with repeated measurement revealed neither group effect (respectively, $F_{(2,42)}$=0.351 and $F_{(2,30)}$=0.543, p=0.7063 and p=0.5864), nor stimulation intensity effect (respectively, $F_{(2,54)}$=0.413 and $F_{(2,60)}$=1.686, p=0.6630 and 0.1939), or group x stimulation intensity interaction (respectively, $F_{(4,84)}$=0.29.6 and $F_{(4,60)}$=0.282, p=0.8800 and 0.8886) (suppl figure 2A and B). Whatever the dose considered, RS67333 did not modify activity of AMPA- or NMDA-mediated current.