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Median and Dorsal Raphe Neurons Are Not Electrophysiologically Identical

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Abstract

The dorsal (DR) and median raphe (MR) nuclei contain 5-hydroxytryptamine (serotonin, 5-HT) cell bodies that give rise to the majority of the ascending 5-HT projections to the forebrain limbic areas that control emotional behavior. In the past, the electrophysiological identification of neurochemically identified 5-HT neurons has been limited. Recent technical developments have made it possible to re-examine the electrophysiological characteristics of identified 5-HT- and non-5-HT-containing neurons. Visualized whole cell electrophysiological techniques in combination with fluorescence immunohistochemistry for 5-HT were used. In the DR, both 5-HT- and non-5-HT-containing neurons exhibited similar characteristics that have historically been attributed to putative 5-HT neurons. In contrast, in the MR, the 5-HT- and non-5-HT-containing neurons had very different characteristics. Interestingly, the MR 5-HT-containing neurons had a shorter time constant and larger afterhyperpolarization (AHP) amplitude than DR 5-HT-containing neurons. The 5-HT_{1A} receptor-mediated response was also measured. The efficacy of the response elicited by 5-HT_{1A} receptor activation was greater in 5-HT-containing neurons in the DR than the MR, whereas the potency was similar, implicating greater autoinhibition in the DR. Non-5-HT-containing neurons in the DR were responsive to 5-HT_{1A} receptor activation, whereas the non-5-HT-containing neurons in the MR were not. These differences in the cellular characteristics and 5-HT_{1A} receptor-mediated responses between the MR and DR neurons may be extremely important in understanding the role of these two 5-HT circuits in normal physiological processes and in the etiology and treatment of pathophysiological states.

INTRODUCTION

The 5-hydroxytryptamine (5-HT, serotonin) neurotransmitter system has been implicated in the etiology and treatment of psychiatric disease states such as depression and anxiety. Two nuclei in the midbrain-pons, the dorsal (DR) and median raphe (MR), contain 5-HT cell bodies that give rise to the majority of the ascending 5-HT projections to the forebrain, including the limbic areas that control emotional behavior (Azmitia and Segal 1978; Molliver 1987; Steinbusch 1982). Distinctions in the projections, morphology, neurotransmitter-mediated effects, and electrophysiological characteristics of the 5-HT- and non-5-HT-containing neurons in the MR and DR may be important in understanding the etiology of neuropsychiatric disorders and in understanding the normal regulatory functions of the 5-HT neurotransmitter system.

Both MR and DR send projections to nuclei considered part of the limbic circuit (Azmitia and Segal 1978; Molliver 1987). Distinct projection areas of the DR include prefrontal cortex, lateral septum, and amygdala, whereas MR innervates medial septum, cingulate, and dorsal hippocampus. The DR fibers are small and fine with pleomorphic varicosities, whereas MR fibers are coarse and large with spherical varicosities (Molliver 1987). Neurotoxic 5-HT-releasing agents selectively destroy DR projection fibers without affecting the dense coarse fibers from the MR (Mamounas and Molliver 1988; Mamounas et al. 1991; Molliver et al. 1990). Studies using microdialysis and voltammetry have also indicated that neurotransmitter-mediated responses may be different, and chronic treatment with agonists may differentially regulate the MR and DR (Blier et al. 1990; Kreiss and Lucki 1997; Tao et al. 1996). These studies demonstrate the selective vulnerability of MR or DR, thereby leading credence to the hypothesis that the development and/or treatment of pathological affective states may be due partly to selective alterations at the level of the MR or DR.

The electrophysiological characterization of 5-HT neurons in the DR has been tentative because most studies did not use neurochemical identification. Two studies are primarily cited as a basis for the identification of putative 5-HT-containing neurons. Using *in vivo* intracellular recording techniques and formaldehyde-induced fluorescence for 5-HT, Aghajanian and Vandermaelen (1982) characterized 5-HT-containing neurons as having a high-input resistance, prominent afterhyperpolarization (AHP) following a single action potential, and long duration action potentials. In a later study that used intracellular recording techniques in a brain slice preparation but did not use neurochemical confirmation, putative 5-HT cells were further defined to have a high-input resistance (150 – 400 M Ω), long duration action potential (1.8 ms), and a large, slow AHP (10–20 mV, 200–800 ms). Also cited as a basis for 5-HT neuron identification, but without neurochemical identification, is that the neuron is hyperpolarized by 5-HT_{1A} receptor activation (Aghajanian and Lakoski 1984). Other studies have defined additional characteristics of putative 5-HT neurons (Hajos et al. 1995b, 1996); however, neurochemical identification was not conducted for confirmation.

A primary goal of this study was to combine whole cell recording techniques with immunohistochemical identification of 5-HT-containing neurons to compare the electrophysiological characteristics of DR and MR 5-HT- and non-5-HT-containing neurons. The electrophysiological characteristics of both 5-HT- and non-5-HT-containing neurons in the DR and MR were identified. The majority of the non-5-HT neurons in the DR exhibited characteristics that were very similar to those of 5-HT-containing neurons in the DR. Also, important differences were identified in the characteristics of 5-HT-containing neurons in the MR and DR.

METHODS

Male Sprague-Dawley rats (75–150 g) from Taconic Farms were used (Taconic, Germantown, NY). All animals were used in accordance with U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals and approved by the institutional IACUC committee. Rats were rapidly decapitated, and the head was placed in ice-cold artificial cerebrospinal fluid (ACSF) in which sucrose (248 mM) was substituted for NaCl. The brain was rapidly removed and trimmed to isolate the brain stem region. The trimmed brain was affixed to a stage of a Leica microslicer (Leica, Allendale, NJ) with cyanoacrylate glue and submerged with ice-cold sucrose ACSF. Coronal slices, 200 μ m thick, were cut and placed in a holding vial containing ACSF at 37°C bubbled with 95% O₂-5% CO₂ for 1 h. After 1 h, the slices were kept in room temperature ACSF bubbled with 95% O₂-5% CO₂ and were transferred one at a time to the recording chamber (Warner Instruments, Hamden, CT). The composition of the ACSF was (in mM) 124 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2.0 MgSO₄, 2.5 CaCl₂, 10 dextrose, and 26 NaHCO₃.

