



SINGLE CELL LASER DISSECTION WITH MOLECULAR BEACON POLYMERASE CHAIN REACTION IDENTIFIES 2A AS THE PREDOMINANT SEROTONIN RECEPTOR SUBTYPE IN HYPOGLOSSAL MOTONEURONS

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Abstract—We hypothesize that sleep state-dependent withdrawal of serotonin (5-hydroxytryptamine, 5-HT) at upper airway (UAW) dilator motoneurons contributes significantly to sleep-related suppression of dilator muscle activity in obstructive sleep apnea. Identification of 5-HT receptor subtypes involved in postsynaptic facilitation of UAW motoneuron activity may provide pharmacotherapies for this prevalent disorder. We have adapted two assays to provide semi-quantitative measurements of mRNA copy numbers for 5-HT receptor subtypes in single UAW motoneurons. Specifically, soma of 111 hypoglossal (XII) motoneurons in 10 adult male rats were captured using a laser dissection microscope, and then used individually in single round molecular beacon polymerase chain reaction (PCR) for real-time quantitation of 5-HT_{2A}, 5-HT_{2C}, 5-HT₃, 5-HT₄, 5-HT_{5A}, 5-HT_{5B}, 5-HT₆ or 5-HT₇ receptor. Receptor mRNA copy numbers from single XII motoneurons were compared to control samples from within the XII nucleus and lateral medulla. All 20 motoneuronal soma assayed for the 5-HT_{2A} receptor had measurable copy numbers (7028 ± 2656 copies/cell). In contrast, copy numbers for the 5-HT_{2A} receptor in XII non-motoneuronal (*n* = 17) and lateral medulla (*n* = 15) samples were 81 ± 51 copies and 83 ± 35 copies, respectively, *P* < 0.05. Seven of 13 XII motoneurons assayed had measurable 5-HT_{2C} receptor copy numbers of mRNA (287 ± 112 copies/cell). XII soma had minimal 5-HT₃, 5-HT₄, 5-HT_{5A}, 5-HT_{5B}, 5-HT₆ or 5-HT₇ receptor mRNA. 5-HT_{2A} receptor mRNA presence within XII motoneurons was confirmed with digoxigenin-labeled *in situ* hybridization.

In summary, combined use of laser dissection and molecular beacon PCR revealed 5-HT_{2A} receptor as the predominant 5-HT receptor mRNA in XII motoneurons, and identified small quantities of 5-HT_{2C} receptor. This information will allow a more complete understanding of serotonergic control of respiratory activity. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: single cell, motor neurons, sleep apnea, upper airway, real-time PCR, taqman, laser capture microdissection, digoxigenin-labeled and *in situ* hybridization.

Obstructive sleep apnea is a highly prevalent disorder (Young et al., 1993), associated with significant cardiovascular (Peker et al., 2000; Shahar et al., 2001) and neurobehavioral morbidity (Young et al., 1993), and this is a disorder for which there are no widely effective pharmacotherapeutics. The pathogenesis of obstructive sleep apnea involves a narrowed collapsible upper airway (UAW) dependent upon UAW dilator muscles for patency and normal respiration (Remmers et al., 1978;

Surratt et al., 1983). While ventilation is normal in waking in persons with obstructive sleep apnea, sleep-related reductions in UAW dilator motoneuronal activity result in collapse of the airway and apneas (Remmers et al., 1978). One of the excitatory influences on UAW dilator motoneurons that is withdrawn in sleep is serotonin (5-hydroxytryptamine, 5-HT). We believe that sleep-related reductions in 5-HT excitation at UAW dilator motoneurons contribute substantially to the pathogenesis of obstructive sleep apnea.

A significant body of research supports the hypotheses that 5-HT plays an important role in the patency of the UAW in beings with obstructive sleep apnea and that sleep-related withdrawal of 5-HT at motoneurons contributes to collapse of the UAW and obstructive breathing. 5-HT locally applied to UAW motoneurons or within brainstem motor nuclei excites motoneurons in adult mammals (McCall and Aghajanian, 1980; Berger et al., 1992; Kubin et al., 1992; Al-Zubaidy et al., 1996; Douse and White, 1996; Bayliss et al., 1997). Second, the activity of serotonergic neurons within the brainstem

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Abbreviations: C_T, threshold cycle; DABCYL, 4-dimethylamino-phenylazobenzoic acid; FAM, 6-carboxyfluorescein; GFAP, glial fibrillary acidic protein; 5-HT, 5-hydroxytryptamine or serotonin; NREM, non-rapid eye movement; NSE, neuron-specific enolase; PCR, polymerase chain reaction; REM, rapid eye movement; RT, reverse transcription; UAW, upper airway; XII, hypoglossal.

declines in sleep (Heym et al., 1982). Activity of serotonergic neurons is less in non-rapid eye movement (NREM) sleep than in quiet wakefulness, and activity is almost ceased in rapid eye movement (REM) sleep. In the carbachol model of REM sleep, microdialysis studies within a motor nucleus for UAW motoneurons show marked reductions in 5-HT delivery during the chemically-induced REM sleep, coincident with suppression of dilator motoneuronal activity (Kubin et al., 1994). Dialysate delivery of 5-HT into motor nuclei for UAW dilators fully attenuates the NREM sleep associated reductions in dilator activity and markedly reduces the REM sleep suppressions in dilator activity in the rat (Jelev et al., 2001). Finally, it is likely that in wakefulness 5-HT delivery is increased to UAW motoneurons in persons with obstructive sleep-disordered breathing. That is, subsets of serotonergic neurons innervating motoneurons show increased firing rates in response to respiratory challenges (Veasey et al., 1995), and long-term facilitation of respiratory activity in response to hypoxia is 5-HT dependent (Bach and Mitchell, 1996). Administration of 5-HT receptor antagonists in an animal model of obstructive breathing produces collapse of the UAW and obstructive breathing (Veasey et al., 1996). Therefore, 5-HT likely contributes to the maintenance of patent airways in persons with obstructive sleep-disordered breathing, and sleep state-dependent withdrawal of 5-HT likely contributes to obstructive sleep apnea.

