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Serotonin excites hippocampal CA1 GABAergic interneurons at the stratum radiatum-stratum lacunosum moleculare border

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Abstract

The hippocampus receives robust serotonergic innervation that is thought to control the excitability of both pyramidal cells and GABAergic interneurons. Previous work has addressed serotonergic regulation of pyramidal cells but considerable gaps remain in our understanding of how serotonin regulates different interneuron subclasses. 5-HT_{2A} receptors (5-HT_{2A}Rs) appear to localize predominantly, if not solely, on interneurons in the hippocampus and have been implicated in the regulation of hippocampal function including mnemonic and novelty recognition processes. Interneurons are functionally diverse. Therefore in the current work, we have used a BAC transgenic mouse line expressing EGFP under the control of the 5-HT_{2A}R promoter to identify the interneuron subtype(s) regulated by serotonin via 5-HT_{2A}Rs. We find that EGFP expression in this mouse identifies a group of interneurons that resides predominantly along the border of the stratum radiatum (SR) and stratum lacunosum moleculare (SLM) of the CA1 region. We then show that these cells are depolarized and excited by serotonin acting through 5-HT_{2A}Rs and appear to belong predominantly to the perforant pathway-associated and Schaffer collateral/commissural pathway-associated subtypes. These results indicate that serotonin interneurons expressing 5-HT_{2A}Rs are localized primarily along the SR-SLM border of the CA1 region and represent a newly identified target for serotonin regulation in the hippocampus.

Keywords

5-HT; hippocampus; GABAergic interneuron; 5-HT_{2A} receptors; SR-SLM border

The mammalian hippocampus is densely innervated by serotonergic fibers originating from the midbrain raphe nuclei (Azmitia and Segal, 1978; Moore and Halaris, 1975). Previous studies have shown that within the CA fields, both pyramidal cells and GABAergic interneurons express serotonin receptors (Andrade, 1998a; Pompeiano et al., 1994). Work during the last three decades has generated a rich understanding of the physiological effects signaled by serotonin through multiple serotonin receptors on pyramidal cells, especially in the CA1 field (Andrade, 1998b; Andrade and Beck, 2010; Barnes and Sharp, 1999; Nichols

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and Nichols, 2008). However, much less is known about the serotonergic regulation of GABAergic interneurons in this region. Previous studies have shown that a group of CA1 interneurons are excited by serotonin acting via ionotropic 5-HT₃ receptors (McMahon and Kauer, 1997; Ropert and Guy, 1991; Varga et al., 2009). In addition, we have previously reported that serotonin increases spontaneous IPSCs in pyramidal cells acting through metabotropic 5-HT₂ receptors, an effect that appears to reflect the excitation of one or more interneuron subtypes (Shen and Andrade, 1998). This is an intriguing finding since whole animal studies suggest an important role for 5-HT_{2A}Rs in the regulation of hippocampal function in memory retrieval and object recognition tests (Bekinschtein et al., 2013; Santini et al., 2011; Schott et al., 2011). Hippocampal GABAergic interneurons form a highly heterogeneous neuronal class comprised of multiple functionally distinct cell types (Freund and Buzsáki, 1996; Klausberger and Somogyi, 2008). Thus, to understand how serotonin acting via 5-HT_{2A}Rs regulates hippocampal circuits, it is essential to identify the interneurons regulated by these receptors. Therefore in the present work, we have taken advantage of the availability of a BAC transgenic mouse line engineered to express EGFP under the control of the 5-HT_{2A}R promoter to localize *Htr2A* gene-expressing neurons in the CA1 area and examine their regulation by serotonin.

Immunohistochemical localization of EGFP in the CA1 region of the *Htr2A*-EGFP mouse revealed labeling of neurons mostly in the SR and the SLM, with a particularly strong concentration at the SR-SLM border. In addition, a few EGFP immunoreactive cells were also detectable over the stratum oriens (SO) of the CA1-CA3 fields (Fig. 1A₁). The hippocampus receives robust serotonergic innervation originating from the midbrain raphe nuclei (Azmitia and Segal, 1978; Freund et al., 1990) that is particularly dense in the SLM of the CA1 region. Consistent with these findings, the EGFP-expressing cells at the SR-SLM border were found to be embedded in a dense plexus of serotonergic axons identified by the expression of the serotonin transporter (Fig. 1A₂).