Electrophysiological recordings

Slices were placed in a recording chamber (Warner Instruments) and continuously perfused with ACSF at 2 ml/min at 32°C maintained by an in-line solution heater (TC-324, Warner Instruments). Neurons were visualized using a Nikon E600 (Optical Apparatus, Ardmore, PA) upright microscope fitted with a 40× water-immersion objective, differential interference contrast (DIC), and infrared filter (IR). The image from the microscope was enhanced using a CCD camera and displayed on a computer monitor. Whole cell recording pipettes were fashioned on a P-97 micropipette puller (Sutter Instruments, Novato, CA) using borosilicate glass capillary tubing (1.2 mm OD, 0.69 mm ID; Warner Instruments). The resistance of the electrodes was 5–10 MΩ when filled with an intracellular solution of (in mM) 130 K-gluconate, 5 NaCl, 1 MgCl₂, 0.02 EGTA, 10 HEPES, 2 MgATP, 0.5 Na₂GTP, and 0.1% biocytin (pH 7.3).

The DR recordings were confined to the ventromedial DR subdivision that contains the densest cluster of 5-HT neurons. A visualized cell was approached with the electrode, a gigohm seal was established, and the cell membrane was ruptured to obtain a whole cell recording using an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA). Once the whole cell recording was obtained, cell characteristics were recorded using current-clamp techniques. If the series resistance of the electrode became >20 MΩ, the cell was discarded. Signals were digitized by a Digidata 1320 A/D converter (Axon Instruments) and stored on-line using pClamp 7/8/9 software (Axon Instruments). The liquid junction potential was approximately 10 mV between the pipette solution and the ACSF and was not subtracted from data obtained.

Drugs were added to the ACSF in known concentrations. A stock solution was made and diluted on the day of the experiment to obtain the desired concentration in the ACSF. All chemicals for making the ACSF, electrolyte solution, and 5-carboxyamodotryptamine maleate (5-CT) were purchased from Sigma-Aldrich (St. Louis, MO). WAY 100,635 was generously donated by Wyeth-Ayerst (Princeton, NJ).

Immunohistochemistry

Standard immunofluorescence procedures were used to visualize the filled cell and neurotransmitter content. Slices were fixed by submersion in 4% paraformaldehyde prepared in 0.1 M phosphate buffer (PB; pH 7.4) overnight and stored in 30% sucrose. Sections were incubated with rabbit anti-5-HT antibody (1:2,000, ImmunoStar, Hudson, WI) at 4°C for a period of 48–96 h in the cold room. Initial experiments (approximately one-third of the neurons) used shorter incubation times. We found that with longer incubation times the signal was greater, with no substantial increase in background noise. Subsequently, immunohistochemical labeling was visualized using a FITC (1:100, Jackson ImmunoResearch, West Grove, PA) or Alexa Fluor 488 (1:200, Molecular Probes, Eugene, OR) conjugated donkey anti-rabbit secondary for 60 min at room temperature. The biocytin was visualized using streptavidin conjugated Cy3 (1:1,000 or 1:2,000, Jackson ImmunoResearch) or Alexa Fluor 633 (1:200, Molecular Probes) for 60 min at room temperature. Initial experiments (approximately one-third) used Cy3 and FITC fluorophores. Between incubations, slices were rinsed with PB solutions (3 times for 10 min), and all incubations were done with mild agitation on a shaker. The sections were mounted on superfrost slides and coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Immunofluorescence label was visualized using a Leica DMR fluorescence microscope and a Leica confocal DMIRE2 microscope (Leica). Images were captured using a digital camera and Openlab 3.09 software (Improvision, Lexington, MA) on the fluorescence microscope and a digital camera and Leica Confocal software (Version 2.5, Leica). When using the confocal microscope sequential collection, i.e., Cy3/Alexa 633 separately from FITC/Alexa488, images 0.6 μm in thickness were acquired at the level of the cell body of the biocytin-labeled neuron. The laser power and emission filters

were adjusted for both the red and green fluorophor so that there was negligible possibility of false positive result. This was done by exciting at the optimal wavelength for the green fluorophor and detecting using the emission spectra for the red fluorophor and vice versa. When Cy3 and FITC were used, the emission spectra were narrowed on the confocal microscope to reduce the likelihood of bleed-through. Images were adjusted to optimal color balance and contrast using Adobe Photoshop 6.0 software (Adobe, San Jose, CA).

Statistics

ANOVA or *t*-test was used to test for statistical significance. A probability of $P \leq 0.05$ was considered significant. All data are reported as means $\pm$ SE.

RESULTS

Whole cell recordings were made from a total of 86 neurons: 49 in the ventromedial DR and 37 in the MR. None of the cells, 5-HT- or non-5-HT-containing, were spontaneously active. Immunohistochemistry visualized with a fluorescence or confocal microscope was used to determine if the neuron was 5-HT- or non-5-HT-containing. The biocytin filled cell body was found, and the immunofluorescence for 5-HT was visualized. The determination that the neuron was non-5-HT-containing was made only if the 5-HT immunofluorescence was not in the biocytin-labeled cell and if other 5-HT-containing cells were found in the same plane of focus. If other 5-HT neurons were not found, then the cell was discarded. This protocol was used primarily to reduce the likelihood of a false negative determination. In many cases, the confocal microscope had to be used to make the determination in the case of non-5-HT-containing neurons. Based on this immunohistochemical identification procedure, 33 DR neurons were classified as 5-HT-containing, 16 as non-5-HT-containing neurons in the DR, 23 as 5-HT-containing neurons in the MR, and 14 as non-5-HT-containing neurons in the MR.

With the microscope used for electrophysiology, DR neurons were easy to visualize, and they were clustered close together (Fig. 1A). It was not easy to distinguish between 5-HT and non-5-HT cells in the DR based on size or shape. In contrast, in the MR, the cells were very difficult to see; the neurons were not clumped together and there appeared to be dense fiber tracts (Fig. 1B). However, once visualized, the non-5-HT-containing cells were much smaller than the 5-HT-containing cells, and it became relatively easy to differentiate and predict the two cell types based on size in the MR. The differentiation was verified by immunohistochemical identification.