The 5-HT receptor subtypes involved in serotonergic modulation of UAW motoneurons are not known. An understanding of the receptor subtypes involved in UAW motoneuronal control is important for the development of safe, effective pharmacotherapeutics for obstructive sleep-disordered breathing. Pharmacological studies to date suggest multiple 5-HT receptor subtypes. Without highly selective agonists to pair with antagonists, the receptor subtypes have not been clearly identified. There are several intracellular changes with 5-HT receptor activation to suggest specific receptor subtypes. 5-HT depolarizes motoneurons through several mechanisms: reductions in resting potassium conductance (gK^+) (Larkman and Kelly, 1992; Garratt et al., 1993), and through enhancement of the hyperpolarization-activated inward current, I_H through both phosphorylation-dependent and -independent mechanisms (Larkman and Kelly, 1997). 5-HT_{2A} receptors are present on motoneurons (Fay and Kubin, 2000) and studies with semi-selective agonists and antagonists suggest a 5-HT_{2A} and/or 5-HT_{2C} receptor effect may result in both the decreased gK^+ and the phosphorylation-dependent cAMP activation, but not the phosphorylation-independent 5-HT receptor activation (Holtman et al., 1987; Aghajanian, 1990; Katakura and Chandler, 1990; Rasmussen and Aghajanian, 1990; Ribeiro-do-Valle et al., 1991; Larkman and Kelly, 1997). It is not known if 5-HT_{2C} receptors are present in UAW dilator motor nuclei. 5-HT₄, 5-HT_{5A}, 5-HT_{5B}, 5-HT₆ and 5-HT₇ receptors have all been shown to activate adenylyl cyclase through a phosphorylation-independent mechanism. Pharmacological studies of 5-HT effects on motoneurons are difficult to interpret given the paucity of selective

serotonergic drugs and the multiplicity of 5-HT receptor subtypes acting pre- or postsynaptically.

The purpose of this present study was to provide a semi-quantitative profile of serotonergic receptor mRNA for a representative population of UAW dilator motoneurons, the hypoglossal (XII) motoneurons. To accomplish this, we have combined laser capture of single cells with single round molecular beacon reverse transcription-polymerase chain reaction (RT-PCR) for eight 5-HT receptor subtypes. Digoxigenin-labeled 5-HT receptor subtype antisense and sense were used to substantiate receptor subtype presence in XII slices.

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague–Dawley rats (300–375 g; Charles River Laboratories, Wilmington, MA, USA) were used in these experiments. Prior to experiments, animals were housed in a temperature- and humidity-controlled University Lab Animal Research colony. They were fed an *ad libitum* diet of pellets and water and were maintained on a 12:12 light:dark schedule with lights on at 07:00 h. The University of Pennsylvania's Institutional Animal Care and Use Committee approved all procedures.

Primer and molecular beacon design

Conserved sequences of rat 5-HT receptor subtypes, α -tubulin, neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP) were amplified. The primer sets and molecular beacon sequences (Table 1) were selected using Primer Express software (Primer Express[®] 1.0) and were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). All 5-HT receptor subtypes selected for study were known excitatory subtypes, or had been shown to increase adenylate cyclase in neuronal tissue. Tubulin and NSE were added to characterize the quality of motoneuron samples, and GFAP was added to substantiate differences in mRNA profiles for single XII motoneurons and the control samples within the XII nucleus and lateral to the nucleus. Primer sets were selected for proximity to the 3'-end, specificity to target mRNA (GenBank), GC content of 30–80%, no runs > 3G, T_m between 57 and 60°C and to allow for positioning of a molecular beacon. In small tissue samples (< 1 mm³), we assayed both 3'-end and 5'-end pairs for both the 5-HT_{2A} receptor and α -tubulin. Prior to ordering molecular beacons, all primer pairs were tested for sensitivity to detect a band by standard PCR on medullary tissue sample sizes < 1 mm³. Each molecular beacon possessed two six- or seven-nucleotide arm sequences that were designed to form a stable stem hybrid at the annealing temperature of the PCR (probe T_m were 66–68°C), ensuring that non-hybridized molecular beacons would not fluoresce. The probe sequences were designed to be complementary to a conserved region within their target amplicon. Each molecular beacon was labeled with 6-carboxyfluorescein (FAM) at its 5'-end and the non-fluorescent quencher 4-dimethylaminophenylazobenzoic acid (DABCYL) at its 3'-end. FAM served as the reporter fluorochrome and DABCYL served as the quencher. Our 5-HT₇ receptor primer/probe set was designed to assay all four isoforms of the receptor mRNA (Heidmann et al., 1997).

Microdissection of hypoglossal motoneurons

Rats were deeply anesthetized with 80 mg/kg pentobarbital i.p. and then perfused with sterile 4°C phosphate-buffered saline (PBS). Brains were immediately removed and frozen in liquid

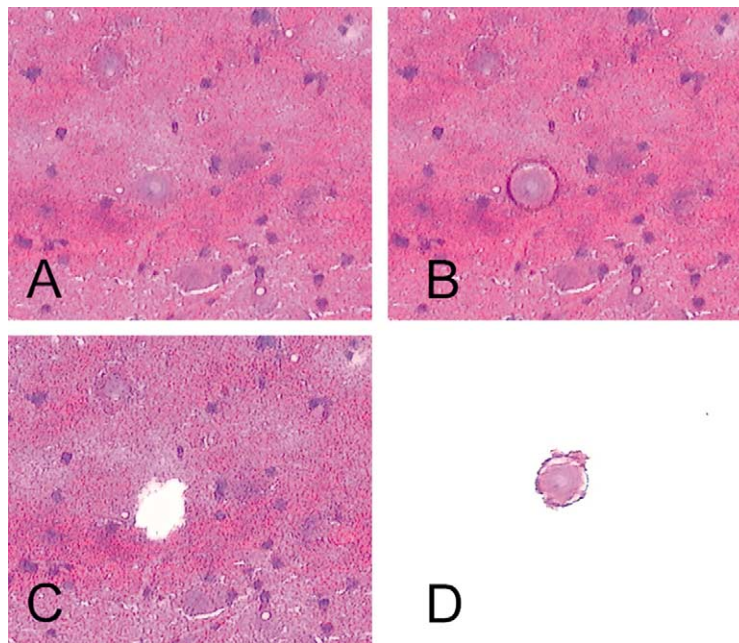


Fig. 1. Laser capture of a single XII motoneuron. Shown in A is the ventrolateral region of the XII nucleus within a 10 μ m hematoxylin- and eosin-stained slice. In B a motoneuron has been targeted, such that the perimeter of the motoneuronal soma has adhered to the melted cap where the dark ring is present. As the cap is raised (C) the cell body is captured onto the cap, and an image is obtained (D) showing capture of the one XII motoneuron.

nitrogen or placed on dry ice. 10 μ m sections were made with a sterilized cryostat blade through the medulla from 1 mm rostral to the calamus scriptorius to 1 mm caudal to the calamus scriptorius and placed on sterilized slide. Slides were immediately stained with hematoxylin and eosin and dried with progressive alcohol followed by xylene (Arcturus H&E protocol: www.arctur.com). Staining was performed in sterilized vessels with RNase inhibitor added to each solution. Slides were air-dried under a hood and then viewed microscopically for adequate sections within the rostral half of the XII nucleus on the stage of a laser capture microdissection scope (PixCell II, Arcturus, Mountain View, CA, USA). Motoneurons were identified by size within the XII nucleus as previously substantiated with electrophysiological recordings (Singer et al., 1996; Larkman and Kelly, 1997; Buck et al., 2000).