To determine whether these interneurons responded to serotonin, we conducted whole-cell recordings in hippocampal slices and targeted EGFP-expressing interneurons using a combination of fluorescence and DIC microscopy. Previous work has shown that many interneurons in the CA1 region, including some located at the SR-SLM border, express 5-HT₃ receptors (McMahon and Kauer, 1997; Morales et al., 2004). Therefore, to simplify the analysis of the actions of serotonin on EGFP-expressing interneurons, these experiments were conducted in the presence of 1 μ M tropisetron to block 5-HT₃ receptors. As illustrated in Figure 1B and 1D, bath application of serotonin (30 μ M) elicited a strong depolarization in most of the interneurons tested (30/32 cells). Overall, 22 of the interneurons were depolarized beyond threshold resulting in sustained spiking (peak firing rate of 6.2 ± 0.3 spikes/s), while the eight remaining cells responded to serotonin with a subthreshold depolarization averaging 11.4 ± 0.8 mV. None of these interneurons spiked prior to serotonin application and the serotonin-induced depolarization/spiking recovered in the majority of cells within minutes of removal of the serotonin from the bath (Fig. 1B). The distribution of all the EGFP-expressing cells included in the present work is illustrated in Figure 1C.

To test the idea that the serotonin-induced depolarization observed in the EGFP-expressing interneurons was mediated by serotonin receptors of the 5-HT_{2A} subtype, we next examined

the effects of the preferential 5-HT_{2A}R agonist α -methylserotonin (α m-5-HT). As illustrated in Figure 2, administration of 30 μ M α m-5-HT depolarized and induced spiking in all 6 cells tested (Fig. 2A₁ and 2A₂, n=6, peak firing rate 8.1 ± 1.9 spikes/s), an effect that recovered within minutes after removing the agonist from the bath. In addition, when recording in voltage clamp, administration of 30 μ M α m-5-HT induced an inward current averaging 79.6 ± 9.2 pA (Fig. 2A₃, n=6 cells). These results suggested the involvement of a receptor in the 5-HT₂ family. Therefore we next examined the ability of the selective 5-HT_{2A}R antagonist MDL 100,907 to block the effect of serotonin. Because 5-HT_{2A}Rs have been reported to desensitize (Araneda and Andrade, 1991; Leysen et al., 1989), a potential confound for this experiment, we first tested the effect of consecutive applications of serotonin. All 7 cells tested in this experiment responded to the first administration of serotonin (30 μ M) with a strong depolarization that initiated sustained spiking. Upon a second administration of serotonin, 6 out of these 7 cells responded with a comparable excitation while the remaining cell was depolarized by 11 mV (Fig. 2B). These results suggested limited desensitization of the serotonin response under our experimental conditions. To test for the involvement of 5-HT_{2A}Rs we used MDL 100,907 at 100 nM, a concentration that should greatly inhibit 5-HT_{2A}R mediated responses but should have only minimal effects on responses mediated via 5-HT_{2B} or 5-HT_{2C} receptors (Baxter et al., 1995). We administered the antagonist between the first and the second applications of serotonin and allowed it to equilibrate for ~ 10 min. As illustrated in Figure 2C, the first serotonin administration excited all 4 cells tested, while the second administration of serotonin, now in the presence of MDL 100,907, failed to excite any of the cells tested and elicited only a small and variable depolarization averaging 2.8 ± 1.3 mV. This residual depolarization could reflect an incomplete blockade of the antagonist or conceivably the presence of an additional serotonin receptor. Altogether, these results point to a key role for 5-HT_{2A}Rs in the depolarization/excitation elicited by serotonin in these cells.

The experiments above identified a strong serotonergic regulation of a subgroup of CA1 interneurons located at the SR-SLM border. Previous studies have identified several interneuron subtypes in this region (Capogna, 2011; Klausberger, 2009; Vida et al., 1998). Since an interneuron's electrophysiological properties provide important clues to its identity (Ascoli et al., 2008; Klausberger and Somogyi, 2008), we examined the passive and active membrane properties of EGFP-expressing cells of the SR and SLM in the CA1 region. The results of this analysis in 53 neurons are summarized in Table 1. Altogether, the electrophysiological properties of these cells resemble those previously reported for several classes of SR-SLM interneurons of the CA1 region including Schaffer collateral/commissural pathway-associated and perforant pathway-associated interneurons (Lacaille and Schwartzkroin, 1988; Vida et al., 1998; Williams et al., 1994).