Figure 2 contains photomicrographs of a 5-HT-containing DR neuron taken on a confocal microscope. Figure 2, A1–C1, shows immunohistochemistry for 5-HT, Fig. 2, A2–C2, shows the immunohistochemistry for the biocytin-filled neuron, and Fig. 2, A3–C3, is the overlay of the 5-HT and biocytin photomicrographs. Using a confocal microscope, a z-series of 79 photomicrographs was obtained at a thickness of $0.6 \mu\text{m}$ through a total depth of $51 \mu\text{m}$. In Fig. 2, A1–A3, the photomicrograph is in the *x-y* plane of axis. The neuron that was recorded from is indicated by an arrow and can be seen in all three panels; the neuron appears orange in A3 because it is labeled for biocytin as well as 5-HT. Figure 2, B1–B3 and C1–C3, shows photomicrographs taken through $51 \mu\text{m}$ of the slice in the *y-z* and *x-z* plane, respectively, through the body of the labeled cell as demarcated by the white lines in Fig. 2A3. The neuron is indicated by the arrow.

Figure 3 contains photomicrographs of a non-5-HT-containing neuron from the DR. Photomicrographs of 5-HT immunoreactivity are seen in A1–C1, of biocytin in A2–C2, and of 5-HT and biocytin together in A3–C3. Figure 3, A1–A3, is the *x-y* axis, B1–B3 is the *y-z* axis, and C1–C3 is the *x-z* axis. The number of sections acquired was 37, through a total depth of

22 μm . The biocytin-filled neuron is indicated by the arrowhead in *A2*, and the dark space where the neuron would be is indicated in *A1*, *B1*, and *C1* by the arrowhead. In *A3*, the neuron is red because it is not double-labeled. In *B1–B3* and *C1–C3*, it is also apparent that the biocytin-labeled cell is not immunoreactive for 5-HT. A neuron that is 5-HT immunoreactive is indicated by an arrow in *A1*, *A3*, and *B1*. 5-HT labeling is seen throughout the extent of the *z* axis. These photomicrographs provide evidence that the labeling of the 5-HT neurons was successful throughout the extent of the slice and that the biocytin-filled neuron was not 5-HT-containing.

Figure 4 contains photomicrographs of two biocytin-filled MR neurons. *A1–C3* were taken at a plane of focus for the cell seen on the *right* of *A1–A3*, and *D1–F3* were taken at a plane of focus for the cell seen on the *left* of *D1–D3*. Using the confocal microscope, a *z*-series of 119 photomicrographs were taken through 72 μm of tissue. Biocytin labeling is seen in Fig. 4, *A2*, *B2*, *C2*, *D2*, *E2*, and *F2*, immunohistochemistry for 5-HT in Fig. 4, *A1*, *B1*, *C1*, *D1*, *E1*, and *F1*, and the biocytin and 5-HT immunohistochemistry combined in Fig. 4, *A3*, *B3*, *C3*, *D3*, *E3* and *F3*. The *x*-*y* axis is in *A1–A3* and *D1–D3*, the *y*-*z* axis in *B1–B3* and *E1–E3*, and the *x*-*z* axis in *C1–C3* and *F1–F3*. The red biocytin-filled cells have processes extending from the cell body that are demarcated by an arrow (*A1* and *A3*) and an arrowhead (*D2* and *D3*). The cell body of the neuron that is immunoreactive for 5-HT is indicated by an arrow in *A1–C1* and appears orange in *A3–C3*. The process of the neuron in *A1* is immunoreactive for 5-HT because it appears orange in *A3*. In *D2*, the cell body and process of the neuron are demarcated by an arrowhead. In *D1–F1*, the arrowhead indicates where the cell body of the biocytin-labeled cell should appear, and an arrow in *D1*, *D3*, and *F1* shows another neuron that is immunoreactive for 5-HT in the same *x* axis as the biocytin-labeled cell. In *D3–F3*, the cell body and process appear red, not orange, indicating that neuron is not double-labeled for 5-HT and biocytin. In *F2*, the arrowhead indicates the biocytin-labeled process that appears red in *F3*.

The 5-HT-containing neurons of the MR and DR were very similar but did differ in two cellular characteristics. Figure 5 contains representative traces recorded from a 5-HT-containing neuron in the MR and DR, showing the voltage response to current injection (Fig. 5, *A* and *D*), the AHP (Fig. 5, *B* and *E*), and action potential (Fig. 5, *C* and *F*). The voltage–current plot of the 5-HT-containing neurons was linear for cells recorded from both the MR and DR; an example recorded from a DR neuron is shown in Fig. 6*A*. The 5-HT-containing neurons of the DR and MR had similar resting membrane potential, input resistance (measured by the slope of a straight-line fit to the voltage–current plot; Fig. 6*A*), action potential amplitude (measured from threshold to the peak), and action potential width (measured at the base of the action potential (Table 1; Fig. 5*C*). The action potential of both MR and DR 5-HT-containing neurons demonstrated a shoulder on the falling phase of the action potential and was approximately 2 ms in duration.

Interestingly, the 5-HT cells of the MR and DR differed in two important characteristics. The time constant (τ) was significantly less in the MR than DR neurons (Table 1). In the DR and MR cells in which a -20 -, -40 -, and $+20$ -pA current pulse was used to measure the time constant, the values were not significantly different within groups and across current magnitudes (DR: 55.0 ± 3.4 , 58.2 ± 3.5 , and 59.0 ± 4.2 , respectively; $F = 0.09$, $P = 0.9$, $n = 26$; MR: 46.0 ± 2.3 , 45.1 ± 2.5 , and 46.7 ± 2.8 , respectively; $F = 0.36$, $P = 0.7$, $n = 14$). The linearity of the voltage–current plot and the consistency of the time constant measurement indicates that voltage-gated ionic conductances are not a significant factor in the measurement of the τ . The 5-HT-containing cells of the MR also had a larger amplitude AHP than the 5-HT-containing cells of the DR (Table 1). However, even though the amplitude of the AHP was greater, the time course was the same because the AHP $t_{1/2}$ and the AHP duration were not significantly different between the neurons of the two subfields (Table 1).

