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Hippocampal 5-HT$_7$ receptors signal phosphorylation of the GluA1 subunit to facilitate AMPA receptor mediated-neurotransmission *in vitro* and *in vivo*

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Summary

Background and purpose: The 5-HT_7 receptor is a G-protein coupled receptor that is the target of a broad range of antidepressant and antipsychotic drugs. Various studies have demonstrated an ability of the 5-HT_7 receptor to modulate glutamatergic neurotransmission and cognitive processes although the potential impact upon AMPA receptors has not been investigated directly. The purpose of the present study was to investigate a direct modulation of the GluA1 AMPA receptor subunit and determine how this might influence AMPA receptor function.

Experimental approach: The influence of pharmacological manipulation of the 5-HT_7 receptor system upon phosphorylation of GluA1 subunits was assessed by western blotting of fractionated proteins from hippocampal neurones in culture (or proteins resident at the neurone surface) and the functional impact assessed by electrophysiological recordings in rat hippocampus *in vitro* and *in vivo*.

Key results: 5-HT_7 receptor activation increased cAMP and relative pCREB levels in cultures of rat hippocampal neurones along with an increase in phosphorylation (Ser845) of the GluA1 AMPA receptor subunit evident in whole neurone extracts and within the neurone surface compartment. Electrophysiological recordings in rat hippocampus demonstrated a 5-HT_7 receptor-mediated increase in AMPA receptor-mediated neurotransmission *in vitro* and *in vivo*.

Conclusions and implications: The 5-HT_7 receptor-mediated phosphorylation of the GluA1 AMPA receptor provides a molecular mechanism consistent with the 5-HT_7 receptor-mediated increase in AMPA receptor-mediated neurotransmission.

Abbreviations

A, adenine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; C, cytosine; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; cAMP, cyclic adenosine monophosphate; cDNA, complementary deoxyribonucleic acid; CREB, cAMP response element-binding protein; 5-CT, 5-carboxamidotryptamine; DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; dNTPs, deoxynucleotides; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; EPSCs, excitatory post-synaptic currents; FBS, foetal bovine serum; FSK, forskolin; G, guanine; GABA, gamma-aminobutyric acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HBSS, Hank's balanced salt solution; HRP, horseradish peroxidase; 5-HT, 5-hydroxytryptamine; IBMX, 3-isobutyl-1-methylxanthine; KO, knock-out; LTP, long-term potentiation; MAP, mitogen activated protein; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione; NMDA, N-methyl-d-aspartate; PBS, phosphate buffered saline; PCR, polymerase chain reaction; pCREB, phosphorylated cAMP response element-binding protein; PDE, phosphodiesterase; PKA, protein kinase A; PVDF, polyvinylidene difluoride; RNA, ribonucleic acid; RT, reverse transcription; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Ser, serine; T, thymine; TAE, Tris acetate EDTA; TTX, tetrodotoxin.

Introduction

The 5-HT_7 receptor has been cloned from various species and is a member of the G-protein coupled receptor superfamily (Hoyer et al., 1994; Alexander et al., 2013). The 5-HT_7 receptor signals via an increase in cAMP production as well as various other pathways (e.g. Shen et al., 1993; for review see Gellynck et al., 2013) and is the focus of various projects to identify novel therapeutics to treat conditions such as depression, sleep disorders and cognitive deficits (e.g. Mnie-Filali et al., 2007). Further clinical interest in the 5-HT_7 receptor arises from a broad range of antidepressant and antipsychotic drugs displaying relatively high affinity for the receptor (Roth et al., 1994). Further, two non-selective 5-HT_7 receptor antagonists have recently received marketing authorisation for the treatment of psychiatric conditions: lurasidone and vortioxetine (Fountoulakis et al., 2015; Sanchez et al., 2015).

Expression of the 5-HT_7 receptor is apparent throughout the body with notable expression in the brain and spinal cord, vascular system and gastrointestinal tract (Waeber and Moskowitz, 1995; Gustafson et al., 1996; Stowe and Barnes, 1998; Neumaier et al., 2001). Within the brain there is prominent expression within the hippocampus (Waeber and Moskowitz, 1995; Gustafson et al., 1996; Neumaier et al., 2001; Mengod et al., 2010), where homodimerisation and heterodimerisation with the 5-HT_{1A} receptor may be relevant (Renner et al., 2012). At the cellular level within the hippocampus, expression in glutamatergic pyramidal neurones is evident (Neumaier et al., 2001), which is consistent with a number of independent studies demonstrating that the 5-HT_7 receptor mediates excitation of glutamatergic pyramidal neurones and associated network activity (Bacon and Beck, 2000; Bickmeyer et al., 2002; Gill et al., 2002; Tokarski et al., 2003; 2005; Costa et al., 2012). Such neurophysiological actions may underlie the improvement in cognitive performance following 5-HT_7 receptor activation (Meneses, 2004; Perez-Garcia and Meneses, 2005; Eriksson et al., 2008; Freret et al., 2014), which is consistent with the reduced cognitive performance following inhibition of 5-HT_7 receptor function by pharmacological antagonism or genetic manipulation (Meneses, 2004; Ballaz et al., 2007; Sarkisyan and Hedlund, 2009; Freret et al., 2014; but see Horisawa et al., 2011 and Waters et al., 2012). However, some types of memory deficit are improved by 5-HT_7 receptor antagonists (McLean et al., 2009; Bonaventure et al., 2011; Nikiforuk et al., 2013), which may be a consequence of different neurochemical mechanisms (Bonaventure et al., 2011) or biased signalling; this is consistent with the beneficial effects of some non-selective 5-HT_7 receptor antagonists used to treat psychiatric conditions (e.g. Horiguchi et al., 2011).

In terms of a molecular mechanism by which the 5-HT_7 receptor might drive neuronal excitation in the hippocampus, recent evidence demonstrates that the 5-HT_7 receptor mediates phosphorylation of NMDA glutamate receptors with a consequential increase in NMDA receptor signalling (Vasefi et al., 2013). Consistent with these findings, 5-HT_7 receptor KO mice display reduced long term potentiation in the hippocampus (Roberts et al., 2004). It should be noted, however, that 5-HT_7 receptor KO mice also display impaired contextual fear conditioning - a hippocampus-dependent phenomenon - but no deficit in spatial learning (Roberts et al., 2004), which implicates 5-HT_7 receptor modulation of AMPA receptor neurotransmission (Zamanillo et al., 1999). In support of this, 5-HT_7 receptors enhance AMPA receptor-mediated transmission in the hippocampus via an action on post-synaptic neurones (Costa et al., 2012).

In the present study we have investigated the ability of the 5-HT_7 receptor to impact glutamatergic neurotransmission in the hippocampus and have demonstrated that the receptor promotes protein kinase A (PKA) -mediated phosphorylation of native GluA1 AMPA

receptor subunits (Alexander et al., 2013) in rat hippocampus, which results in an increase in phosphorylated (S845) GluA1 AMPA receptor subunit insertion into the neuronal membrane; this likely contributes to the evident increase in AMPA receptor-mediated neurotransmission in the hippocampus both *in vitro* and *in vivo*.

Materials and methods

Materials

5-HT and papaverine were purchased from Sigma-Aldrich, St. Louis, MO, USA; SB258719, GR113808, SB399885, AS-19, Rolipram, H89 were from Tocris Bioscience, Ellisville, MO, USA. Laboratory reagents were from Sigma-Adrich unless otherwise specified. Cell culture reagents were purchased from Gibco/Invitrogen (Life Technologies, Paisley, UK) except where otherwise indicated.

Use of animals

All animal protocols were in accordance with the European Communities Council Directive 2010/63/EU and Italian or UK legislation acts concerning animal experimentation. The experimental protocol was approved by the Local Ethics Committees. Efforts were made to minimise animal suffering and to reduce the number of animals used. All *in vivo* experiments were performed in accordance with ARRIVE guidelines. (Kilkenny et al., 2010; McGrath et al., 2010).

Preparation of rat neurone or astrocyte cultures

Hippocampal predominantly neuronal cultures were obtained from embryos at day 18/19 of gestation (E18/19) of Sprague-Dawley rats (embryos killed by decapitation). Hippocampi were quickly isolated into Hank's Balanced Salt Solution (HBSS; pH 7.3 buffer containing Hepes (10 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) 4°C) before incubation of trypsin (0.1%; 37°C, 10 min (for the last 5 min of this incubation, DNAse I (166 µg/ml) was added)), washed with HBSS buffer containing 10% foetal bovine serum (FBS) and cells dissociated by triturating. Cells were placed in Poly-L-lysine (MW >30000 kDa) coated plates (density of 700-800 cells/mm^2) in serum-free NEUROBASAL™ Medium supplemented with B-27® Supplement, glutamine (500 µM), glutamate (12.5 µM), penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were grown at 37°C, 5% CO_2 and half the volume of the medium was replaced after 4 days with complete medium minus glutamate. For all assays, primary cells were used between 8-10 days in culture.

Phenotype and purity of cell cultures

Purity of cultured cells was assessed by phenotyping with primary antibodies (rabbit anti-GFAP; 1:1000 (Sigma-Aldrich) or anti-NeuN (mouse 1:200 (Chemicon, Merck Millipore)). with 5 fields per coverslip acquired using a LEICA microscope (IM 50 program; 20 x objective) with positive cells counted manually.