Laser capturing was performed with a 7.5 μ m spot size, 200 mV target and 50 mW power for 1 ms. Images of the cell, capturing and adherence to the cap were obtained for each sample. An example of laser acquisition of one motoneuron is shown in Fig. 1. The entire cap surface was reviewed after capture for any cellular debris, and caps were discarded when debris was noted. Caps with the isolated cells were placed on centrifuge tubes containing 100 μ l Trizol (Molecular Research Center, Cincinnati, OH, USA).

RNA extraction and reverse transcription protocols

Total RNA was extracted by a single step using Trizol and chloroform. After centrifugation at 13 000 r.p.m. for 15 min, the RNA-containing aqueous phase was precipitated in isopropanol. The RNA pellet was then washed once in 75% ethanol and re-suspended in 6 μ l of RNase-free water. Total RNA (5 μ l) was subjected to RT using Superscript[®] II RNase H⁻ reverse transcriptase (Gibco BRL, Invitrogen, Carlsbad, CA, USA) with specific primers (tubulin and one of the following 5-HT subtypes: 2A, 2C, 3, 4, 5A, 5B, 6 and 7; or tubulin and NSE or GFAP) for 50 min at 42°C. Heating at 72°C for 15 min terminated the RT reaction. 4 μ l (40%) of the resulting cDNA was used as a template for PCR and real-time PCR amplification. The specific antisense primers used in RT are listed in Table 1.

PCR analysis

The PCR amplification was performed with 4 μ l (40%) of cDNA for 50 cycles with Amplitaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT, USA) in a GeneAmp PCR System 2400 (Perkin-Elmer). The PCR reaction mixture contained 0.2 mM of dNTPs, 20 pmol of each of two primers and 1.5 units of Amplitaq Gold DNA polymerase in 1 \times reaction buffer in a total volume of 50 μ l. The PCR cycle consisted of heat activation of the DNA polymerase for 9 min at 95°C followed by 45 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and elongation at 72°C for 7 min. One fourth of each PCR-amplified product (12.5 μ l) was electrophoresed on 3.5% ethidium bromide-stained agarose gels.

Real-time PCR analysis

A portion (40%) of each cDNA sample was amplified using 1 \times Amplitaq buffer (Perkin-Elmer), 5 mM MgCl₂, molecular beacon (25 pmol), primers (50 pmol), and 1.5 U of Amplitaq Gold DNA polymerase (Perkin-Elmer) in a total volume of 50 μ l. One cycle of denaturation (95°C for 10 min) was followed by 45 cycles of amplification (95°C for 15 s, 60°C for 1 min). PCR was carried out in a 96-well spectrofluorimetric thermal cycler (ABI Prism 7700; Applied Biosystem, Foster City, CA, USA) that monitors changes in the fluorescence spectrum of each reaction tube during the annealing phase while simultaneously carrying out programmed temperature cycles.

Standard curve generation for primer/probe sets

Gene-specific primer pairs were checked for efficient amplification using each receptor product DNA as a standard for our real-time PCR analysis. PCR-amplified fragments were separated on 3.5% NuSieve 3:1 agarose. Gels were isolated and purified using the Wizard PCR Preps purification system (Promega, Madison, WI, USA). The purified fragments were measured with spectrophotometry as μ g/ml and then converted using molecular weight to copy number. Standard curves were generated for each primer/probe set using serial dilutions of known quantities of DNA: 100 000, 10 000, 1000, 100 and 10 copies per

well, using methods of Medhurst et al. (2000). Standard curves for each set were plotted C_T versus the log of the initial number of DNA copies, where C_T is the threshold cycle, a cycle number where the PCR reaction is both exponential and above the baseline variance for the first 15 cycles.

Data analysis

Absolute sensitivity of the primer/probe sets was defined as the number of copies of DNA detectable at the lowest copy number on the standard curve. Relative sensitivity to other primer/probe sets was measured as the C_T for 25 000 copies of rat DNA. Efficiency for each primer/probe set was quantified as the slope (m) of the standard curve ($C_T = m(\log Q) + c$). Relative quantity of receptor subtypes within motoneurons was normalized to the tubulin C_T per cell. Copy numbers of mRNA for each receptor subtype were compared across three conditions: XII soma samples, XII non-soma similarly-sized samples within the dorsolateral XII nucleus and similarly-sized lateral medulla samples from just outside the XII nucleus. Sample sizes for statistical analyses in each case were \geq six rats. Single factor analysis of variance (ANOVA) with Neumann-Keuls was used with statistical significance at < 0.05 .

RESULTS

Relative efficiencies and sensitivities for 5-HT receptor subtype primer/probe sets for medullary tissue

The sensitivity and efficiency of each primer/probe set

(tubulin and all 5-HT receptor subtypes tested) were established using the C_T values for known quantities of specific cDNA. Sensitivities at 100 000 copies of message for each receptor primer/probe set ranged from 26 to 31 cycles and sensitivities for 100 copies ranged from 35 to 45 cycles. The efficiency and linearity for each primer/probe set were determined by plotting C_T versus log starting copy numbers ($10^1, 10^2, 10^3, 10^4, 10^5$). Linearity and efficiency values for receptor subtypes 5-HT_{5A}, 5-HT₆, 5-HT₇ were poor when data for 10 copies were included. Thus, for these receptor subtypes, standard curves were drawn using 100 to 100 000 copies. For all receptor subtypes and tubulin, slopes ranged from -3.0 to -3.3 . Standard curves are shown in Fig. 2. Thus, maximal efficiency was achieved with several sets, and was acceptable in all sets. Correlation coefficients ranged from 0.94 to 1.0. Efficiency and sensitivities are presented with primer/probe sets in Table 1.