The non-5-HT-containing neurons could be grouped into three different cell types as seen in Fig. 7. The first type of cell, referred to here as type I (Fig. 7, A–C), had characteristics that were very similar to 5-HT-containing cells. Type I neurons had a large input resistance that was linear (Fig. 6B). The most prominent feature of the second cell type, referred to here as type II (Fig. 7, D–F), was the rectification seen in the voltage responses to hyperpolarizing current pulses and the rebound excitation following the offset of the current pulse (Fig. 7D). The voltage–current plots of type II neurons exhibited a distinct rectification (Fig. 6C). The third cell type, referred to here as type III (Fig. 7, G–I), was differentiated based on the presence of a sag in the voltage responses elicited by hyperpolarizing current pulses. Type III neurons had voltage–current plots that were generated by measuring the peak voltage elicited by the hyperpolarizing current pulse and the voltage at the end of the current pulse just prior to the current pulse termination (Fig. 6D). Whereas the voltage–current plots were linear for the voltage response generated at the beginning of the current pulse, the voltage–current plot for the voltage response at the end of the current pulse rectified. The resistance of the neuron was clearly decreased at the end of the current pulse as measured by the shallow slope.

A direct comparison of the 5-HT and non-5-HT-containing neurons in the DR revealed that they were very similar. In the DR, 13/16 non-5-HT neurons exhibited cell characteristics of type I neurons (Fig. 7, A–C), and 3/16 exhibited characteristics of type II neurons (Fig. 7, D–F). The resting membrane potential of the 5-HT-containing and non-5-HT-containing cell types was not significantly different. To compare the resistance of the non-5-HT neurons that exhibited rectification (type II), only the linear portion of the current–voltage plot was used (Fig. 6C). The time constant or tau of the non-5-HT-containing neurons was smaller than that of the 5-HT-containing neurons (Table 1). The amplitude and the shape of the AHP recorded in the 5-HT and non-5-HT neurons with whole cell techniques were not statistically different. Nine of the non-5-HT-containing neurons had a depolarizing after potential at the peak of the AHP (Fig. 7, B and E). The action potential duration was statistically smaller in the non-5-HT neurons compared with the 5-HT neurons (Table 1). Even though the duration of the action potential was smaller, the type I non-5-HT cells exhibited a shoulder on the falling phase of the action potential, similar to that recorded from 5-HT-containing neurons of the DR (Fig. 7C).

In the MR, both 5-HT- and non-5-HT-containing cells were found. As previously stated, it was more difficult to visualize any neuron in the MR compared with the DR. However, when cells were found, it was easier to distinguish the two cell types visually since the non-5-HT cells were smaller in size than the 5-HT cells as seen through the microscope used for electrophysiology. In the MR, 6/14 non-5-HT neurons had characteristics of type I neurons (Fig. 7, A–C), 3/14 non-5-HT-containing neurons exhibited characteristics of type II neurons (Fig. 7, D–F), and 5/14 had characteristics of type III neurons (Fig. 7, G–I). Even though the non-5-HT neurons could be distinguished based on the shape of the voltage–current plots, there were no statistical differences in their cell characteristics, and therefore the data were pooled for comparison to 5-HT-containing neurons in the MR. All of the characteristics were different between the non-5-HT and 5-HT neurons except for the action potential amplitude (Table 1). The membrane resistance of the non-5-HT cells was smaller, the tau was shorter, the AHP amplitude, $t_{1/2}$, and duration were smaller, and action potential duration was shorter than those of the 5-HT-containing cells. Also, a characteristic that differentiated 5-HT from non-5-HT-containing cell types was the presence of a depolarizing after potential in the AHP of the majority, i.e., 6/14, of the non-5-HT-containing neurons (Fig. 7E). The action potential shape was much different in 5-HT (Fig. 5F) and non-5-HT-containing neurons (Fig. 7, F and I), i.e., much shorter in duration without a shoulder in the falling phase.

The largest category of non-5-HT-containing neurons were type I in both the DR and MR. This category of neuron is characterized by a linear *I*-*V* plot. A direct comparison of the

characteristics of the type I cells in the DR and MR revealed differences in the input resistance (DR 572 ± 40 , MR 335 ± 54 ; $t = 3.15$, $P = 0.006$), tau (DR 41 ± 2.1 , MR 19 ± 2.1 ; $t = 7.28$, $P = 0.000002$), AHP $t_{1/2}$ (DR 126 ± 12 , MR 51 ± 18 ; $t = 3.6$, $P = 0.002$), and AHP duration (DR 345 ± 20 , MR 194 ± 37 ; $t = 3.84$, $P = 0.001$).

The response to 5-HT_{1A} receptor activation was also measured in both non-5-HT- and 5-HT-containing neurons of the DR and MR. To characterize the 5-HT_{1A} receptor-mediated response, the agonist 5-CT was used. 5-CT has previously been shown to be a potent 5-HT_{1A} receptor agonist (Beck et al. 1992; Williams et al. 1988). In the DR, both the non-5-HT and the 5-HT neurons exhibited a hyperpolarizing response to bath perfusion of 5-CT. The response was significantly smaller in non-5-HT neurons compared with 5-HT neurons (Table 2, Fig. 8). Figure 8 contains two chart recordings, one from a DR non-5-HT-containing neuron (Fig. 8A) and one from a DR 5-HT-containing neuron (Fig. 8B1). These chart recordings show the concentration-dependent hyperpolarization of the membrane potential in response to bath administration of 5-CT. Concomitant with the change in membrane potential was a large decrease in membrane resistance as shown by the decrease in the magnitude of the membrane potential response to the injection of a -30-pA current pulse (downward deflections). There was a significant difference in the magnitude of the response elicited by a 100 nM 5-CT between the non-5-HT- and 5-HT-containing neurons (Table 2). The response in the non-5-HT-containing neurons was around one-half the magnitude of the response elicited in 5-HT-containing neurons. Also, in Fig. 8B2 is a current trace from the same cell in Fig. 8B1, depicting the response to 5-CT (100 nM) administration when the neuron was voltage clamped at -60 mV.

In the MR, the magnitude of the hyperpolarization in response to administration of a saturating concentration of 5-CT was also measured in both 5-HT-containing and non-5-HT-containing neurons (Fig. 9, Table 2). The non-5-HT-containing neurons did not exhibit any change in membrane potential or membrane resistance in response to 5-CT administration (Fig. 9A). The magnitude of the response elicited by 100 nM 5-CT was significantly smaller in the non-5-HT-containing neurons of the MR than the 5-HT-containing neurons (Table 2). The 5-HT-containing neurons had a concentration-dependent response to 5-CT (Fig. 9B).

The magnitude of the response to 100 nM 5-CT was also significantly different between the type I non-5-HT neurons in the DR and MR (DR 6.7 ± 1.7 , MR 0.0 ± 0.0 ; $t = 2.57$, $P = 0.02$).