RNA extraction and quantification

Total RNA from cultured cells was extracted using RNeasy mini kit from Qiagen (Qiagen Inc) following the manufacturer's instructions. Genomic DNA was removed using RNAse-free DNase I (Applied Biosystems, Life Technologies). Purified RNA was quantified using an Agilent 2100 Bioanalyzer following the Agilent RNA 6000 Nano kit protocol (Agilent Technologies Inc).

Reverse Transcribed PCR

cDNA was generated by reverse transcription of total RNA (100 ng) using TaqMan Reverse Transcription Reagents (Applied Biosystems, Life Technologies); 1x RT buffer, $MgCl_2$ (2.5 mM), dNTPs (1 mM), random hexamers (2.5 µM), RNase Inhibitor (0.4 U/µl), MultiScribe RT (1.25 U/µl). The mixture was incubated consecutively at 25°C (10 min), 48°C (30 min) and 95°C (5 min). PCR was performed using HotStarTaq(R) PCR from Qiagen: 10 ng cDNA, 1x PCR buffer, 200 µM dNTPs, 300 nM Primers, 1 unit Taq in DNase-free water. After 15 min incubation (95°C), 40 cycles of amplification were performed (30 sec denaturation at 95°C, 30 sec annealing at 60°C, and 40 sec extension at 72°C) before termination with 10 min extension at 72°C. Primer pairs were as follows:

5-HT_7 receptor:
Forward; ATCTTCGGCCACTTCTTCTGCAACG
Reverse; CAGCACAAACTCGGATCTCTCGGG

5-HT_6 receptor:
Forward; CCATCTGCTTCACCTACTGC
Reverse; TCTGAATCTGAGTTTGGCGG

5-HT_4 receptor:
Forward; TTGGCTGCTTTGGTCTCTGTCCGC;
Reverse; TGCAAGGCTGGAACAACATCGGC.

PCR products were separated electrophoretically (1.5% agarose gels in 1x TAE buffer (Tris 40 mM, glacial acetic acid 1 mM, 0.1% EDTA, pH 8)) with SYBR safe DNA gel stain (Invitrogen, Life Technologies) running alongside DNA molecular weight markers (100 bp ladder, Invitrogen, Life Technologies) for 1 hour at 100 V.

Assessment of cAMP concentration

cAMP was quantified using the cAMP Hi Range kit (Cisbio) according to the manufacturer's instructions.

Sample preparation and measurement of total CREB, pCREB, and phospho-p38 MAPK

Cells were removed by scraping in lysis buffer (Tris (10 mM; pH 7.4), NaCl (100 mM), EDTA (1 mM), EGTA (1 mM), NaF (1 mM), $Na_4P_2O_7$ (20 mM), Na_3VO_4 (2 mM), 1% Triton X-100, 10% glycerol, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, phenylmethanesulfonyl fluoride (2 mM), protease inhibitors (Complete Mini, Roche Life Science, Sigma-Aldrich) and 1% phosphatase inhibitors I and II (Sigma-Aldrich)). Cells lysates were incubated at 4 °C for 30 min before centrifugation (10 min at 13000 x g) and the supernatants quantified for total CREB and Serine^{133}-phosphorylated CREB by ELISA (Invitrogen, Life Technologies) accodrding to the manufacturer's instructions. Phospho-p38 MAP Kinase levels were also measured by ELISA (Cell Signaling Technology) following the manufacturer's instructions.

Isolation of cell surface proteins

Cell surface proteins were isolated using EZ-link sulfo-NHS-SS-Biotin (Pierce) at 4°C. Cells were incubated with biotin (1 mg/ml; 20 min) dissolved in PBS. After three rinses in ice-cold glycine (50 mM) in PBS, cells were scraped into homogenization buffer (0.1% SDS, 0.5% NP40, 0.5% sodium deoxycholate, 150 mM NaCl, Complete mini protease inhibitors, phosphatase inhibitors cocktail I and II in PBS, pH 7.4), centrifuged (10,000 x g, 20 min) and the supernatant represented the total protein extract. For purification of the biotinylated surface protein fraction, 50 µg of total proteins were incubated with 80 µl of 50% slurry Neutravidin resin (Pierce) for 2 h at 4ºC. After washing, biotinylated proteins were eluted (100 µl of elution buffer; Invitrogen NuPage LDS sample buffer with ditiothreitol (50 mM)), heated at 95ºC for 5 min before centrifugation (1,000 x g for 2 min).

Protein concentration was determined using Bio-Rad reagents (Bio-Rad Laboratories Inc., Hercules, CA, USA) based on the Bradford assay. When protein concentration was expected to be low, the micro BCA kit was adopted (Pierce).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)-Western blotting

Proteins were separated using SDS-PAGE in an XCell SureLock™ Mini-Cell (Invitrogen, Life Technologies). Equal amounts of cell lysates (~6-10 µg protein) in 4 x NuPAGE® LDS Sample Buffer and 10 x NuPAGE® Sample Reducing Agent were boiled for 5 min, centrifuged briefly and loaded in pre-cast gels (1.5 mm 4-12% Novex Bis-Tris Pre-Cast Mini Gels, Invitrogen, Life Technologies). Prestained protein molecular weight markers (Novex® Sharp Pre-stained Protein Standard) were loaded in parallel. Electrophoresis was performed (50V for 1 h) and proteins transferred to polyvinylidene difluoride (PVDF) membranes (Amersham) using a Mini Trans-Blot module (Bio-Rad) in Transfer Buffer (25 mM Tris, 192 mM Glycine, 20% methanol, pH 8.3) at 40-50V for 2 h. PVDF membranes were blocked (5% skimmed dried milk in Tris Buffered Saline (TBS: 50 mM Tris HCl, 280 mM NaCl, 2.7 mM KCl, pH 7.6; 1 hr) containing 0.1% Tween-20) and then incubated with the relevant primary antibodies; either rabbit anti-GluA1 Ser845P (Sigma-Aldrich; 1:1000), rabbit anti-GluA1 (Millipore; 1:1000), rabbit anti-pan Cadherine (Abcam; 1:1000) or mouse anti-GAPDH (Santa Cruz Biotechnology; 1:10000). Membranes were washed (3 x 10 min) with 0.1% Tween-20 in TBS before incubation (1 h at room temperature) with peroxidase-conjugated

secondary antibodies (goat anti-rabbit IgG-HRP or goat anti-mouse IgG-HRP, Santa Cruz; 1:5000). The chemiluminescence reaction was developed with ECL Plus (Amersham) and detected with Luminescent Image Analyzer LAS-4000mini (Fujifilm). Optical densities were determined by densitometric analysis with Quantity One software (Bio-Rad).

Electrophysiologyical recording in the hippocampus in vivo

Adult male Sprague-Dawley rats (250-350 g, n=12) were initially anaesthetized with isoflurane and subsequently with chloral hydrate (400 mg/kg i.p.) with anaesthetic depth maintained by constant intravenous infusion (80-120 mg/kg/hr) adjusted dependent on corneal reflex, withdrawal response to paw-pinch and the stability of monitored cardiovascular variables. Core body temperature was maintained at 37 ± 1°C. The right femoral vein, artery and the trachea were cannulated to permit, respectively: administration of supplemental anaesthetic; the recording of arterial blood pressure via a pressure transducer and amplifier (Neurolog module NL108, Digitimer), and the maintenance of a clear airway. Animals were placed in a stereotaxic frame (Narishige ST-7) and the dorsal brain surface overlying the hippocampus exposed by craniotomy. A small incision was made in the dura and a multi-barrel recording electrode (Kation Scientific) was lowered vertically through the cortex to the CA3 pyramidal layer of the hippocampus according to the following stereotaxic boundaries (Paxinos & Watson, 1998): Bregma -3.9 – 4.4 mm, lateral 3.7 – 4.2 mm, depth 3.6 – 4.2 mm below pial surface. Unitary activity was recorded through an extracellular carbon fibre microelectrode and, using the Neurolog system, the signal was amplified (x10 k, module NL104) and filtered (bandwidth 1 – 30 kHz, module NL125), with the conditioned output being captured on a PC using a micro1401 interface with Spike 2 software (CED). Multi-barrel electrodes were filled with 5-CT (DP-5-CT, 1-10 mM), WAY-100,635 (1-10 mM), AMPA (5 mM in 195 mM NaCl; pH 8) and NBQX (disodium salt, 1 mM in ddH_2O; pH 8) and for current balancing and recording site marking, Chicago Sky Blue. Initially, baseline CA3 pyramidal neurone activity was examined during iontophoretic administration of 5-CT alone and in the presence of the $5\text{-}HT_{1A}$ receptor antagonist WAY-100,635. Subsequent experiments examined the response to 5-CT, with and without concomitant application of WAY-100,635, on neuronal bursts of firing induced by cyclical (5/30 s) iontophoretic administration of AMPA. NBQX was iontophoretically administered at the end of experiments to confirm that AMPA-induced excitations were selective. Stable baseline or AMPA-evoked neuronal activity was recorded for a minimum of 5 minutes before test substance examination. Neurones with unstable activity or whose recording location lay outside the pyramidal layer were excluded from analyses. Neuronal activity was measured in spikes per second (Hz) for a minimum period of 60 s before and during test compound treatment.

Subsequent to electrophysiological recordings *in vivo*, rats were killed without recovery of consciousness by an overdose of sodium pentobarbitone (200 mg/kg), followed by cervical dislocation.