Isolation of single hypoglossal motoneuronal soma, and control samples

Single XII motoneurons, identified by size and location within the XII nucleus, were targeted with laser capture microdissection (25 μ m diameter) and affixed to the capture film. 111 captures of individual XII motoneuron soma, 88 non-soma samples within the XII

Table 1. The Primers and molecular beacon sequences used for RT-PCR

Gene	Primer and molecular beacon	Position (bp)	Expected length (bp)	10 ⁵ copies C_T and slope	Acc. no.
Tubulin	sense: AGCGCAGCATCCAGTTTGT	95–110	135	28	V01226
	antisense: CTGTGGTGTGGTCTCAGCATA GA	229–210			
	molecular beacon: GCGAGTCAGCCTCCCACCGTGGTACCTGGTACTCGC				
NSE	sense: CATGGGAAGGTCACAGAAAGG	1965–1986	117	N/A	AF019973
	antisense: GGGAGAGAGTTATAGGAGAA	2082–2063			
	molecular beacon: GCGAGTCACTAACTGAAGCTCGGTACTTTACAACCTCGC				
GFAP	sense: CTGAAAGTGTCCCTCAGTT	8483–8502	112	N/A	AF028784
	antisense: ACAGTACTGCTCTGAAGGTTAG	8595–8574			
	molecular beacon: GCGAGTACTTTTCCCAGGACATCGTACACACTCGC				
5HT2A	sense: CTACATTACTCTGACAAATCCCAAT	3458–3483	134	31	M64867
	antisense: TCA ACAGTAATGTGCTCACAGT	3592–3571			
	molecular beacon: GCGAGTTGGCTACTACAGACCAACAAGACCAGCACTCGC				
5HT2C	sense: AATTTACCGAAGGGCTTTCTCTAAA	1372–1396	148	31	U35315
	antisense: CACGTTTCATTGGTATGCCGATA	1520–1499			
	molecular beacon: GCGAGTCAGATTTCCTAGGGTTGCTGCCACTGC ACTCGC				
5HT3	sense: TGGAGAAGCGGATGAGATG	1301–1320	93	29	D49395
	antisense: GCCAGCAGGTAGATGCGAAACA	1394–1373			
	molecular beacon: GCGAGTAGCACATATCCCACCCGCAACCAACTCGC				
5HT4	sense: TTACCGTATCTATGTCACTGCTAAGGA NM012853	656–683	141	26	
	antisense: CTTGGCTGCTTTGGTCTCTGT	797–777			
	molecular beacon: GCGAGTCCACCTCTGAAAGCAGGCCCCAGACTCGC				
5HT5A	sense: CACAGGCCTGGCCACAACGT	1459–1479	99	28	L10072
	antisense: TGTCTCTTCTCTCTCTCTCTCT	1558–1537			
	molecular beacon: GCGAGTCCACTCCAGCGGGACCATGAGAAACTCGC				
5HT5B	sense: CTAGTGAATTGTGCTACATTTCT	1631–1653	109	30	L10073
	antisense: CAACTCTGCCACATGTGGAGTCT	1740–1718			
	molecular beacon: GCGAGTCCGCCAAACCCTTTCCGGGTGACTCGC				
5HT6	sense: AGCTCACAGCCAGCTTCTGCT	1172–1193	113	29	L41146
	antisense: GTATCTCAGGCTCCACAGAGTCTG	1285–1262			
	molecular beacon: GCGAGTCCACCACCAGGGCCACCCTACTACTCGC				
5HT7	sense: CTTCTTCCAGATGTCCTGATGTCTA	2825–2850	127	30	L22558
	antisense: TGGTCAGTCTAGCAAACTGCA	2951–2931			
	molecular beacon: GCGAGTCACATTCTGAAAGGACAGTGCCTGCCTACTCGC				

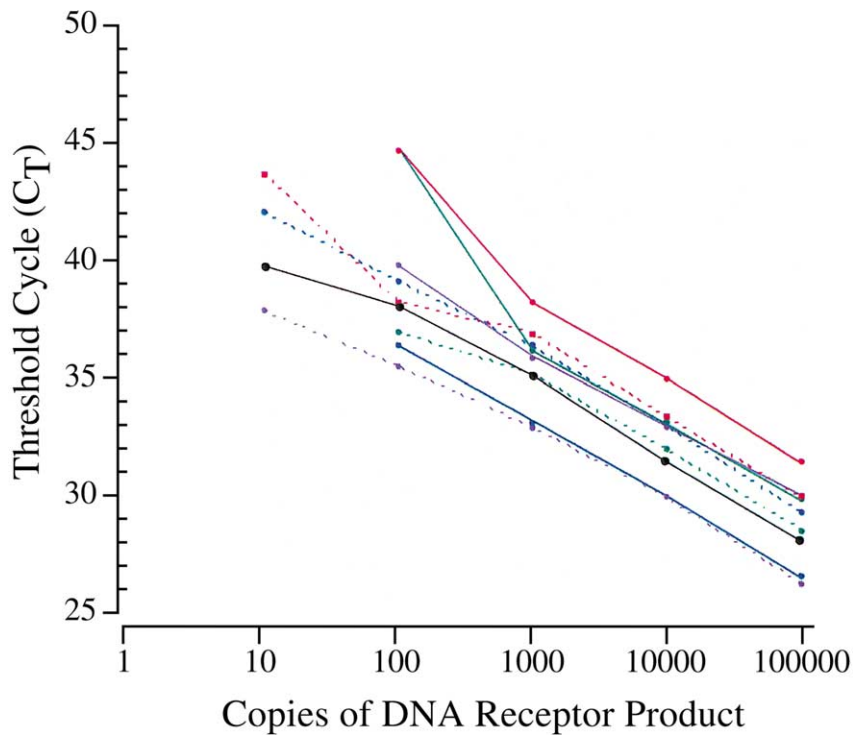


Fig. 2. Standards curves for primer/molecular beacon probe sets for all 5-HT receptor subtypes assayed. Standard curves are generated from the C_T values plotted against estimated copy numbers of DNA. Our primer/probe sets had similar efficiencies (slope range -3.0 to -3.3) and minimal differences in sensitivities (absolute C_T for a given copy number). Primer/probe legend: 5-HT_{2A}: dashed red line; 5-HT_{2C}: solid red line; 5-HT₃: dashed blue line; 5-HT₄: solid blue line; 5-HT_{5A}: dashed green line; 5-HT_{5B}: solid green line; 5-HT₆: dashed purple line; 5-HT₇: solid purple line.