A direct comparison of the hyperpolarization elicited by different concentrations of 5-CT is summarized in Fig. 10 for the 5-HT-containing neurons of the DR and the MR. The data for the construction of concentration-response curves was collected for 5-HT-containing neurons of the DR and MR using current-clamp techniques. The summarized means of the hyperpolarizing responses elicited by the different concentrations of 5-CT for the DR and MR neurons were fit to a logistic equation to obtain estimates of $E_{\max}$, EC_{50} , and slope. For the DR neurons, the $E_{\max}$ was equal to 14 mV, EC_{50} was equal to 7.4 nM, and slope was equal to 1.3. For the neurons in the MR, the $E_{\max}$ was 8.6 mV, the EC_{50} was 7.0 nM, and the slope was equal to 1.2. The response elicited by 100 nM 5-CT ($t = 2.31$; $P = 0.03$; $n = 16$ for DR and 10 for MR) was significantly less in 5-HT-containing cells of the MR compared with the neurons of the DR. The responses to 100 nM 5-CT were completely blocked by the selective 5-HT_{1A} antagonist WAY 100,635 (0.1 nM) in 5-HT-containing neurons in both the MR ($n = 4$) and DR ($n = 3$) and non-5-HT-containing neurons in the DR ($n = 4$).

Voltage-clamp experiments were also conducted to measure the magnitude of the outward current elicited by 5-CT in the DR and MR 5-HT-containing neurons. The maximum current elicited by 100 nM 5-CT was greater in the DR, i.e., 53.3 ± 5.1 pA ($n = 6$) than the MR, i.e., 26.0 ± 9.4 ($n = 6$; $t = 2.66$, $P = 0.03$). These results are in agreement with the results obtained

using current-clamp recording (Fig. 10, Table 2), demonstrating a significant difference in the response elicited by 100 nM 5-CT in the 5-HT-containing neurons of the MR and DR.

Figure 11 contains current–voltage plots of the outward current elicited by 100 nM 5-CT. The neurons were voltage clamped at -60 mV. A control current–voltage response (Fig. 11, A and C) was elicited by injecting a voltage ramp from -110 to -40 mV (1 mV/s). The 5-CT was administered in the bath, and a current–voltage response was collected again in the presence of 5-CT (Fig. 11, A and C). The amount of current elicited by 5-CT is shown in Fig. 11, B and D, by subtracting the amount of current elicited in the absence of 5-CT from the amount of current elicited in the presence of 5-CT. The reversal potential for neurons recorded from the DR was -90 ± 3.6 mV ($n = 6$), and from the MR, it was -90 ± 3.7 mV ($n = 4$). When the junction potential of 10 mV is taken into account, the reversal potential is approximately -100 mV, in agreement with the estimated Nernst potential, i.e., -103 mV, for potassium, given a $[K]_o$ of 2.5 mM and $[K]_i$ of 130 mM. The shape of the current–voltage plots and the reversal potentials are similar for the neurons recorded from the MR and DR.

DISCUSSION

The electrophysiological characteristics and 5-HT_{1A} receptor-mediated response were determined in DR and MR 5-HT and non-5-HT-containing neurons as identified by immunohistochemistry. The primary findings of this study were that 1) the characteristics of 5-HT-containing neurons in the DR and MR are not the same, but have important differences in active and passive cell characteristics as well as in 5-HT_{1A} receptor-mediated hyperpolarization; 2) there are non-5-HT-containing neurons that have characteristics that are very similar to those of 5-HT-containing neurons in the DR; and 3) the non-5-HT-containing neurons of the MR have completely different cell characteristics from 5-HT-containing neurons of the MR and no 5-HT_{1A} receptor-mediated response. These results provide important background information toward understanding the selective regulation of forebrain structures by the MR and DR.

One of the important findings of this study was the high degree of similarity in the characteristics of the 5-HT and non-5-HT-containing neurons of the DR. Both cell types exhibited the cellular characteristics of a putative 5-HT-containing neuron, in agreement with results we reported previously using intracellular recording techniques (Kirby et al. 2003). Historically 5-HT-containing neurons, as identified by formaldehyde-induced fluorescence techniques, of the DR are characterized by exhibiting a high membrane input resistance, a long time constant, a large afterhyperpolarization, and a broad action potential that has a shoulder on the falling phase (Aghajanian and Vandermaelen 1982). In this study, immunohistochemistry with whole cell recording was used. All neurons included in this study were identified using immunohistochemistry with fluorescent or confocal microscopy. The data from the biocytin-labeled neurons were included only if 5-HT-containing neurons were found in the same plane of focus as the labeled neuron and it was clear that the 5-HT antibody had penetrated to the depth of the biocytin-labeled cell (Figs. 2-4). In many cases, a neuron that appeared to be unlabeled under the fluorescent microscope was found to be labeled when examined by confocal microscopy. For this reason, all non-5-HT-containing neurons in the DR included in this study were confirmed by confocal microscopy.

The only defining characteristics that differentiated the non-5-HT- and 5-HT-containing neurons in the DR were the tau and the action potential width. Another characteristic that was present in most of the non-5-HT neurons was a depolarizing after potential at the peak of the AHP. The proportion of 5-HT- to non-5-HT-containing neurons is in agreement with estimates in the literature, i.e., approximately one-third to two-thirds (Descarries et al. 1982; Jacobs and Azmitia 1992; Stein-busch et al. 1980, 1981).

One possible explanation for these results is that the non-5-HT cells are 5-HT cells that have stopped producing 5-HT. Previous studies have shown that the levels of the mRNA for the 5-HT synthesizing enzyme tryptophan hydroxylase decreases from postnatal days 22 to 61 (Rind et al. 2000). The decrease is attributed to a loss in 5-HT cells because the density of tryptophan hydroxylase mRNA per cell did not decline with age. It is possible that the levels of the enzyme protein decrease within 5-HT-containing cells; as the rat matures, some cells that made 5-HT at postnatal day 22 no longer make significant quantities of the enzyme and therefore there is a reduction in the amount of neurotransmitter. Even if true, the end result is the same. The non-5-HT-containing neuron that resembles the 5-HT-containing neuron does not produce 5-HT and therefore it will not be secreted as a neurotransmitter in projection areas.