Electrophysiologyical recording in the hippocampus in vitro

Male Sprague Dawley rats (6-8 weeks old) were killed by anaesthetic overdose using isoflurane inhalation. Transverse hippocampal slices (400 μm thickness) were cut using a vibratome (Leica VTS1000) in chilled (<4°C) carbogenated (95% O_2 / 5% CO_2) artificial cerebrospinal fluid (aCSF; NaCl, 127 mM; KCl, 1.9 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 2.4 mM; $MgCl_2$, 1.3 mM; $NaHCO_3$, 26 mM; D-glucose, 10 mM). Slices were maintained in aCSF at room temperature for a minimum of 1 hr before transferring to a custom-built recording chamber to commence whole-cell recordings from hippocampal CA3 pyramidal neurones with an Axopatch 1D amplifier (Molecular Devices LLC), using the blind version of the patch-clamp technique (see Pickering *et al*., 1991). Patch pipettes were pulled from thin-walled borosilicate glass (GC150-TF10, Harvard Apparatus) with resistances between 3 and 8 MΩ when filled with intracellular solution of the following composition: Kgluconate, 130 mM; KCl, 10 mM; EGTA-Na, 1 mM; HEPES, 10 mM; Na_2ATP, 4 mM; Na_2GTP, 0.3 mM and Lucifer yellow, 2 mM; pH-adjusted to 7.4 with KOH, osmolarity-adjusted to 310 mOsm with sucrose. Data was filtered at 2-5 kHz (current-clamp) or 1 kHz (voltage-clamp), digitised at 10 kHz (Digidata 1322, Molecular Devices LLC) and recorded on a PC running Clampex 9.1 software (Molecular Devices LLC). After achieving whole-cell access current/voltage relations were generated to identify intrinsic membrane conductances consistent with CA3 pyramidal neurones. In voltage-clamp experiments, mossy fibre pathway synaptic inputs were stimulated using a concentric bipolar stainless steel electrode and AMPA receptor-mediated excitatory postsynaptic currents (EPSCs) pharmacologically isolated using D-AP5 (25 μM), bicuculline (10 μM) and CGP-55845 (200 nM) to block NMDA, $GABA_A$ and $GABA_B$ receptor-mediated synaptic inputs, respectively. Where relevant, 5-HT_{1A} receptors were also blocked with WAY-100,635 (1 μM) prior to obtaining a stable baseline of at least 5 minutes and then examining the effect of 5-CT (10 μM) application, before a final period of wash and confirmation of AMPA-mediated responses with NBQX (10 μM). In current-clamp experiments, a minimum of two stable responses were obtained to AMPA application (10 – 20 μM, 2-6 s) in the presence of WAY-100,635 (1 μM) and tetrodotoxin (TTX, 1 μM), before repeating in the presence of 5-CT (10 μM), a period of wash and, where possible, perfusion of SB258719 (20 μM) and a further 5-CT response examination. After completion of experiments, slices were fixed in 4% paraformaldehyde for a minimum of 48 hrs, before clearing with DMSO (>4 hrs), and visualisation of recorded cells using fluorescence microscopy to confirm their location within the CA3 pyramidal layer and characteristic morphology.

Data Analysis

Drug concentration-response curves were fitted to a four parameter logistic equation using GraphPad Prism 5 (GraphPad Software Inc). Agonist potency was expressed as pEC_{50} (-log EC_{50}). The inhibition of antagonists was expressed as the pIC_{50} (–logIC_{50}), where IC_{50} represents the concentration of drug inhibiting 50% of agonist maximal response. pK_B values for antagonists were determined using the equation: pK_B =(-log([antagonist]/(concentration ratio-1)) where concentration ratio = ratio of the agonist EC_{50} in the presence and in the absence of the antagonist. Where variance is indicated, data represent mean±SEM.

For biochemical assays, where occurring, normalisation was a % change from the control group's individual value for a given experiment with individual values then used to generate mean±SEM. Statistical analysis was only performed if the n value was 5 or greater. Where

more than two groups were compared, a Kruskal-Wallis ANOVA was performed. If $P<0.05$ from the Kruskal-Wallis ANOVA, or where just two groups were to be compared, a Mann-Whitney U test was performed with $P<0.05$ considered a statistically significant difference between groups.

Analysis of electrophysiological data was carried out using Clampfit 9.1 software (Molecular Devices LLC). For voltage-clamp data, the mean maximum electrically-evoked EPSC amplitude measured from a minimum of 5 consecutive sweeps (60 s) was compared with a temporally-matched period during the peak response to 5-CT. For current-clamp data, the area under the curve (in $V.ms^{-1}$) of AMPA-evoked responses was the primary measurement, the baseline was taken as the response immediately preceding 5-CT perfusion. All data are represented as mean±SEM. Comparisons between treatment periods were made using the paired student's t-test. Probability (P) values < 0.05 were considered significant.

Results

5-HT_7 receptor mRNA expression in primary culture of hippocampal cells

The purity of primary cell cultures after 8-10 days *in vitro* was evaluated by immunocytochemistry for the neuronal marker NeuN and the astrocyte marker glial fibrillary acidic protein (GFAP); the percentage of hippocampal NeuN- and GFAP-positive cells vs. DAPI-stained cells was around 80% and 10%, respectively (Supplementary Figure S1). At the same end-point of 8-10 days *in vitro*, expression of 5-HT_7 receptor mRNA was evident by RT-PCR with appropriate sized product detected from cultured hippocampal cells (and also cultured cerebral cortex cells and adult hippocampus; Figure 1). Hereafter the hippocampal primary cells enriched considerably for neurones using appropriate culture conditions will be termed hippocampal neurones.

Ability of 5-HT to increase intracellular cAMP levels in hippocampal neurones and pharmacological definition of the receptor

5-HT displayed a concentration-dependent ability to increase cAMP levels in hippocampal neurones (pEC_{50} of 7.2±0.2; Hill slope of 1.2±0.2; Figure 2A). Neither the selective 5-HT_4 receptor antagonist, GR113808, nor the selective 5-HT_6 receptor antagonist, SB399885 (either at concentrations up to 1.0 µM) prevented the ability of a sub-maximal concentration of 5-HT (100 nM) to increase cAMP levels (the increase in cAMP levels evident at the relatively high micromolar concentrations of SB399885 may be a consequence of engagement with additional receptors; Hirst et al, 2006). In contrast, the selective 5-HT_7 receptor antagonist, SB258719, prevented the ability of 5-HT (100 nM) to increase cAMP levels in a concentration dependent manner (pIC_{50} of 7.95±0.09, n=3; Figure 2B). Furthermore, SB258719 displayed competitive antagonism of the 5-HT-evoked response (pKb of 8.23±0.1; Figure 2C).

Influence of 5-HT_7 receptors upon pCREB levels in hippocampal neurones

5-HT (1.0 µM) induced an increase in relative pCREB levels (pCREB:CREB ratio) above basal levels although the signal was transient (evident at 5 min of incubation with 5-HT but

not at 10 minutes and longer; Figure 3A). Prior incubation with SB258719 (100 nM – 10 μM) inhibited the ability of 5-HT (5 min) to increase relative pCREB levels in a concentration-dependent manner (Figure 3B).

5-HT (1.0 μM) failed to modify phosphorylated p38 MAP kinase levels in the hippocampal neurones (Supplementary Figure S2). This contrasted the ability of 5-HT (1.0 μM) to increase phosphorylated p38 MAP kinase levels in rat primary astrocytes (Supplementary Figure S2), the latter being consistent with previous studies (e.g. Lieb et al., 2005).

Influence of 5-HT$_7$ receptors upon GluA1 expression and post-translational modification in hippocampal neurones

In hippocampal neurones, either 5-HT (1.0 μM; 5 min) or FSK (10 μM; 5 min) increased the relative total GluA1 protein level and to a greater extent the relative protein level of pGluA1 (Ser845) (Supplementary Figure 3; Figure 4).

The prior application of either GR113808 (up to10 μM) or SB399885 (up to 10 μM) failed to prevent the impact of 5-HT upon relative total GluA1 or pGluA1 (Ser845) protein levels, whereas SB258719 (10 μM) reduced the ability of 5-HT to increase relative pGluA1 (Ser845) protein levels (Figure 4) although not total GluA1 protein levels (Figure 4B).

Similar to 5-HT, the selective 5-HT$_7$ receptor agonist AS-19 (1.0 μM) increased relative pGluA1(Ser845) levels and the action of AS-19 was prevented completely by prior application of SB258719 (10 μM; Figure 4). However, AS-19 (1.0 μM) did not increase total GluA1 protein levels (Figure 4C). This lack of activity associated with AS-19, and the failure of SB258719 (10 μM) to prevent the 5-HT-induced increase in total GluA1 protein levels suggests the latter effect was not mediated via the 5-HT$_7$ receptor.

Influence of 5-HT$_7$ receptors upon cell surface GluA1 AMPA receptor expression in hippocampal neurones

Treatment of hippocampal neurones with 5-HT (1.0 μM) increased cell surface membrane expression of pGluA1(Ser845) for the three time points investigated (5, 10 and 30 min; Figure 5). In contrast, no difference in cell surface levels of GluA1 was detected (Figure 5).

The ability of 5-HT (1.0 μM) to increase cell surface membrane expression of pGluA1(Ser845) was prevented by prior application of either SB258719 (10 μM; Figure 6) or the protein kinase A inhibitor, H89 (10 μM; Figure 6). As demonstrated in other experiments, SB258719 (10 μM) also inhibited the 5-HT-induced increase in pGluA1(Ser845) in total cell extracts, as did H89 (10 μM; Figure 6).