Table 2. Characteristics of mRNA copies for eight 5-HT receptor subtypes measured in single XII motoneurons and control samples

5-HT receptor mRNA	Mean copy number \pm S.E.M.	Copy number range	Sample size (<i>n</i>)	% samples with measurable copies
5-HT _{2A}	XII MS: 7028 \pm 2656	XII MS: 100–20 000	XII MS: 20	XII MS: 100%
	XII NMS: 81 \pm 45	XII NMS: 0–120	XII NMS: 17	XII NMS: 33%
	LM Con: 319 \pm 281	LM Con: 0–500	LM Con: 15	LM Con: 22%
5-HT _{2C}	XII MS: 287 \pm 105	XII MS: 0–750	XII MS: 13	XII MS: 75%
	XII NMS: 50 \pm 33	XII NMS: 0–200	XII NMS: 8	XII NMS: 38%
	LM Con: 142 \pm 80	LM Con: 0–150	LM Con: 10	LM Con: 33%
5-HT ₃	XII MS: 7 \pm 6	XII MS: 0–30	XII MS: 7	XII MS: 0%
	XII NMS: 475 \pm 335	XII NMS: 0–2000	XII NMS: 7	XII NMS: 29%
	LM Con: 0	LM Con: 0–0	LM Con: 7	LM Con: 0%
5-HT ₄	XII MS: 65 \pm 35	XII MS: 0–100	XII MS: 7	XII MS: 29%
	XII NMS: 0	XII NMS: 0	XII NMS: 8	XII NMS: 0%
	LM Con: 0	LM Con: 0	LM Con: 8	LM Con: 0%
5-HT _{5A}	XII MS: 81 \pm 11	XII MS: 0–90	XII MS: 8	XII MS: 63%
	XII NMS: 0	XII NMS: 0	XII NMS: 9	XII NMS: 0
	LM Con: 533 \pm 271	LM Con: 100–2000	LM Con: 7	LM Con: 57%
5-HT _{5B}	XII MS: 21 \pm 21	XII MS: 0–21	XII MS: 4	XII MS: 25%
	XII NMS: 0	XII NMS: 0	XII NMS: 7	XII NMS: 0
	LM Con: 29 \pm 29	LM Con: 0–29	LM Con: 5	LM Con: 20%
5-HT ₆	XII MS: 35 \pm 25	XII MS: 0–78	XII MS: 8	XII MS: 38%
	XII NMS: 0	XII NMS: 0	XII NMS: 6	XII NMS: 0
	LM Con: 0	LM Con: 0	LM Con: 7	LM Con: 0
5-HT ₇	XII MS: 17 \pm 17	XII MS: 0–17	XII MS: 7	XII MS: 29%
	XII NMS: 60 \pm 60	XII NMS: 20–300	XII NMS: 8	XII NMS: 13%
	LM Con: 4800 \pm 2800	LM Con: 0–20000	LM Con: 9	LM Con: 78%

XII MS, hypoglossal motoneuron soma; XII NMS, hypoglossal non-motoneuron soma control sample; LM Con, control sample obtained lateral to the XII nucleus.