Hippocampal interneurons along the SR-SLM border are most readily identified based upon their pattern of dendritic and especially axonal arborizations (Capogna, 2011; Vida et al., 1998). Therefore, to identify the *Htr2A* gene-expressing interneurons more precisely, we recorded from EGFP-expressing interneurons using biocytin-containing electrodes and reconstructed them using HRP/DAB histochemistry. Upon light microscopic examination, satisfactory fills with extensive dendritic and axonal labeling were obtained from 10 cells. Eight cells were located in the border of the SR-SLM. Four of these cells extended axons

predominately along the SLM near the SR border and likely correspond to SR-SLM perforant path-associated interneurons (Fig. 3A). Three additional cells extended their axons predominantly into the SR and thus could be classified as apical dendrite projecting or SR-SLM Schaffer collateral/commissural pathway-associated interneurons (Fig. 3B). The last of these cells extended its axon into the SR, stratum pyramidale (SP) and SO and is not a clearly identifiable cell type but may correspond to a basket cell (Fig. 3C). The membrane and firing properties of all these filled SR-SLM cells were similar and matched those of the *Htr2A*-EGFP interneuron group as a whole (Fig. 3A-C, insets). Two additional satisfactory fills were obtained from cells located in the SR proper. The electrophysiological properties of one of these cells resembled those found along the SR-SLM border while the other fired as a stutterer. All the filled cells extended dendrites into both the SLM and SR and sometimes through the SP into the SO.

In the present study we identified a population of hippocampal interneurons in the SR-SLM border of the CA1 region that are regulated by serotonin. These cells are depolarized and excited by serotonin through the activation of 5-HT_{2A}Rs and correspond to at least two of the SR-SLM interneuron subtypes previously identified in this stratum. Since SR-SLM interneurons receive and help integrate extrinsic input to the hippocampus, including from the entorhinal cortex, the identification of these cells suggests a role for serotonin acting via 5-HT_{2A}Rs in the control of extrinsic afferents to the CA1 region.

To localize cells expressing 5-HT_{2A}Rs in the CA1 region, we took advantage of the availability of a BAC mouse expressing EGFP under the control of the 5-HT_{2A}R promoter. Cells expressing EGFP were localized predominately in the SR-SLM border and were surrounded by SERT immunoreactive fibers suggesting that these cells are well placed to receive synaptic innervation from serotonergic neurons. The restricted expression of EGFP to a subset of GABAergic interneurons noted in this study is consistent with the overall low levels of 5-HT_{2A}R mRNA, 5-HT_{2A}R binding and 5-HT_{2A}R immunoreactivity, as detected using a 5-HT_{2A}R KO mouse-validated antibody, previously reported in the rodent hippocampus (Pompeiano et al., 1994; Tanaka et al., 2012; Weber and Andrade, 2010; Wright et al., 1995).

Electrophysiological recordings from EGFP-expressing cells along the SR-SLM border indicated that these interneurons are depolarized by serotonin as well as by α m-5-HT, and these effects are blocked by the selective 5-HT_{2A}R antagonist MDL 100,907. These results indicate that serotonin depolarizes and excites these cells by acting primarily through 5-HT_{2A}Rs and thus identify a discrete subgroup of interneurons in the CA1 region that are specifically regulated by serotonin acting via these receptors. The serotonin-induced excitation of these interneurons identified in the current study could account for the 5-HT₂ receptor-mediated increase in spontaneous GABAergic synaptic transmission previously detected in adult pyramidal cells of the CA1 region (Shen and Andrade, 1998).

Previous work has shown that serotonin excites a group of hippocampal interneurons in the SR and SR-SLM border through the activation of ionotropic 5-HT₃ receptors (McMahon and Kauer, 1997). While the identity of these interneurons was not established in the original study, subsequent work suggests that 5-HT_{3A} receptor mRNA is expressed in a variety of

interneurons derived from the caudal ganglionic eminence (CGE) (Chittajallu et al., 2013). In the current work we have shown that, similarly, serotonin excites a heterogeneous population of interneurons at the SR-SLM border via metabotropic 5-HT_{2A}Rs. It will be interesting to find out if these cells, as is the case with 5-HT₃ receptor-expressing interneurons, also share a common embryological origin. The availability of the *Htr2A*-EGFP and *Htr2A*-Cre driver mice should facilitate such studies.