Ability of PDE_4 and PDE_{10} to regulate 5-HT-induced pGluA1(Ser845) in hippocampal neurones

In hippocampal neurones, either the phosphodiesterase (PDE4) inhibitor, rolipram (10 μM) or the PDE10 inhibitor, papaverine (10 μM) increased pGluA1(Ser845) levels (Supplementary Figure 4), which were further increased by combination with 5-HT (1.0 μM) to levels above those detected with 5-HT (1.0 μM) alone (Supplementary Figure 4). In contrast, neither rolipram nor papaverine impacted significantly total GluA1 when applied alone or in combination with 5-HT (Supplementary Figure 4).

Ability of the 5-$HT_{1A/7}$ receptor agonist 5-CT to alter the firing pattern of hippocampal CA3 neurones in vivo

Extracellular recordings were performed in anaesthetized rats (n=12) examining the effects of iontophoretic administration of the 5-$HT_{1A/7}$ receptor agonist, 5-CT (for electrode placement see Supplementary Figure 5). In all, recordings were made from 16 hippocampal CA3 pyramidal neurones, of which 50% (8/16) were active (firing rate of 7.9±3.0 Hz), whilst the remaining were quiescent. In 7 of these active neurones, 5-CT administration was tested on baseline activity, with neuronal activity significantly reduced from 15.1±2.8 to 9.7±3.7 Hz in 4 of these 7 neurones, and activity increasing from 0.8±0.4 to 4.9±1.7 Hz in the remaining 3 neurones (Figure 7A and 7C). To remove the potentially confounding effect of 5-HT_{1A} receptor activation by 5-CT, the selective 5-HT_{1A} receptor antagonist, WAY-100,635, was applied to 7 neurones, which revealed an almost exclusively excitatory response to 5-CT; the firing rate of 6 of these 7 neurones increasing significantly from a mean of 6.2±2.2 to 11.5±2.5 Hz (P<0.05, n=6, Figure 7A and 7D). In only a single neurone (1/7) was activity reduced (from 12.7 to 6.2 Hz; Figure 7A).

The impact of 5-CT administration was also examined on bursts of action potential firing of hippocampal CA3 pyramidal neurones induced by cyclical administration of AMPA. When tested with 5-CT alone, AMPA-evoked firing was significantly reduced in the majority (6/7) of neurones (from 29.0±12.0 to 13.6±6.2 Hz; Figure 7B) and in the remaining neurone firing increased from 6.3 to 7.9 Hz (Figure 7B). However, in the presence of the selective 5-HT_{1A} receptor antagonist WAY-100,635, a predominant excitatory action of 5-CT was revealed, with AMPA-induced neuronal firing increased significantly in 5 of 7 neurones (from 28.6±8.7 to 50.1±13.8 Hz; Figure 7B and 7E) with the activity of the remaining 2 neurones being reduced from 13.2 to 3.9 Hz (Figure 7B).

Ability of the 5-HT_7 receptor to modify synaptic input into hippocampal CA3 pyramidal neurones in vitro

To determine whether 5-HT_7 receptor activation could modify the magnitude of AMPA receptor-mediated synaptic input to CA3 pyramidal neurones, whole-cell patch-clamp recordings were performed from rat hippocampal slices, electrically stimulating mossy fibre inputs to evoke EPSCs (for electrode placement see Supplementary Figure S5). In the presence of the 5-HT_{1A} receptor antagonist, WAY100,635 (1.0 μM), the 5-$HT_{1A/7}$ receptor agonist, 5-CT (10 μM) revealed a mixed response with EPSC amplitude reduced to 62.4±1.6% of control levels in 3 of 9 neurones (from 77.2±23.0 to 48.8±15.6 pA; Figure 8A, 8C top and 8D), and enhanced to 150.8±14.5% of control levels in 6 of 9 neurones (from

69.6±15.2 to 101.9±20.5 pA; P<0.05, n=6, Figure 8B, 8C bottom and 8D). Both these responses partially recovered to pre-5-CT application levels upon washout of 5-CT (to 72.4±24.3 and 86.2±17.5 pA, respectively). EPSCs were confirmed as AMPA receptor-mediated via administration of the selective AMPA receptor antagonist, NBQX (10 μM) which abolished EPSC amplitude (Figure 8A, 8B, 8C and 8D).

Further patch-clamp experiments were performed to determine the effects of 5-HT_7 receptor activation upon isolated postsynaptic AMPA receptor-mediated activity by examining the effects of 5-CT (10 μM) administration, in the presence of tetrodotoxin (1.0 μM) and WAY-100,635 (1.0 μM), upon short periods of depolarisation induced by brief perfusion with AMPA (20 μM). These current-clamp experiments revealed an almost exclusive augmentation of AMPA-induced depolarisation by 5-CT (10 μM), with the AUC of 8 neurones enhanced significantly from 1169±167 to 1827±284 $V.ms^{-1}$ (156.8±10.4% of control; P<0.01, n=8, Figure 9A, 9B and 9D), with recovery following washout of 5-CT (to 1262±236 $V.ms^{-1}$, 104.7±10.0% of pre-5-CT application; Figure 9D). In 3 neurones, AUC analysis revealed a similar mean control 5-CT increase to 157.2 ± 4.7% of AMPA-responses (n=3), which was reduced to 111.0±14.7%, when repeated in the added presence of the 5-HT_7 receptor antagonist SB258719 (1.0 μM; Figure 9C and 9D).

Discussion

The present studies originated to better understand the mechanisms that might underlie the ability of the 5-HT_7 receptor to modulate glutamate-mediated neurotransmission (Bacon and Beck, 2000; Bickmeyer et al., 2002; Gill et al., 2002; Tokarski et al., 2003; 2005; Costa et al., 2012), which may have relevance to the ability of the 5-HT_7 receptor to modulate memory and learning (Meneses, 2004; Perez-Garcia and Meneses, 2005; Ballaz et al., 2007; Eriksson et al., 2008; Sarkisyan and Hedlund, 2009; Horisawa et al., 2011; Waters et al., 2012; Freret et al., 2014) and relevant physiological phenomena such as LTP.

Initially our investigations used primary cultures of rat hippocampus under suitable conditions to favour the culture of neurones. The cultured hippocampal neurones expressed 5-HT_7 receptor transcripts and pharmacological studies demonstrated that the 5-HT_7 receptor increased cAMP levels as well as the phosphorylation of the downstream signalling transcription factor, CREB (cAMP response element-binding protein). A key finding in the present study was that 5-HT_7 receptor activation signalled via the cAMP-dependent kinase, protein kinase A (PKA), via phosphorylation of serine 845 within the C-terminal tail of the GluA1 AMPA receptor subunit. This post-transcriptional modification promoted expression of the phosphorylated GluA1-S845 subunit in the neuronal surface membrane.

Glutamate AMPA receptors are the primary means of relaying excitatory synaptic neurotransmission and regulation of their function impacts synaptic plasticity and associated memory function. It is well established that phosphorylation of AMPA receptor subunits modifies their activity (e.g. Song and Huganir, 2002); the GluA1 subunit forms homomeric receptors and also contributes a major component of the predominant heteromeric AMPA receptor (GluA1/2) in the hippocampus (Lu et al., 2009). The GluA1 AMPA receptor subunit possesses two phosphorylation sites (serine 831 and 845; S831 and S845); S831 is a substrate for calcium/calmodulin-dependent protein kinase (CaMKII) and protein kinase C (Roche et al., 1996; Barria et al., 1997; Mammen et al., 1997) whereas S845 is a substrate for PKA (Roche et al., 1996). Relevant to the present study, phosphorylation of S845 within GluA1 subunits by directly enhancing cAMP levels (via either direct stimulation of adenylate

cyclase by forskolin or inhibition of phosphodiesterase [PDE] by rolipram) promotes cell surface expression of the phosphorylated S845 GluA1 subunit (Esterban et al., 2003; Oh et al., 2006) and promotes AMPA receptor function (Roche et al., 1996; Lee et al., 2000; Oh et al., 2006; He et al., 2009; Makino and Malinow, 2009). In the present studies activation of the 5-HT_7 receptor increased cell surface expression of phosphorylated S845 GluA1 via a PKA-dependent mechanism, consistent with the 5-HT_7 receptor elevating cAMP levels. The involvement of the 5-HT_7 receptor was indicated by a number of lines of evidence. Thus, 5-HT_7 receptor transcripts were expressed by the primary neurones in culture and their functional involvement verified pharmacologically by the comparable action to 5-HT (pEC_{50} = 7.2) of the selective 5-HT_7 receptor agonist AS-19 at a just-maximal concentration to activate the 5-HT_7 receptor (1.0 μM; Brenchat et al., 2009); the apparent lower efficacy of AS-19 compared to 5-HT may reflect the partial agonist action of AS-19 demonstrated in other assays *in vitro* (Brenchat et al., 2009). Furthermore, the relatively selective 5-HT_7 receptor antagonist, SB258719 (Thomas et al., 1998; 1999) antagonised the 5-HT-evoked responses; SB258719 also displayed competitive receptor antagonism of the 5-HT-induced increase in cAMP levels within the primary hippocampal cultures generating a pKb of 8.23, consistent with the affinity of SB258719 for the 5-HT_7 receptor (Thomas et al., 1998; 1999). Furthermore, two additional 5-HT receptor subtypes known to increase cAMP production, the 5-HT_4 and 5-HT_6 receptors, did not appear relevant to the responses detected in the present studies since the selective 5-HT_4 and 5-HT_6 receptor antagonists, GR113808 (Gale et al., 1994) and SB399885 (Hirst et al., 2006), failed to inhibit 5-HT-induced elevated cAMP levels in the hippocampal primary cell cultures at pharmacologically relevent concentrations (upto 1 μM). Involvement of cAMP was also indicated by the effect of phosphodiesterase inhibition; thus 5-HT_7 receptor-mediated phosphorylation of GluA1 was increased by the non-selective PDE4 and PDE10 inhibitors, rolipram and papaverine, respectively, with both PDE4 and PDE10 known to be expressed in hippocampus (Menniti et al., 2006).