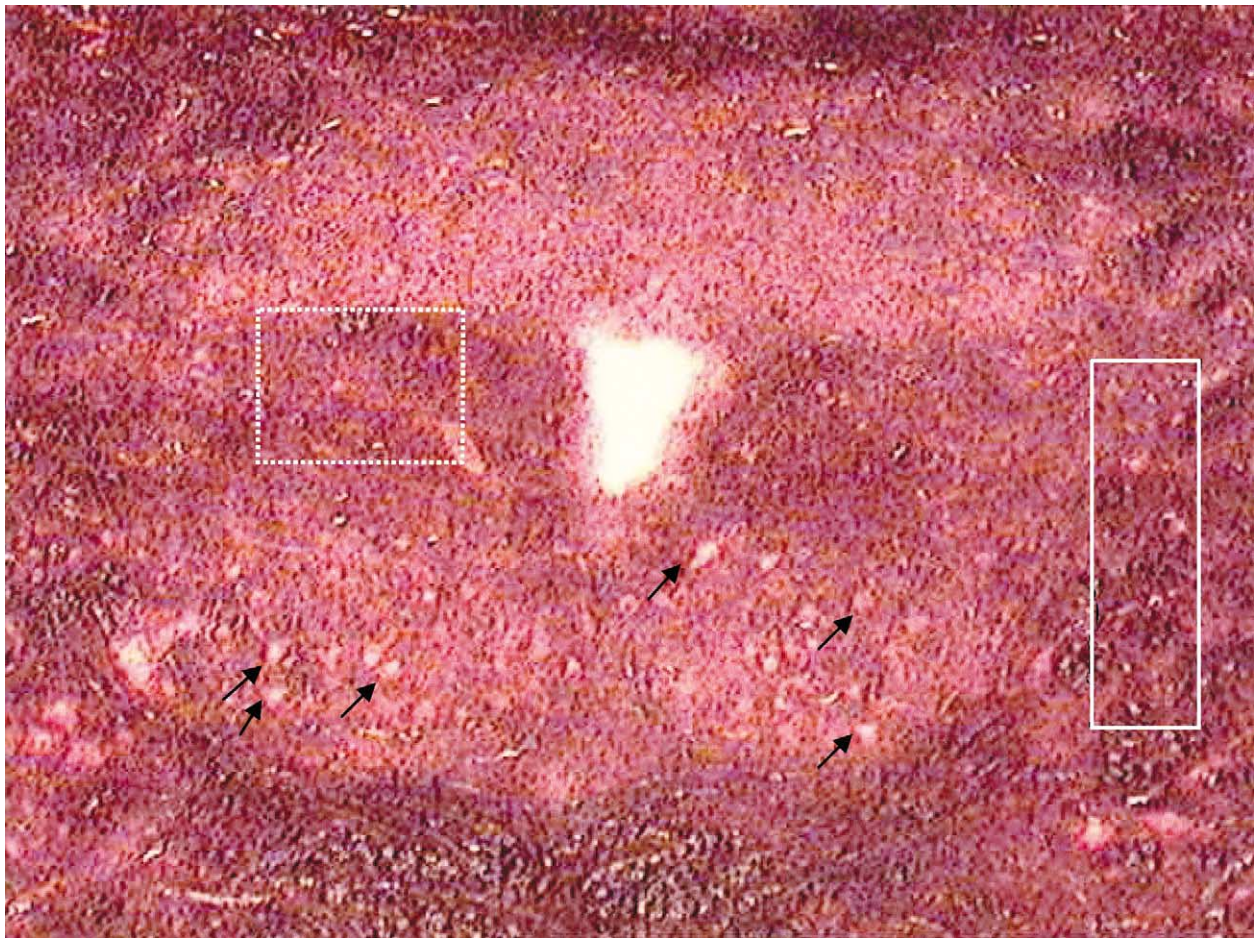


Fig. 3. Delineation of regions sampled for real-time measurement of mRNA. 10 μm slices through the rostral-caudal portions of the XII nucleus were sampled for single XII motoneuronal cell bodies. In this section, the black arrows highlight six cells sampled in this section. The dashed line box surrounds the areas from which control non-motoneuronal samples were obtained, and the solid box marks the perimeter of the lateral medulla control sample sites.

nucleus and 85 samples outside the XII nucleus in a total of 10 rats were successfully performed such that additional tissue was not transferred to the cap and Trizol effectively dissolved cellular contents from the cap. Images of a targeted XII motoneuron before and after capture are presented in Fig. 1. Multiple samples were obtained from each rat from XII motoneuronal cell bodies, similarly-sized samples within area of the nucleus most concentrated with motoneuronal dendrites and lateral to the nucleus. Regions sampled are shown in Fig. 3.

Comparison of tubulin, neuronal specific enolase and GFAP mRNA yields across the three medullary sites

C_T values were obtained for tubulin, NSE and GFAP. Tubulin was detected in 76 of 90 (84%) XII soma samples. XII motoneuron soma contained $11\,230 \pm 644$ copies of tubulin, while non-soma samples within the XII nucleus (82 samples) and lateral to the nucleus (70 samples) contained 1224 ± 387 copies and 5230 ± 1695 copies, respectively. NSE was detected in 98% of all XII soma samples. Relative quantities (measured as C_T) for tubulin and NSE were greater in motoneurons compared to XII non-motoneuron or lateral medulla samples, $P < 0.05$,

while GFAP quantities trended towards greater in the dendritic (27 samples, $P = 0.06$) and lateral medulla samples (25 dissections, $P = 0.05$) than in XII motoneuron cell bodies (27 soma samples).

Quantitation of mRNA for 5-HT receptor subtypes in hypoglossal motoneurons, and hypoglossal non-motoneuron samples and outside the hypoglossal nucleus

These data are presented in Table 2 and Fig. 4. 5-HT_{2A} receptor mRNA was detected in all XII motoneuronal soma in which tubulin mRNA was also present. There was significantly more 5-HT_{2A} receptor mRNA in soma samples than in either the XII non-motoneuronal control samples ($P < 0.05$) or lateral medulla sample ($P < 0.01$). The only other receptor subtype observed in XII motoneuron(s) in quantities > 250 was the 5-HT_{2C} receptor. Copy numbers for 5-HT_{2C} receptor mRNA were higher in XII motoneuron(s) than in the XII non-motoneuronal samples, $P < 0.05$, but not significantly greater than the lateral medulla controls. Although the 5-HT₇ receptor was not seen in XII motoneurons, this receptor subtype was found in large numbers in the majority of lateral medulla samples. Also

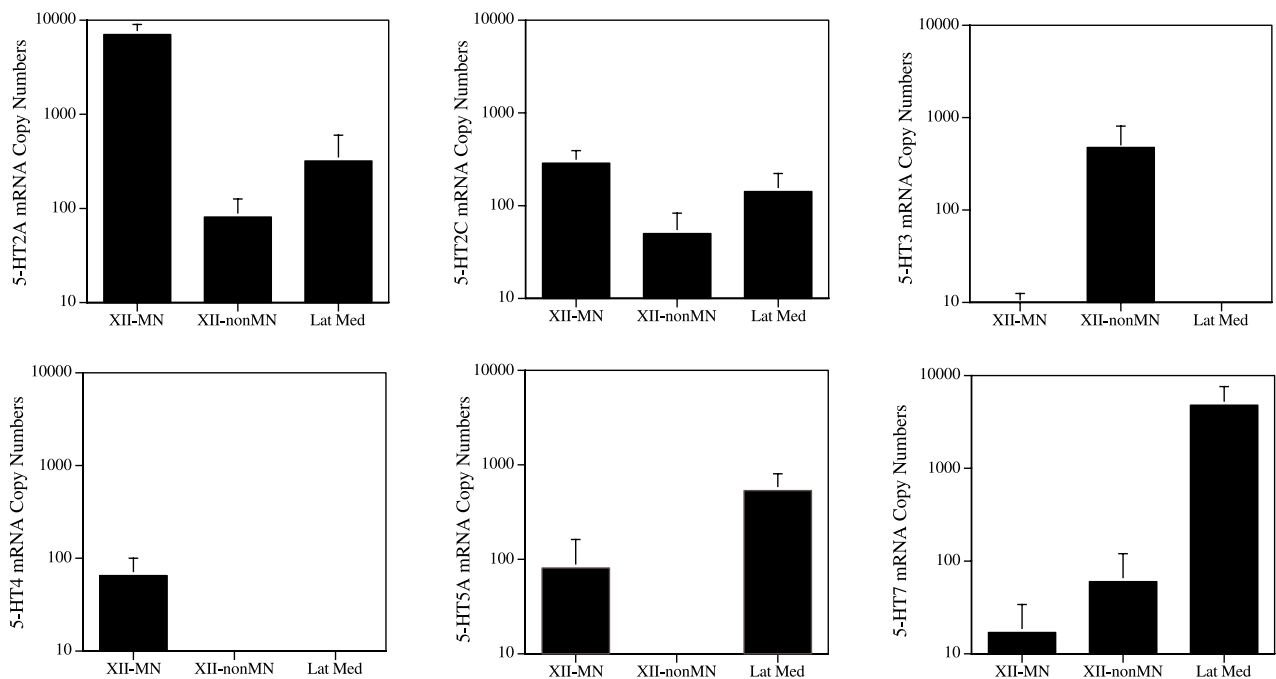


Fig. 4. 5-HT receptor subtype mRNA expression in XII motoneurons and control areas. Plotted are the mean estimated mRNA copy number (log scale) for each of three sites, single XII motoneurons (XII-MN); similarly-sized samples within the XII nucleus without a motoneuronal soma evident (XII-nonMN); and similarly-sized samples from the region just lateral to the XII nucleus (Lat Med). 5-HT_{2A} is present within XII motoneurons in substantially larger quantities than any other receptor subtype. Within XII motoneurons, 5-HT_{2C} is the only other receptor subtype detected with copy numbers over 100/motoneuron. 5-HT₃ is present in XII non-motoneuron samples, and 5-HT₇ is present in large quantities adjacent to the XII nucleus.

found in lateral medulla samples, at a lesser frequency, was the 5-HT_{5A} receptor mRNA.