Another explanation that a 5-HT neuron could be misidentified as non-5-HT-containing is that the cell is being dialyzed by the contents of the recording pipette. However, there was no correlation with the length of time of the recording with the 5-HT or non-5-HT nature of the neuron. Many cells that were found to be 5-HT-containing were recorded for prolonged periods of time, i.e., several hours.

Previously there have been reports that the voltage-current or current-voltage plots of the 5-HT-containing neurons of the DR were linear (Crunelli et al. 1983) or demonstrated a degree of rectification (Williams et al. 1988). In this study, the 5-HT-containing cells of both the MR and DR were found to have linear voltage-current plots. Also, the majority of the non-5-HT-containing cells of the DR, i.e., type I, were also found to have linear voltage-current plots, whereas a minority, type II, demonstrated a nonlinear degree of rectification. The defining characteristic for the 5-HT cells in the study that reported nonlinear rectification is that the neuron exhibit a hyperpolarization in response to 5-CT (Williams et al. 1988). We report here, as well as in a previous study (Kirby et al. 2003), that non-5-HT neurons in the DR also exhibit responses to 5-CT and express 5-HT_{1A} receptors. It is possible that the cells previously identified as 5-HT-containing cells with nonlinear current-voltage plots were in fact non-5-HT-containing neurons.

In contrast, the characteristics of the 5-HT- and non-5-HT-containing neurons in the MR were very different. Even the type I non-5-HT neurons had characteristics that were different from 5-HT-containing neurons in the MR. Also, the type I non-5-HT neurons had different characteristics from the type I non-5-HT neurons in the DR, i.e., membrane resistance, tau, and AHP duration. The classic description of a putative 5-HT neuron compared with a non-5-HT neuron is more appropriately applied to the neurons in the MR than the DR.

The 5-HT_{1A} receptor-mediated hyperpolarization and increase in outward current was also measured in both 5-HT-containing and non-5-HT-containing neurons in the DR and MR. Previously, a 5-HT_{1A} receptor-mediated response was used as one of the defining characteristics of 5-HT-containing neurons (Aghajanian and Lakoski 1984). A 5-HT_{1A} receptor-mediated response was present in the 5-HT-containing neurons in the DR and MR as well as non-5-HT-containing neurons in the DR, but was absent in the non-5-HT-containing neurons in the MR. The magnitude of the response elicited in the non-5-HT-containing neurons in the DR was significantly less than that elicited in the 5-HT-containing neurons. However, these data are important because they confirm our previous report that there are 5-HT_{1A} receptors on non-5-HT-containing neurons in the DR (Kirby et al. 2003) and extend those findings by revealing that non-5-HT-containing neurons in the MR do not have a 5-HT_{1A} receptor-mediated response.

Previous studies have assumed that 5-HT_{1A} receptors were only on 5-HT-containing neurons in the DR. Interpretation and conclusions garnered from these studies may need to be re-evaluated in light of the evidence that some of the neurons may not be 5-HT neurons. Both

extracellular and intracellular electrophysiological studies have been conducted looking at the effects of various drug treatments such as antidepressants, corticosterone, behavioral stress paradigms, or 5-HT selective agonists and antagonists on 5-HT_{1A} receptor-mediated responses in putative 5-HT-containing neurons from the DR (Dong et al. 1997; Laaris et al. 1995; Le Poul et al. 1997, 2000; Pineyro and Blier 1999). Putative 5-HT neurons were identified using the criterion defined by the classic studies (Aghajanian and Vandermaelen 1982; Vandermaelen and Aghajanian 1983). The results were discussed in terms of affecting only 5-HT neurotransmission and its role in etiology and treatment of mood disorders. However, the drug effects were most likely also on non-5-HT-containing neurons. The implications are that non-5-HT neurons in the raphe are also affected by drug treatments, and therefore to understand the mechanism of action of the drugs, the effects on both 5-HT- and non-5-HT-containing neurons must be considered.

The concentration–response curve characteristics of 5-HT activation of 5-HT_{1A} receptor were not the same in the DR and MR 5-HT-containing neurons. The efficacy or magnitude of the response was greater in the DR than in the MR, whereas the potency appeared to be the same. These results may be attributed to a difference in the density of the 5-HT_{1A} receptor in the DR and MR or due to a difference in coupling between the 5-HT_{1A} receptor and its second messenger system cascade. Previous studies have reported that the density of 5-HT_{1A} receptors is lower in the MR than DR (Khawaja 1995), but this may be due to the smaller number of cells in the MR as compared with the DR. It is unknown whether the density of the receptors per individual neuron is smaller in the MR compared with the DR. Previous studies, primarily microdialysis studies, have implicated a difference in the efficacy of the 5-HT_{1A} receptor-mediated response between the MR and DR (Casanovas and Artigas 1996; Kreiss and Lucki 1994; Romero and Artigas 1997). However, to our knowledge, no studies have directly measured the 5-HT_{1A} receptor-mediated response in individual 5-HT-containing neurons in the median and dorsal raphe. The importance of these results is that the MR is going to be under less autoreceptor regulation than the DR 5-HT-containing neurons. This difference in the receptor-mediated response also provides a potential site for differential regulation by pathophysiological processes, such as stress, or by drugs, such as antidepressants. These data provide yet another important piece of evidence demonstrating differences between the DR and the MR.

Two of the cellular characteristics were different between 5-HT-containing neurons of the DR and MR. The tau was less in the 5-HT-containing neurons of the MR than the DR. Since the voltage–current plots were linear, the time constant can be used as an indication of the capacitance of the neuron that is a direct consequence of the morphology of the cell (Hille 1992). The difference in the time constant of the MR and DR neurons indicates that there may be differences in their morphology. Also, the processing of synaptic input will be different. Whereas the rise time and amplitude of synaptic input may be greater in the MR, the possibility of synaptic input temporally summing will be less in MR than in the DR neurons. However, it is important to remember that even though whole cell recording techniques were used, the dendritic processes of the 5-HT-containing cells are extensive, and the neuron is not isopotential. The measurement of the neuronal time constant will be primarily influenced by the morphology of the cell near the soma. An in-depth analysis of cell morphology needs to be conducted to test the hypothesis that the morphology of the 5-HT-containing neurons of the MR and DR are different.