Consistent with the biochemical studies demonstrating the 5-HT_7 receptor promoted expression of phosphorylated GluA1-S845 subunit in the neuronal membrane of cells derived from all fields of the hippocampus, electrophysiological recordings in the CA3 field of the hippocampus *in vivo* revealed 5-HT_7 receptor activation increased spontaneous activity and enhanced AMPA-induced increases in action potential firing. Furthermore, electrophysiolgical recordings *in vitro* demonstrated that whilst selective AMPA receptor-mediated synaptic transmission via mossy-fibre inputs to CA3 pyramidal neurones could be enhanced or depressed in the presence of 5-CT, AMPA-induced whole-cell postsynaptic currents were consistently increased by 5-HT_7 receptor activation.

The present studies confirmed the role of 5-HT_7 receptors in the regulation of CREB phosphorylation in hippocampal neurones (Mahgoub et al., 2006). In contrast, no effect was apparent by 5-HT upon p38 MAP kinase in the same preparation, despite studies run in parallel that demonstrated an effect of 5-HT to increase p38 MAP kinase phosphorylation in rat cortical astrocytes in culture, which also expressed 5-HT_7 receptor mRNA (data not shown). The latter is consistent with previous studies (Lieb et al., 2005; Mahe et al., 2005) and hence our data suggests differential cell signaling for the 5-HT_7 receptor in neurones compared to astrocytes.

As well as the relatively short-term actions of 5-HT_7 receptors investigated in the present studies, relevant longer term actions mediated via the 5-HT_7 receptor are also evident including elevation in glutamate levels *in vivo* indicating enhanced excitatory synaptic tone (Canese et al., 2015) and plastic neuronal remodelling (Kvachnina et al., 2005; Kobe et al., 2012; Speranza et al., 2013; Canese et al., 2015), which may underlie structural changes

associated with memory formation.

Interestingly, a previous report suggests the 5-HT_{1B} receptor evokes CaMKII mediated phosphorylation of the GluA1 subunit at a further phosphorylation site in the C-terminus, S831, which also contributes to 5-HT-induced potentiation of neurotransmission via a post-synaptic mechanism (Cai et al., 2013).

In summary, the present study has demonstrated that 5-HT_7 receptor activation in rat hippocampal neurones signals a protein kinase A-dependent phosphorylation of serine 845 within the GluA1 AMPA receptor subunit that results in increased levels of phosphorylated S845 GluA1 subunits within the neuronal membrane that likely manifests the evident enhanced AMPA receptor mediated neurotransmission within the rat hippocampus *in vitro* and *in vivo*.

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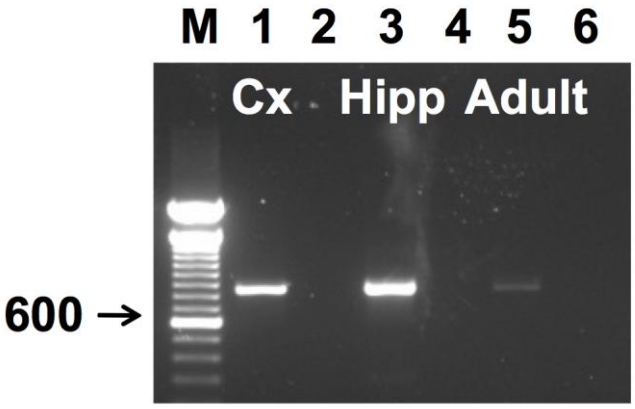

Figure 1. Expression of the 5-HT_7 receptor by hippocampal primary cells in culture in comparison to expression by primary cerebral cortex cells and adult rat hippocampal tissue. Representative RT-PCR of cDNA products of around 850 bp from adult rat hippocampi (Adult; lanes 5 and 6) and primary cells cultured from the hippocampi (Hipp; lanes 3 and 4) and cerebral cortices (Cx; lanes 1 and 2) of E18/19 rat embryos. Lanes 2, 4 and 6 show lack of product when the enzyme was omitted from the RT step. Each line of the ladder (M) represents a band size increase of 100 bp. Similar results were obtained from three independent experiments.

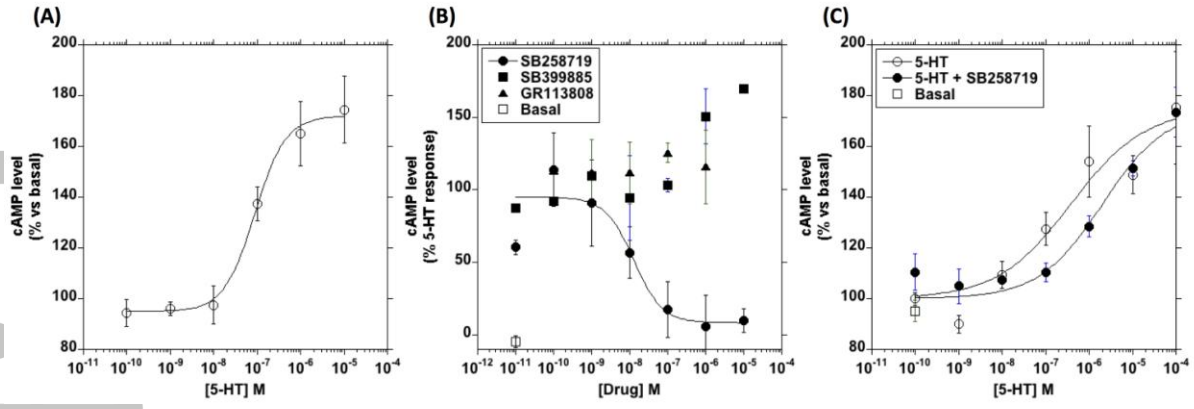

Figure 2. Ability of the 5-HT_7 receptor to increase cAMP levels in hippocampal neurones. A: Concentration-dependent ability of 5-HT to increase intracellular cAMP levels. B: Concentration-dependent ability of the selective 5-HT_7 receptor antagonist, SB258719, to inhibit the 5-HT (100 nM) induced increase in cAMP levels and lack of inhibition by the selective 5-HT_4 and 5-HT_6 receptor antagonists (GR113808 and SB399885, respectively). Data represent the percentage of cAMP increase after 5-HT addition vs. basal levels (mean ± SEM, n=3). C: Concentration-dependent ability of 5-HT to increase intracellular cAMP levels in the absence and presence of the selective 5-HT_7 receptor antagonist, SB258719 (100 nM); the rightward shift in the 5-HT concentration-response curve is indicative of competitive antagonism by SB258719. Data represent mean ± SEM, n=3.