In situ hybridization for 5-HT receptor subtypes detected in motoneurons with real-time PCR

In situ hybridization histochemistry with antisense probes for 5-HT_{2A} revealed hybridization-positive motoneuronal cell bodies in the XII nucleus with low background (Fig. 5A). In contrast the sense probe for the 5-HT_{2A} receptor did not label motoneurons (Fig. 5B), thereby supporting the specificity of the antisense probe. *In situ* hybridization for the 5-HT_{2C} receptor did not reveal labeling with either the sense or antisense probes within the XII nucleus, or anywhere within the slices. There was, however, faint labeling within the choroid plexus for the 5-HT_{2C} receptor antisense probe, without detectable labeling using the 5-HT_{2C} receptor sense probe.

DISCUSSION

A semi-quantitative profile of eight 5-HT receptor subtypes mRNA for single XII motoneurons has been characterized and compared with profiles in surrounding neuronal tissue. We have shown a presence and concentration of the 5-HT_{2A} and 5-HT_{2C} receptor in XII motoneurons, with very low levels (< 100 copies/cell) for the 5-HT₄, 5-HT_{5A}, 5-HT₆ and 5-HT₇ receptors and no detectable message for the 5-HT₃ or 5-HT_{5B} receptor

in XII motoneurons. These data suggest that the predominant 5-HT receptor subtype contributing to 5-HT postsynaptic facilitation of XII motoneurons in the normal adult rat is the 5-HT_{2A} receptor; although the 5-HT_{2C} receptor is present in small quantities in XII motoneurons and may, therefore, contribute to XII excitation. This semi-quantitative technique provides a window into serotonergic control of respiratory activity and a focus for pharmacologic studies for obstructive sleep apnea. The technique may be helpful in the analysis of 5-HT receptor subtype plasticity in respiratory motoneurons.

Detection of mRNA in single hypoglossal motoneurons

Isolation of RNA from a single cell for detection of specific mRNA transcripts has been a formidable challenge (for review, O'Dell et al., 1999; Sucher et al., 2000). In most cases, a preamplification process is required to detect mRNA in single cells (Phillips and Lipski, 2000). *In situ* transcription provides linear amplification and thus quantitation of mRNA. However, in whole live cells, only 40–50% of genes may be assayed with this technique (Kacharmina et al., 1999). Single cell RT-PCR can detect message levels (Sucher et al., 2000), but this technique does not allow quantitation. In the present study, we have demonstrated that laser capture of single motoneurons, paired with molecular beacon real-time PCR, is a sensitive technique for assaying message levels in single motoneurons. Tubulin, NSE and GFAP were measured to evaluate the integrity of our

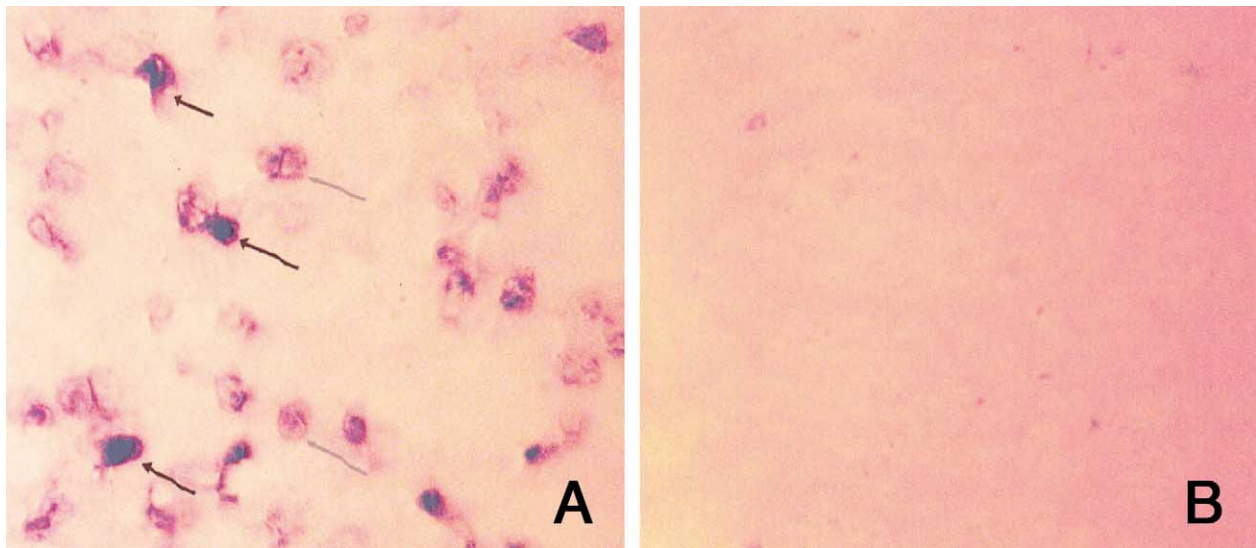


Fig. 5. *In situ* hybridization histochemistry for 5-HT_{2A} in the XII nucleus. Sections (10 μ m) of the medulla through the entire XII nucleus were hybridized with antisense (A) and sense (B) for the 5-HT receptor subtype 5-HT_{2A}. XII motoneurons throughout the nucleus were positive for antisense, although the intensity of hybridization varied with some motoneurons demonstrating (arrows) individual motoneuronal cells with strong labeling and others showing less intense.