The magnitude of the AHP following a single action potential was greater in MR than DR 5-HT-containing neurons. The AHP in the DR is mediated by activation of calcium-dependent potassium channels (Aghajanian 1985; Crunelli et al. 1983; Freedman and Aghajanian 1987). The late phase of the AHP is mediated in part through the release of intracellular calcium through activation of calcium induced calcium release (Pan et al. 1994). The ion channels

mediating the AHP in the MR have not been defined. The difference in the magnitude of the AHP between the MR and DR 5-HT-containing neurons could be attributed to many factors. There may be different ion channels that underlie the AHP, a greater density of calcium dependent potassium channels, a difference in channel kinetics, or a larger calcium current. Since there was no difference in the duration of the AHP, the release of intracellular calcium is probably similar in the MR and DR 5-HT-containing neurons. The firing rate of MR neurons is significantly lower than the firing rate of DR neurons (Hajos et al. 1995a). A greater AHP magnitude could lengthen the interspike interval, leading to a lower firing frequency. The greater AHP magnitude in the MR than the DR could be partly responsible for the reported difference in firing frequency.

In conclusion, the important advances of this study are that we examined both 5-HT- and non-5-HT-containing neuron characteristics in the DR and the MR. Three types of non-5-HT-containing neurons were identified. We found that the characteristics of the 5-HT- and non-5-HT-containing neurons of the DR were very similar. In contrast, the differences between the 5-HT and non-5-HT neurons of the MR were numerous. Also, we found important differences in the characteristics of 5-HT-containing neurons in the DR and MR. The tau was smaller and the AHP amplitude larger in 5-HT-containing neurons of the MR compared with those in the DR. Furthermore, a 5-HT_{1A} receptor-mediated response was present in 5-HT-containing neurons of the MR and DR and non-5-HT-containing neurons in the DR, but absent from non-5-HT neurons in the MR. The magnitude of the 5-HT_{1A} receptor-mediated hyperpolarization was larger in 5-HT-containing neurons of the DR compared with the MR. In concert with the evidence that the DR and MR have different projections to forebrain brain regions, these distinctions may be very important in understanding the role of the MR and DR 5-HT circuits in normal physiological processes as well as in the etiology and treatment of pathophysiological states such as affective disorders.

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FIG. 1. Photomicrograph taken through the microscope used for electrophysiology of the dorsal raphe (A) and median raphe (B). In both panels, a recording electrode can be seen attached to a neuron. In B, the neuron is indicated by an arrow. Scale bar, 50 μm .

FIG. 2.

Photomicrographs of a 5-hydroxytryptamine (5-HT)-Containing dorsal raphe (DR) neuron. Fluorescent photomicrographs taken on the confocal microscope depicting green 5-HT-containing neurons (A1–C1), a red biocytin-filled cell (A2–C2), and the overlay of the 2 (A3–C3). A z stack of 79 photomicrographs 0.6 μm in thickness were taken, extending through 51 μm of tissue. In A1–A3, the photomicrograph is in the *x-y* axis; in B1–B3, the photomicrographs are in the *y-z* axis; and in C1–C3, the photomicrographs are in the *x-z* axis. Scale bar in A2, 20 μm . Arrow points to the biocytin-filled cell that is also 5-HT immunoreactive.

FIG. 3.

Photomicrographs of a non-5-HT-containing DR neuron. Fluorescent photomicrographs taken on the confocal microscope depicting green 5-HT-containing neurons (A1–C1), a red biocytin-filled cell (A2–C2), and the overlay of the 2 (A3–C3). A z stack of 37 photomicrographs $0.6\ \mu\text{m}$ in thickness were taken, extending through $22\ \mu\text{m}$ of tissue. In A1–A3, the photomicrograph is in the x – y axis; in B1–B3, the photomicrographs are in the y – z axis; and in C1–C3, the photomicrographs are in the x – z axis. Arrowhead points to the non-5-HT-containing biocytin-filled cell and the black space where the cell should appear if it was 5-HT immunoreactive. Arrow points to a different neuron in the same plane of focus as the

biocytin-filled cell that contains 5-HT. Note that the 5-HT immunoreactivity appears throughout the extent of the z axis in *B1* and *C1*. Scale bar in *A2*, 20 μm .

FIG. 4.

Immunohistochemistry of a 5-HT-containing and non-5-HT-containing cell in the medial raphe (MR). In *A1–C3*, the photomicrographs were taken at the plane of focus for the neuron that is on the *right* of *A1–A3*, whereas *D1–F3* were taken at a plane of focus for the neuron on the *left* of *D1–D3*. A z stack of 119 photomicrographs $0.6\ \mu\text{m}$ in thickness were taken, extending through $72\ \mu\text{m}$ of tissue. The *x-y* axis is shown in *A1–A3* and *D1–D3*, the *y-z* axis in *B1–B3* and *E1–E3* and the *x-z* axis in *C1–C3* and *F1–F3*. Fluorescent photomicrograph depicting red biocytin-filled cells (*A2*, *B2*, *C2*, *D2*, *E2*, and *F2*), green 5-HT-containing neurons (*A1*, *B1*, *C1*, *D1*, *E1*, and *F1*), and the overlay of the two (*A3*, *B3*, *C3*, *D3*, *E3*, and *F3*). The arrow indicates the cell body and process of the neuron (*A1*, *B1*, and *C1*) that appears orange and is therefore double-labeled for biocytin and 5-HT in *A3*, *B3*, and *C3*. Arrowhead in *D2*, *D3*, *E1*, *F1*, and *F2* indicates the cell body and process of the neuron that is not double-labeled. The arrow in *D1*, *D3*, and *F1* demarcates a 5-HT-containing neuron in the same *x* plane of focus of the biocytin-labeled neuron.

FIG. 5.

Characteristics of 5-HT-containing neurons of the DR (A–C) and MR (D–F). In A and D, hyperpolarizing and depolarizing current pulses were injected into the cell, and the voltage response was measured. In B and E, a depolarizing current pulse of sufficient magnitude to elicit a single action potential was used to generate an afterhyperpolarization following the action potential. In C and F, a current pulse of sufficient magnitude was used to generate a single action potential. In A, B, D, and E, the action potentials are truncated due to the sampling rate.

FIG. 6.

Voltage–current plots for a 5-HT–containing neuron from the DR (A) and non–5-HT–containing neurons of type I from the DR (B), type II from the MR (C), and type III from the MR (D). Data were obtained by measuring the maximum voltage response to hyperpolarizing current pulses. In D, maximum voltage response (●) and the voltage at the end of the current pulse (■) were measured, due to the sag in the membrane potential during the hyperpolarizing current pulse. In each case, the voltage–current plot was fit by a straight line to obtain measurements of input resistance and resting membrane potential. In C, only the 1st 4 points were fit due to rectification of the voltage–current plot.