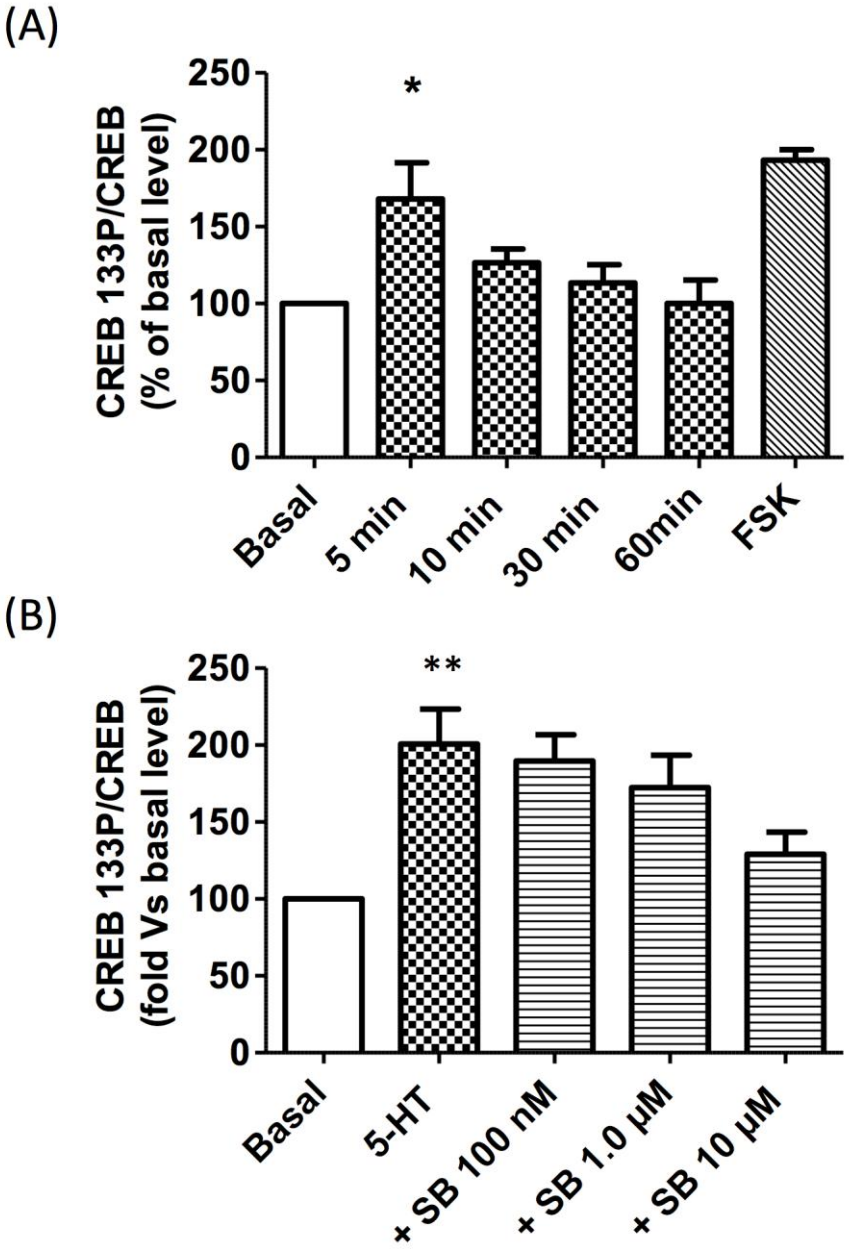

Figure 3. Ability of the 5-HT_7 receptor to increase CREB phosphorylation in hippocampal neurones. A: Time-dependent effect of 5-HT (1.0 µM) upon CREB phosphorylation (forskolin [10 µM] applied for 30 min). Data were the mean±SEM of eight (5-HT [5 min]) or three (5-HT [10-60 min] or forskolin [FSK]) independent experiments and represent the pCREB/total CREB ratio of treated cells vs. basal levels (**P*<0.05, ***P*<0.01 5-HT [5 min] vs. basal levels; Mann-Whitney U test). B: Concentration-dependent ability of the selective 5-HT_7 receptor antagonist, SB258719, to inhibit 5-HT (1.0 µM; 5 min) induced CREB phosphorylation. Data represent the mean±SEM from three independent experiments.

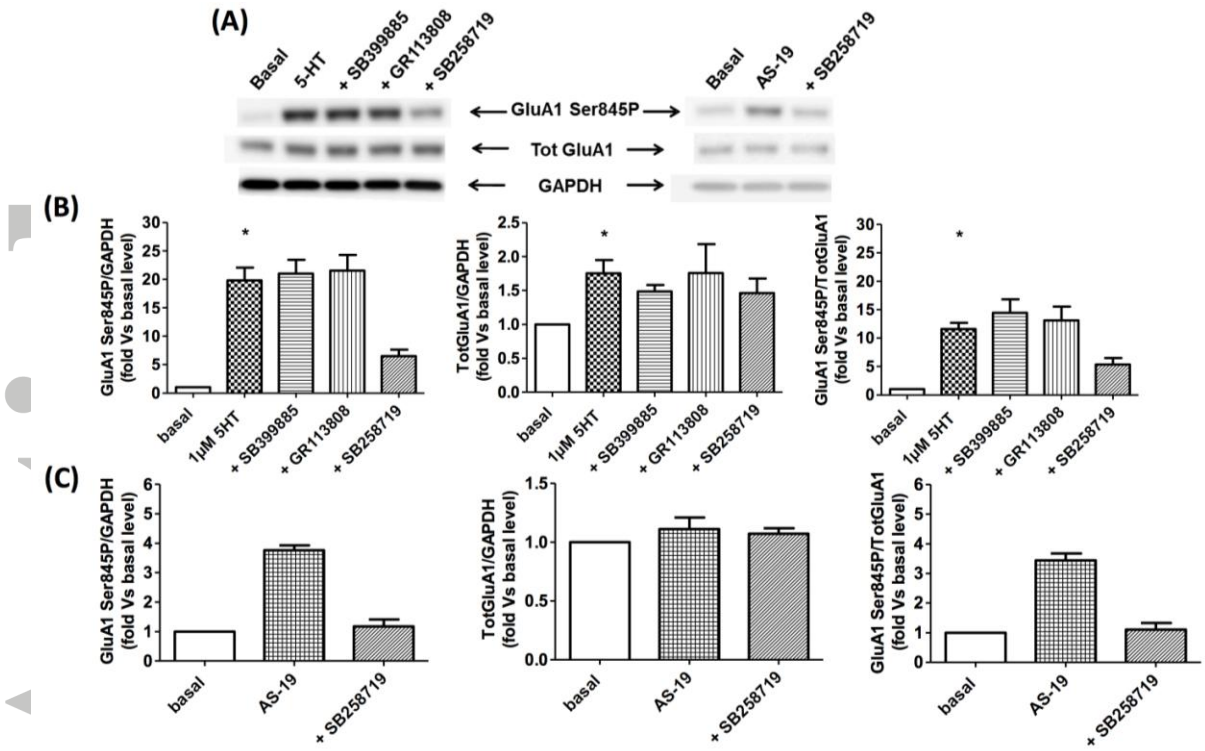

Figure 4. 5-HT_7 receptor activation increases phosphorylation of GluA1 at serine^{845} in hippocampal neurones A: Representative immunoblots of hippocampal neurones treated for 15 min with vehicle (DMSO 0.1%), GR113808 (1.0 µM; 5-HT_4 receptor antagonist) SB399885 (1.0 µM; 5-HT_6 receptor antagonist), or SB258719 (10 µM; 5-HT_7 receptor antagonist) followed by treatment with 5-HT (1.0 µM; left hand panel) or AS19 (1.0 µM; right hand panel) for 5 min. B-C: Quantification of pGluA1 (Ser845) and total GluA1. Ratio of pGluA1 (Ser845)/GAPDH (glyceraldehyde 3-phosphate dehydrogenase), total GluA1/GAPDH and pGluA1 (Ser845)/total GluA1 are expressed as fold increase ± SEM with respect to basal levels from seven (5-HT), six (5-HT plus SB258719), four (AS-19) or three independent experiments ($P<0.01$ Kruskal-Wallis ANOVA; *$P<0.05$ versus basal Mann-Whitney U test).

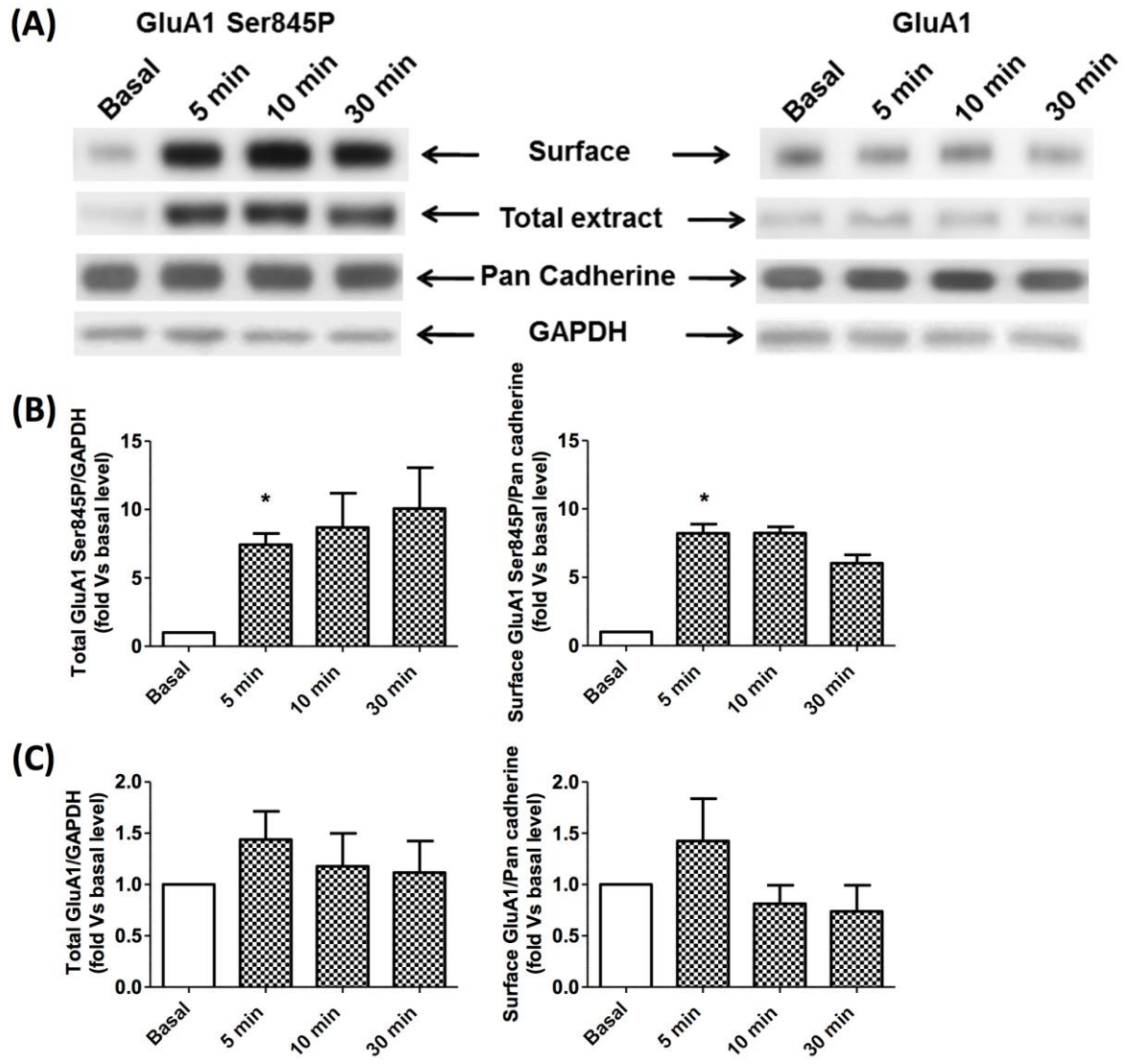

Figure 5. Ability of 5-HT to affect cell surface membrane expression of pGluA1 (Ser845). A: Representative immunoblots of neurones treated with 5-HT (1.0 μM) for 5-30 mins. B-C: Quantification of pGluA1 (Ser845) and total GluA1 in the total cell extract (left) and in the surface fraction (right). Amount of proteins were normalised using GAPDH and pan-cadherine in the total cell extract and in the cell surface fraction, respectively. Value are expressed as fold increase ± SEM with respect to basal levels from seven (5-HT [5 min]) or four independent experiments (**P*<0.01 5-HT [5 min] vs. basal; Mann-Whitney U test).