Hippocampal interneurons are highly heterogeneous and include multiple anatomically, molecularly and functionally defined subtypes (Klausberger and Somogyi, 2008). Reconstruction of the axonal arborizations of biocytin filled EGFP-expressing interneurons indicated that most *Htr2A*-EGFP cells in the CA1 region correspond to perforant pathway-associated interneurons and Schaffer collateral/commissural pathway-associated interneurons. GABAergic interneurons of the SR-SLM border, including these two interneuron subtypes, are thought to help structure the integration of extrinsic inputs onto CA1 neurons, including those inputs originating from the entorhinal cortex (Capogna, 2011). Thus the current results suggest a role for serotonin and 5-HT_{2A}Rs in the regulation of these processes. More broadly, they add to our growing appreciation of how serotonin can regulate hippocampal networks through the cell-specific expression of different serotonin receptor subtypes.

Detailed Methods

Animals and brain slice preparation

All experiments described were conducted in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. BAC transgenic mice expressing EGFP under the control of the 5-HT_{2A}R promoter [strain Tg(*Htr2a*-EGFP)^{118Gsat/Mmcd}, GENSAT, *Htr2A*-EGFP mouse] were obtained from the NIH's Mutant Mouse Regional Resource Centers (MMRRC) and have been characterized previously (Weber and Andrade, 2010). Brain slices were prepared as previously described (Shen and Andrade, 1998; Villalobos et al., 2011). Briefly, male and female mice, postnatal day 15 to 32 (P15-P32), were anesthetized with isoflurane and decapitated. Brains were extracted and cooled in Ringer's solution of standard composition (in mM: 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 2.5 CaCl₂, 1 NaH₂PO₄, 26.2 NaHCO₃, and 11 glucose) supplemented with 10 mM HEPES and bubbled to saturation with 95% O₂ and 5% CO₂. The brains were blocked and horizontal slices (300 μ m nominal thickness) containing the hippocampus were obtained using a vibratome. Slices were immediately placed into oxygenated Ringer's solution at 33 $\pm$ 1 $^{\circ}$ C and then allowed to cool to room temperature and recover for at least 1 h before recording.

Electrophysiological recordings

Hippocampal slices were transferred one at a time to a recording chamber on the stage of an upright microscope (Olympus BX50WI). Slices were held in place on the chamber by a platinum and nylon grid and continuously perfused with Ringer's solution at 30 $\pm$ 1 $^{\circ}$ C bubbled to saturation with 95% O₂ and 5% CO₂. Slices were imaged using differential interference contrast (DIC) and fluorescence. *Htr2A* gene-expressing cells were identified

by their expression of EGFP. Patch electrodes were pulled from 1.2 mm o.d. borosilicate glass using a Flaming-Brown model P-97 horizontal puller (Sutter Instruments, Novato, CA) and filled with a potassium-based intracellular solution (composition in mM: 130 KMeSO₄, 5 KCl, 5 NaCl, 0.02 EGTA, 1 MgCl₂, 10 Na₂ phosphocreatine, 4 MgATP, 0.3 NaGTP, and 11 HEPES, pH 7.3-7.4). Patch electrodes filled with this intracellular solution exhibited resistances in the range of 2 to 4 MΩ. Electrical signals were recorded using an EPC10 amplifier (HEKA) and data were collected using Patchmaster (HEKA). Electrophysiological recordings were obtained from EGFP-expressing interneurons located predominantly along the border of the SR and SLM of the CA1 region. Experiments using serotonin were conducted in the presence of 1 μM tropisetron to block 5-HT₃ receptors (Richardson et al., 1985).