samples. In neuronal tissue, mRNA for both tubulin and NSE is abundant and likely to be present in quantities greater than for 5-HT receptor subtypes, while GFAP should be present in larger quantities in samples without neuronal soma. In the present study, tubulin and NSE mRNA were detectable in most XII motoneuronal samples in relatively large quantities, and GFAP mRNA copies were greater in non-soma samples. The presence of digoxigenin-labeled 5-HT_{2A} receptor antisense in XII motoneurons coupled with an absence of label in control sample areas supports the real-time finding of the 5-HT_{2A} receptor in XII motoneurons and not in surrounding tissue. At the same time, a lack of digoxigenin-labeled 5-HT_{2C} receptor in XII motoneurons and weak labeling within the choroid plexus with three different 5-HT_{2C} probes, highlights the relative sensitivity and ease of this real-time technique. The combination of laser capture microdissection of single cells with real-time PCR with molecular beacons, as we have described in this paper, provides a highly sensitive, specific and relatively straightforward method for quantitation of mRNA in single neurons. A present limitation with this technique is that from one cell, just two message levels may be assayed, rather than a large profile that is feasible if the sample was pre-amplified.

We designed 3'-end primer pairs for 5-HT_{2A}, 5-HT_{2C}, 5-HT₃, 5-HT₄, 5-HT_{5A}, 5-HT_{5B}, 5-HT₆ and 5-HT₇ receptor mRNA with high sensitivity for PCR studies on small samples of medullary tissue. 3'-end primer pairs were significantly more sensitive than 5'-end sets. Qualitatively, use of selective primers in cDNA synthesis enhanced sensitivity. The technique of laser capture of single XII motoneurons, followed by our protocols for real-time PCR, is sensitive enough to detect NSE mRNA in all single cell samples. Tubulin is captured in almost all motoneuron samples, and thus, either NSE or tubulin can provide an index of sampling adequacy. Normaliza-

tion to tubulin copy number should be discouraged as the correlations between tubulin and the 5-HT_{2A} receptor and tubulin and NSE in XII motoneurons soma have never been substantiated with other techniques and in our study the correlations were weak. Ideally, standard curves should be performed on known copy numbers of the nucleic acid being assayed. Without available known copy number samples, spectrophotometry may be used to measure concentrations of dsDNA. This technique, although excellent in providing stable DNA, can be limited by suboptimal accuracy in concentration measurement. To improve accuracy, we used samples with high OD values and for several primer pairs we ran duplicate samples. In duplicates, concentration values were remarkably similar (differences < 0.01%). However, with this concern and with the concern that the entire cytoplasm may not be lasered for one cell, we believe the measurements should be described as semi-quantitative rather than quantitative.

5-HT receptor subtypes in hypoglossal motoneurons

Pharmacologic trials of 5-HT receptor agonists and antagonists support 5-HT_{2A} and 5-HT_{2C} receptor postsynaptic facilitation of XII motoneurons (McCall and Aghajanian, 1980; Berger et al., 1992; Kubin et al., 1992; Al-Zubaidy et al., 1996; Douse and White, 1996; Bayliss et al., 1997; Inoue et al., 1999). Data from these studies, however, could not discern 5-HT_{2A} from 5-HT_{2C} effect and vice versa. Further, due to non-specificity of drugs, other receptor subtypes could not be ruled out. Our data suggest that 5-HT_{2A} receptor is likely the predominant receptor for postsynaptic 5-HT modulation of XII respiratory activity. Further, it is far less likely that the minimal quantities of other receptor subtypes contribute to facilitation.

The 5-HT innervation pattern in the XII nucleus is

unique relative to other brainstem motor nuclei. 5-HT terminals surrounding the motoneuronal bodies in the XII nucleus are sparse (Arita et al., 1993). Rather, the majority of terminals appose the distal dendrites. Within some neurons there exists a topical distribution of mRNA (Kacharina et al., 1999), with message for distal dendrite receptors found differentially in distal dendrites. Our sample size was too large to isolate individual dendrites, but the dorsolateral XII nucleus is rich in protruder dendrites, and in our sampling we avoided motoneuron cell bodies. We sampled this area to determine if dendritic cytoplasm contained copies of specific 5-HT receptors. The 5-HT_{2C} receptor was found in a minority of dendrite-rich samples and similarly in samples lateral to the XII nucleus. Our data suggest that the 5-HT_{2C} receptor mRNA is concentrated primarily in XII cell bodies.

A recent immunologic study suggests that 5-HT₃ receptors on XII and other brainstem motoneurons (Morales et al., 1996). Immunolocalization studies may be hampered by non-specific binding on motoneuronal membranes. We have recently shown a lack of effect for a 5-HT₃ receptor agonist and two antagonists locally within the XII nucleus (Veasey et al., 1999). In the present study we have minimal message for the 5-HT₃ receptor in XII motoneurons. In contrast, mRNA copy numbers were high in regions abutting the dorsal motor nucleus of vagus. It is possible then that single cell mRNA quantitation with comparison to surrounding tissue, as a specific method to corroborate presence of receptor mRNA, may provide a complementary assay for the presence of postsynaptic receptor subtype

mRNA. Functional studies for the receptors, however, are still necessary.

XII motoneurons innervate several of the larger UAW dilators, but do not innervate all muscles involved in UAW control. Pharmacologic effects of partially selective 5-HT receptor agonists and antagonists suggest similar 5-HT receptor subtype functional profiles for facial (McCall and Aghajanian, 1980; Larkman and Kelly, 1997), trigeminal (Ribeiro-do-Valle et al., 1991; Hsiao et al., 1997) and XII nerves (Berger et al., 1992; Kubin et al., 1992; Al-Zubaidy et al., 1996; Douse and White, 1996). Characterization of 5-HT receptor subtypes in the other brainstem motoneuronal groups should be performed for comparison.

In the present study we have determined that the postsynaptic 5-HT receptor subtype most likely to contribute to 5-HT facilitation of XII motoneuronal respiratory activity is the 5-HT_{2A} receptor; although the 5-HT_{2C} receptor may also contribute to respiratory control of XII motoneurons. Which of these contributes and is modulated by obstructive sleep apnea will require further study. The 5-HT mediated increase in cellular adenylyl cyclase (Larkman and Kelly, 1997) is not explained by our findings and suggests a 5-HT_{2A}/5-HT_{2C} receptor effect on second receptor through activation of second messenger systems. It is hoped that this information will advance our understanding of 5-HT control of respiratory activity.

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