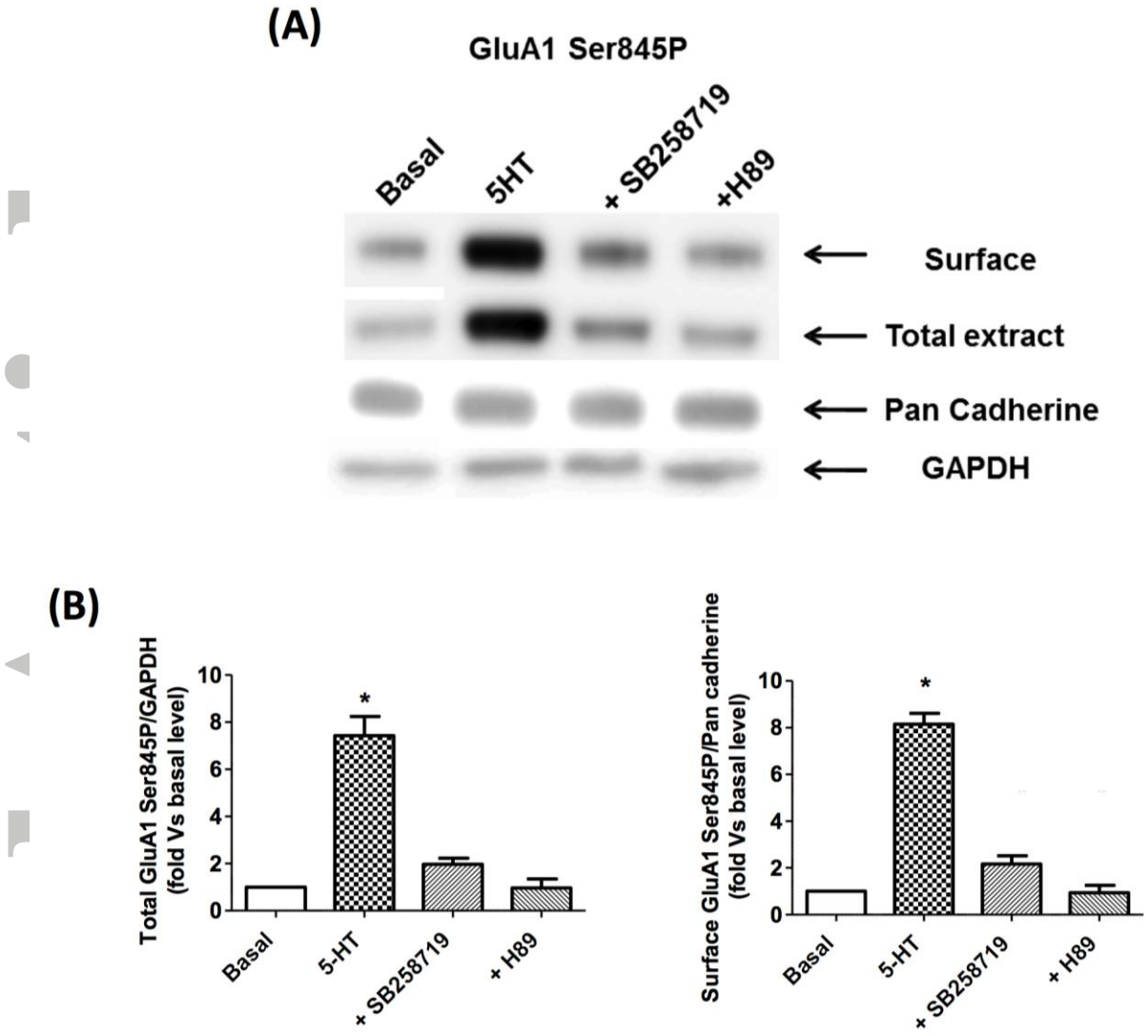

Figure 6. The 5-HT$_7$ receptor increases cell surface expression of GluA1(Ser845) via protein kinase A in hippocampal neurones A: Representative immunoblots of hippocampal neurones treated with vehicle (Basal and 5-HT) SB258719 (10 µM) or H89 (10 µM) for 15 min followed by addition of 5-HT (1.0 µM) or vehicle (Basal) treatment for 5 min. B Quantification of pGluA1(Ser845) levels in total cell extracts and in cell surface fractions. Amount of proteins were normalised using GAPDH and pan-cadherin in the total extract and in the cell surface fraction, respectively. Values are expressed as fold increase ± SEM with respect to basal levels from seven (5-HT; **P*<0.01 5-HT vs. basal, Mann-Whitney U test) or three independent experiments .

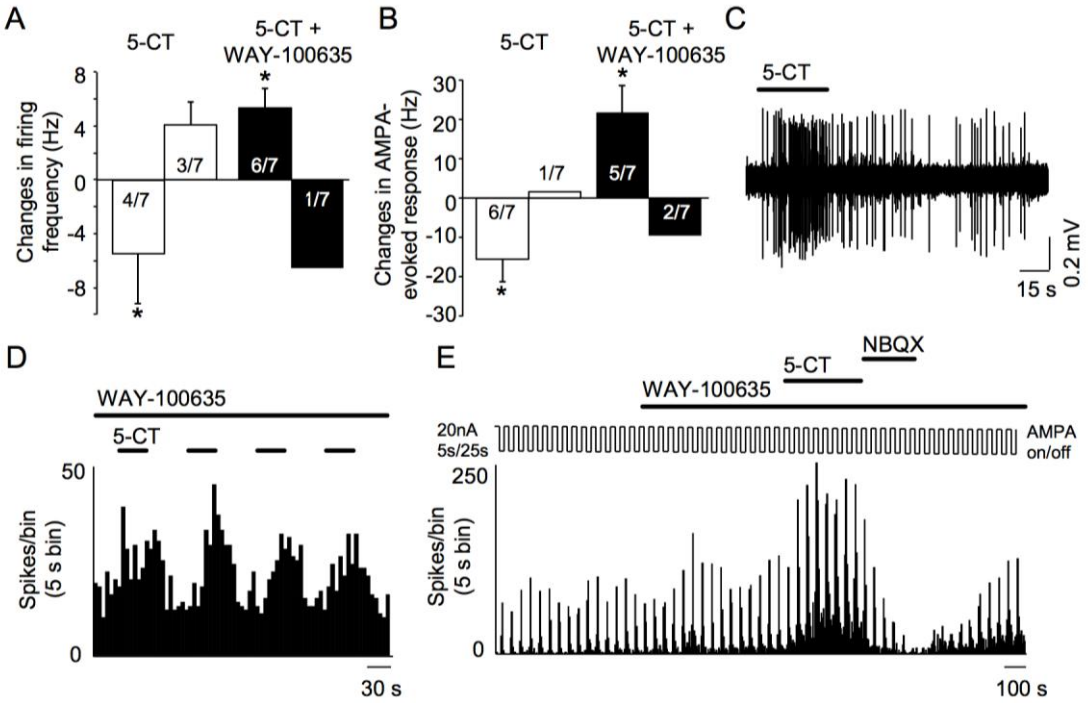

Figure 7. Iontophoretic administration of the 5-$HT_{1A/7}$ receptor agonist 5-CT induced a predominant excitation in the baseline and AMPA-induced firing of hippocampal CA3 pyramidal neurones in the presence of the 5-HT_{1A} receptor antagonist WAY-100,635, *in vivo*. A: Group data revealed a mixed excitatory/inhibitory response to 5-CT administration on baseline neurone firing in the absence of WAY-100,635, which was modified to an almost exclusively excitatory response during 5-HT_{1A} receptor blockade; neurone numbers are overlaid histograms of change in firing rate (in Hz). B: Similarly, in the absence of WAY-100,635 5-CT application depressed AMPA-evoked firing, whereas in the presence of WAY-100,635, 5-CT predominantly enhanced AMPA-evoked bursts of activity; neurone numbers are overlaid histograms of change in firing rate (in Hz). C-E: Typical examples of 5-CT inducing: an increase in baseline neuronal activity in the absence of WAY-100,635 (C); an increase in neuronal activity in the presence of WAY-100,635 (D); an enhancement of AMPA-evoked firing in the presence of WAY-100,635, with demonstration of AMPA receptor mediation using NBQX also shown (E). * P<0.05 vs. basal levels; paired student's t-test.