Immunohistochemistry and histology

Mice were deeply anesthetized with sodium pentobarbital and perfused transcardially with 4% paraformaldehyde. Brains were extracted and postfixed overnight in 4% paraformaldehyde at 4°C. After washing in PBS, horizontal or coronal 50 μm thick slices were obtained using a vibratome. Immunohistochemistry was performed as previously described (Weber and Andrade, 2010). Anti-EGFP primary antibodies were obtained from Invitrogen (rabbit) and from Aves (chicken). The goat anti-serotonin transporter (anti-SERT) antibody was obtained from Santa Cruz Biotechnology. This antibody labels serotonin fibers as indicated by the elimination of the staining by pretreatment of the animals with 5,7-dihydroxytryptamine (Xie et al., 2006). Secondary antibodies were obtained from Molecular Probes/Invitrogen (Alexafluor 488 or Alexafluor 568) and from Jackson Immunoresearch (Dylight 488 or Cy3).

For reconstructions of neuronal morphology, whole-cell recordings were conducted using an intracellular solution containing 0.25% biocytin. Following the recordings, slices were fixed overnight in 4% paraformaldehyde at 4°C and rinsed with PBS. Slices were then treated with a solution containing H₂O₂ for 30 min to inactivate endogenous peroxidases and then permeabilized using 1% Triton X-100. Slices were processed using a Vectastain ABC kit (Vector Labs) with nickel-enhancing diaminobenzidine (DAB) chromogen. Sections were then mounted, dehydrated and cleared in graded alcohol/xylene, and coverslipped using Krystalon. To analyze morphologically recovered cells, brain slices were first imaged using DIC as implemented in an Olympus Fluoview laser scanning confocal microscope equipped with an Argon laser. The obtained image stacks were then imported into NIH ImageJ, where the cells were reconstructed manually. Axons were differentiated from dendrites based on their anatomical characteristics such as the presence of a single, long, relatively uniform and thin process from or very near the soma and the presence of branches at greater angles than those attained by dendrites.

Reagents

Drugs were prepared as concentrated stock solutions in H₂O and kept frozen. Immediately before each experiment, drugs were thawed and administered to the slice dissolved in Ringer's solution at the concentrations stated in the text. All drugs were obtained from Sigma-Aldrich with the exception of tropisetron, which was a kind gift from Sandoz (Basel,

Switzerland), and MDL 100,907, which was a kind gift from Dr. K. C. Rice of the Laboratory of Medicinal Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD.

Electrophysiological parameters and data analysis

Only cells with stable recordings were used for data analysis. Input resistance (R_i) was calculated as the slope of the steady-state current-voltage (I - V) plot at steady-state after a series of hyperpolarizing current pulses of 600 ms duration, where the voltage used for the I - V plot was measured as the voltage deflection during the last 50 ms of the current pulse. The membrane time constant (τ_m) was estimated by fitting an exponential function to the voltage deflection elicited by 200 ms long hyperpolarizing current pulses in neurons devoid of observable inward rectification. The action potential threshold was calculated as the deflection of the membrane potential preceding the first spike which exceeded 75 mV/ms. Action potential amplitudes and half-widths were calculated based on the first spike elicited by depolarizing current steps. The action potential amplitude was measured from action potential threshold to the peak of the first action potential. The action potential half-width corresponds to the duration of the action potential at half-amplitude. A fast afterhyperpolarization (AHP) following the spike was measured from its peak amplitude to action potential threshold. Interspike interval (ISI) was calculated based on the time between action potentials and the instantaneous frequency of action potentials is reported as the reciprocal of the ISI. Spike frequency adaptation measurements were taken as the ratio of the first to the last ISI during spiking induced by a current pulse of an amplitude approximately equal to 2 $\times$ the current necessary to reach threshold. Data was analyzed using MATLAB (The Mathworks Inc.) and reported as mean $\pm$ SEM.

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Figure 1.

Figure 2.

Figure 3.

Table 1
Intrinsic electrophysiological properties of *Htr2A*-EGFP cells at the SR-SLM border of the CA1 region

Property	Value	n
Resting membrane potential (mV)	-63.1 ± 1.3	53
Input resistance (M Ω)	196.4 ± 11.0	53
Membrane time constant (ms)	17.5 ± 1.2	18
Action potential threshold (mV)	-38.1 ± 0.2	53
Action potential amplitude (mV)	82.1 ± 1.4	53
Action potential half-width (ms)	0.49 ± 0.03	53
Afterhyperpolarization (mV)	19.4 ± 0.9	53
Adaptation ratio	0.29 ± 0.03	53

Values are means $\pm$ SEM