Non-5-HT Containing

Type I

Type II

Type III

FIG. 7.

Three different non-5-HT neuron types. In A–C, characteristics of a type I neuron from the DR is shown; in D–F, characteristics of a type II neuron from the MR is shown; and in G–I, characteristics of a type III neuron from the MR are shown. In A, D, and G, voltage responses to injection of hyperpolarizing and depolarizing current pulses are plotted. In B, E, and H, afterhyperpolarizations following a single action potential are shown; and in C, F, and I, action potentials are shown. Note the presence of a depolarizing afterpotential at the peak of afterhyperpolarization in B and E. In A, B, D, E, G, and H, action potentials are truncated due to sampling rate.

FIG. 8.

Membrane potential responses to bath perfusion of 5-carboxyamodotryptamine maleate (5-CT) in non-5-HT-containing (A) and 5-HT-containing (B1) neurons in the DR. B2: chart recording of current elicited by bath application of 5-CT as recorded in voltage clamp with the cell held at -60 mV. Length of line above each chart recording depicts the amount of time the drug was in the chamber. Downward deflections are voltage response to intracellular injection of a -30 -pA current pulse used to monitor changes in membrane resistance. Resting membrane potential for cell A was -72 mV and for cell B was -56 mV.

FIG. 9.

Membrane potential responses to bath perfusion of 5-CT in non-5-HT-containing (A) and 5-HT-containing (B) neurons in the MR. Downward deflections are voltage response to intracellular injection of a -30 -pA current pulse used to monitor changes in membrane resistance. Length of line above each chart recording depicts the amount of time the drug was in the chamber. Resting membrane potential for cell A was -58 mV and for cell B was -63 mV.

FIG. 10.

Concentration-response curves for the averaged data of the neurons recorded in the DR (squares) and the MR (triangles). Number of observations per data point is presented in parentheses on graph. Data were fit to a logistic equation to obtain estimates for EC₅₀, $E_{\max}$, and slope. For the DR neurons, the $E_{\max}$ was equal to 14 mV, EC₅₀ was equal to 7.4×10^{-9} M, and slope was equal to 1.3. For the neurons in the MR, the $E_{\max}$ was 8.6 mV, the EC was 7.0×10^{-9} M, and slope was equal to 1.2. Response elicited by 100 nM 5-CT ($t = 2.31$; $P = 0.03$; $n = 16$ for DR and 10 for MR) was significantly less in 5-HT-containing cells of the MR compared with the neurons of the DR.

DR 5-HT Containing

MR 5-HT Containing

FIG. 11. current-voltage plots for 5-CT (100 nM) activation of 5-HT_{1A} receptor-mediated outward current in 5-HT-containing neurons of the DR (A and B) and MR (C and D). In A and C, current-voltage plots elicited by voltage ramp from -110 to -40 mV (1 mV/s) in the absence and presence of 5-CT. In B and D, current elicited by 5-CT obtained by subtracting amount of current in the absence of 5-CT from the current elicited in the presence of 5-CT.

TABLE 1

Cellular characteristics of neurons recorded from the ventromedial DR and MR

Area	RMP (mV)	Res (MΩ)	Tau (ms)	AHP amp (mV)	AHP t 1/2 (ms)	AHP dur (ms)	AP amp (mV)	AP dur (ms)	AP thresh (mV)
DR 5-HT (N = 33)	-63 ± 1.7	637 ± 32	51 ± 3.0	16 ± 0.8	132 ± 5.5	338 ± 13	69 ± 1.4	2.0 ± 0.1	-22 ± 1.7
DR Non 5-HT (N = 16)	-68 ± 1.5	531 ± 38	42 ± 2.6*	14 ± 1.0	117 ± 10	325 ± 25	63 ± 3.5	1.5 ± 0.1*	-23 ± 2.4
MR 5-HT (N = 23)	-66 ± 1.6	688 ± 35	44 ± 2.2 [‡]	20 ± 0.7 [‡]	137 ± 12	307 ± 22	72 ± 1.5	2.1 ± 0.1	-20 ± 1.9
MR Non-5-HT (N = 14)	-58 ± 2.8**	432 ± 57 [‡]	22 ± 2.1 [‡]	15 ± 1.6 [‡]	64 ± 12 [‡]	178 ± 20 [‡]	70 ± 2.0	1.5 ± 0.2 [‡]	-27 ± 3.0 [‡]

Values are mean ± SE.

* DR 5-HT different from DR non-5-HT: tau $t = 2.1$, $P = 0.04$; AP duration $t = 3.5$, $P = 0.001$.

[‡] MR 5-HT different from MR non-5-HT: RMP $t = 2.6$, $P = 0.01$, Res $t = 4.0$, $P = 0.0003$; tau $t = 6.7$, $P = 0.00000009$; AHP amplitude $t = 3.3$, $P = 0.002$; AHP t 1/2 $t = 4.6$, $P = 0.000005$; AHP duration $t = 4.8$, $P = 0.00003$; AP duration $t = 3.2$, $P = 0.003$; AP threshold $t = -2.2$, $P = 0.04$.

[‡] DR 5-HT different from MR 5-HT: tau $t = 2.2$, $P = 0.03$, AHP amplitude $t = -2.8$, $P = 0.008$.

TABLE 2

Magnitude of hyperpolarization elicited by 100 nM 5-CT in neurons recorded from ventromedial DR and MR

Area	5-CT hyper (mV)
DR 5-HT (<i>N</i> = 16)	13.0 ± 1.4
DR non-5-HT (<i>N</i> = 15)	7.1 ± 1.4 [*]
MR 5-HT (<i>N</i> = 10)	8.7 ± 1.3 [‡]
MR non-5-HT (<i>N</i> = 11)	0.0 ± 0.0 [‡]

Values are mean ± SE.

^{*} DR 5-HT different from DR non-5-HT: *t* = 2.8, *P* = 0.01.[‡] MR 5-HT different from MR non-5-HT: *t* = 8.6, *P* = 0.000000005.[‡] DR 5-HT different from MR 5-HT: *t* = 2.2, *P* = 0.03.