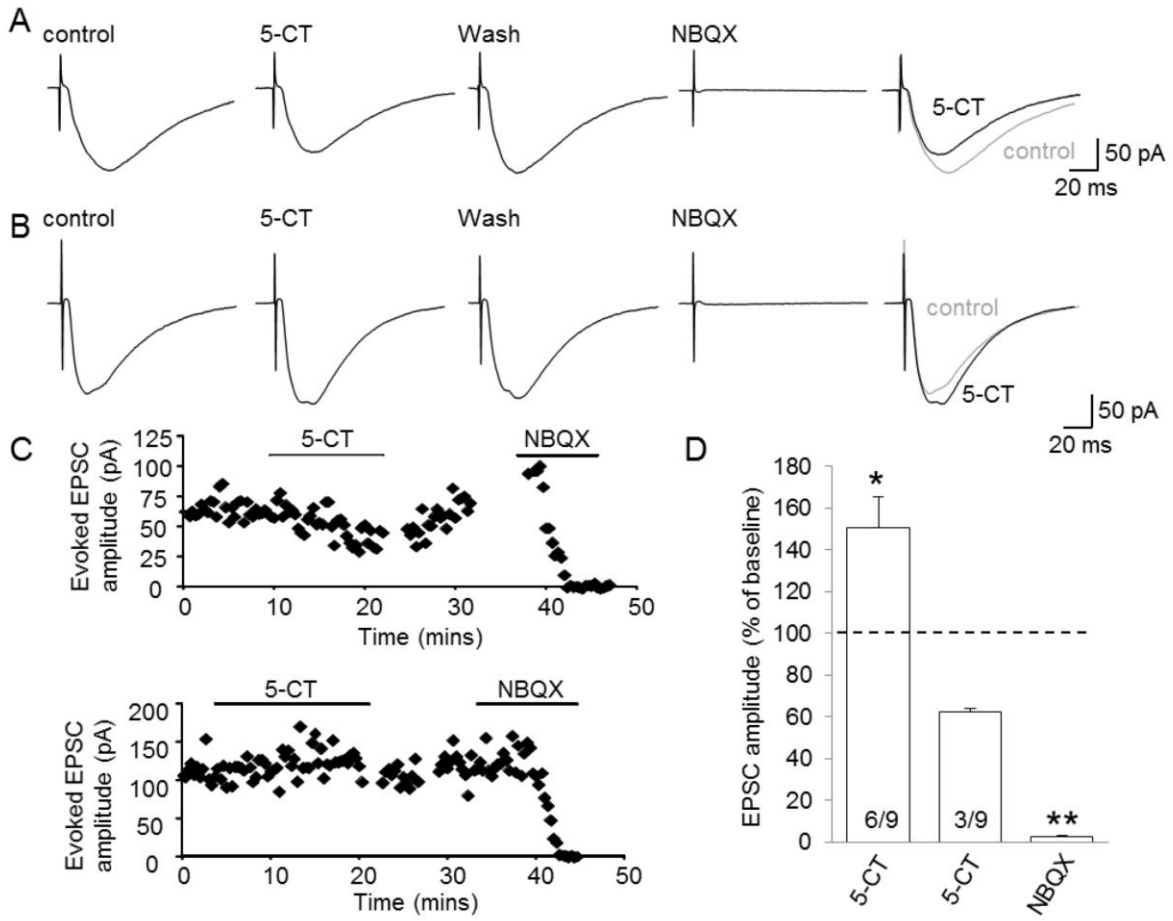

Figure 8. The 5-$HT_{1A/7}$ receptor agonist 5-CT induced both enhancements and reductions in the amplitude of electrically-evoked AMPA-mediated EPSCs, in hippocampal CA3 pyramidal neurones in the presence of the 5-HT_{1A} receptor antagonist WAY-100,635, *in vitro*. Typical CA3 pyramidal neurone in which 5-CT induced a reduction (A) and an enhancement (B) in the magnitude of EPSCs, recorded in the presence of AP-5, Bicuculline, CGP55845 and WAY-100,635, with typical traces during baseline, 5-CT, wash and NBQX shown, each trace is the average of 5 sweeps. C: Scatter plots representing EPSC amplitude over time of two CA3 pyramidal neurones in which 5-CT induced a reduction (top) and an enhancement (bottom), with the subsequent administration of NBQX also shown. D: Group data of the effect of 5-CT and NBQX on AMPA-mediated EPSC activity in the presence of WAY-100,635; neurone numbers are overlaid histograms of change in EPSC amplitude as a percentage of baseline. * P<0.05 vs. basal levels; paired student's t-test.

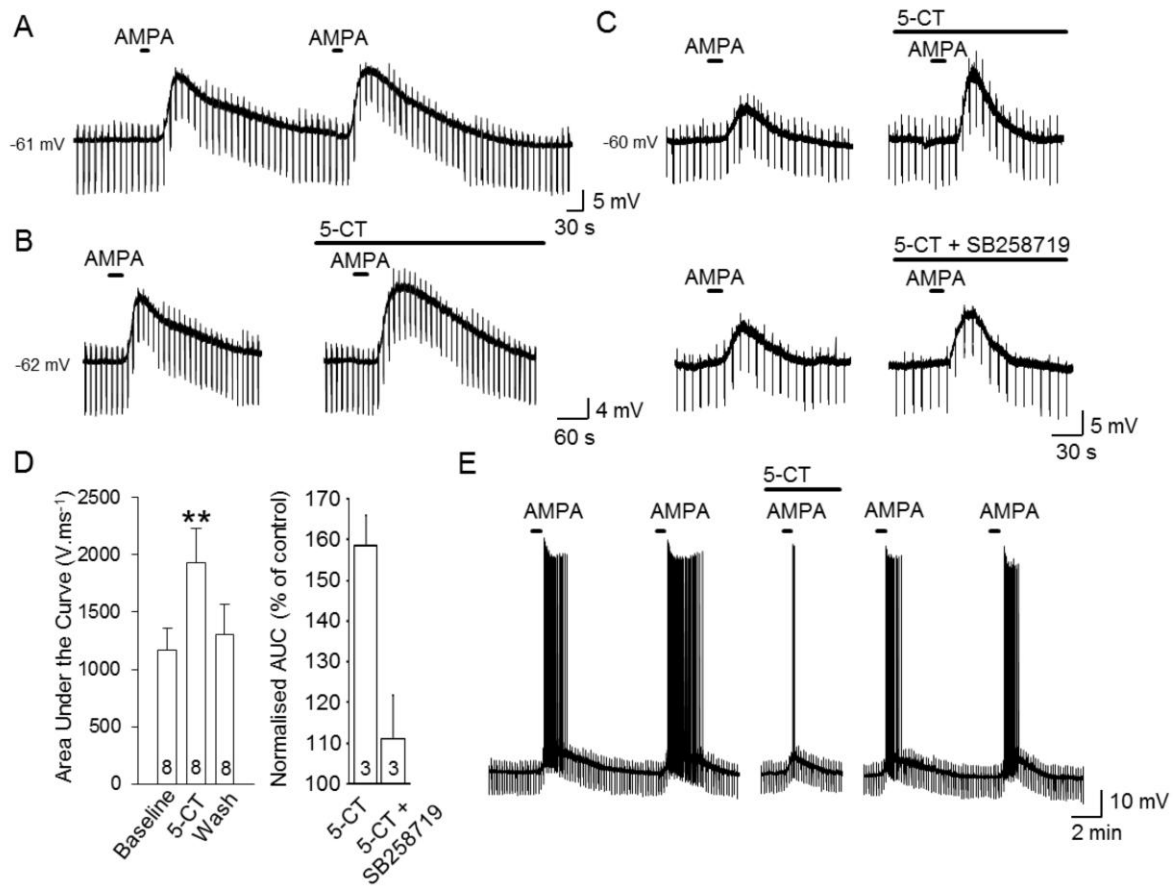

Figure 9. The 5-$HT_{1A/7}$ receptor agonist 5-CT enhanced excitatory responses of hippocampal CA3 pyramidal neurons *in vitro* induced by short-term application of AMPA, in the presence of the 5-HT_{1A} receptor antagonist and the sodium channel blocker, WAY-100,635 and tetrodotoxin, respectively. The 5-CT-induced enhancement was antagonized by the 5-HT_7 receptor antagonist, SB258719. A-C: CA3 pyramidal neurones recorded in current-clamp in the presence of WAY-100,635 and TTX demonstrating A: Repeatable AMPA perfusion-induced depolarisations; B: The enhancement in AMPA responses induced by 5-CT and C: The reduced enhancement in the added presence of SB258719. D: Left: group data of the effect of 5-CT on the AMPA-evoked excitation of six CA3 pyramidal neurones, in the presence of WAY-100,635 and TTX and measured using area under the curve analysis. Right: group data of the normalised increase in AMPA-evoked responses induced by 5-CT in the absence and presence of SB258719 recorded from 3 of these neurons. E: A CA3 pyramidal neurone in which the effect of AMPA has been examined in the absence of WAY-100-635 and TTX. ** P<0.05/0.01 vs. basal levels; paired student's t-test.