Impaired Chemosensitivity of Mouse Dorsal Raphe Serotonergic Neurons Overexpressing Serotonin 1A (Htr1a) Receptors

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Abstract

Background: Serotonergic system participates in a wide range of physiological processes and behaviors, but its role is generally considered as modulatory and noncrucial, especially concerning life-sustaining functions. We recently created a transgenic mouse line in which a functional deficit in serotonin homeostasis due to excessive serotonin autoinhibition was produced by inducing serotonin 1A receptor (Htr1a) overexpression selectively in serotonergic neurons (Htr1aRO mice). Htr1aRO mice exhibit episodes of autonomic dysregulation, cardiovascular crises and death, resembling those of sudden infant death syndrome (SIDS) and revealing a life-supporting role of serotonergic system in autonomic control. Since midbrain serotonergic neurons are chemosensitive and are implicated in arousal we hypothesized that their chemosensitivity might be impaired in Htr1aRO mice.

Principal findings: Loose-seal cell-attached recordings in brainstem slices revealed that serotonergic neurons in dorsal raphe nucleus of Htr1aRO mice have dramatically reduced responses to hypercapnic challenge as compared with control littermates. In control mice, application of 9% CO₂ produced an increase in firing rate of serotonergic neurons (0.260±0.041 Hz, n = 20, p = 0.0001) and application of 3% CO₂ decreased their firing rate (0.142±0.025 Hz, n = 17, p = 0.0008). In contrast, in Htr1aRO mice, firing rate of serotonergic neurons was not significantly changed by 9% CO₂ (0.201±0.034 Hz, n = 16, p = 0.49) and by 3% CO₂ (0.012±0.046 Hz, n = 12, p = 0.97).

Conclusions: Our findings support the hypothesis that chemosensitivity of midbrain serotonergic neurons provides a physiological mechanism for arousal responses to life-threatening episodes of hypercapnia and that functional impairment, such as excessive autoinhibition, of midbrain serotonergic neuron responses to hypercapnia may contribute to sudden death.

Introduction

Serotonergic system activity participates in a wide range of basic physiological processes regulating cardiovascular and respiratory autonomic responses, arousal, sleep-wake cycle, nociception, food intake and energy balance as well as in higher brain functions such as emotion and cognition. However, the role of serotonin has traditionally been considered as modulatory and nonessential for life-sustaining responses. Recent findings using transgenic mice with functional or anatomical alterations of the serotonergic system have substantially changed this view by revealing a key role of serotonergic system in the regulation of life-sustaining autonomic functions. We created Htr1aRO transgenic mice by reversible overexpression of the Htr1a selectively in serotonergic neurons [1]. This mouse line has a functional deficit in the serotonergic system due to increased negative feedback of serotonin on somatodendritic Htr1a autoreceptors. Unexpectedly, Htr1aRO mice showed episodes of autonomic dysregulation, life-threatening cardiovascular crises and sudden death. A similar panel of symptoms have been found in mutant mice with structural deficits in the serotonergic system. In mice lacking serotonergic neuron-restricted Pet-1 transcription factor (Pet-1−/− mice), the majority of serotonergic neuron precursors fail to differentiate while the remaining ones show multiple deficit in serotonergic-specific gene expression [2]. Pet-1−/− mice exhibit increased neonatal mortality and, immediately after birth, their respiratory control is susceptible to environmental conditions, such as exposure to hypoxia or anoxia, suggesting a critical developmental window, analogous to that of sudden infant death syndrome (SIDS) [3,4]. Another mouse line, Lmx1bf/f/p, in which nearly all serotonergic neurons are genetically deleted, also exhibits compromised autonomic functions, shows high mortality.
[5] and lacks arousal response to inhalation of CO2 [6]. In addition, pharmacological lesion of serotonergic neurons in neonatal rat pups, which reduced serotonin content by ~ 90%, increased their mortality in response to repeated environmental anoxia, when tested at P7-10, suggesting a physiological role of serotonergic neurons in autoregulation [7].

In humans, functional and/or structural serotonergic system alterations resulting in the dysregulation of life-supporting-autonomic responses are suspected to participate in SIDS, the leading cause of death for infants aged 1–12 months in developed countries [5].

Several genetic studies have revealed association of SIDS with genes involved in serotonergic function, including the serotonin transporter gene, SLC6A4 [9–12], but see [13], and Htr1a gene [14]. Post-mortem studies have also suggested that serotonergic system abnormalities might be implicated [15–17] and that incomplete arousal from sleep might be the actual cause of death [8,18]. The activity of serotonergic neurons in dorsal raphe nucleus (DRN) correlates with behavioral arousal and sleep-waking states [19–21]. Together with the median raphe nucleus, the DRN is considered to be part of the wake-promoting ascending arousal system (see [22]). Since acute hypercapnia is a powerful stimulus for arousal from sleep in infants and adults [23,24] and DRN serotonergic neurons are chemosensitive [25,26], it has been proposed that midbrain serotonergic neurons initiate the arousal response to hypercapnia and that impairment in CO2 chemoreception due to serotonergic system dysfunction might be the primary defect in a subset of SIDS [27].

The physiological mechanism by which altered serotonin homeostasis in Htr1aKO mice compromises life-sustaining functions are unknown. We hypothesized here that excessive serotonin autoinhibition in Htr1aKO mice may interfere with CO2 chemosensitivity of serotonergic neurons. To test this hypothesis we used loose-seal cell-attached recording to examine chemosensitivity of DRN serotonergic neurons in brainstem slices from Htr1aKO mice and control littermates. We particularly focused on responses to hypercapnia, which may have a crucial role in survival response to a life-threatening event in Htr1aKO mice and may be related to SIDS.

**Results**

Using loose-seal cell-attached voltage-clamp recordings in brain slices obtained from Htr1aKO and control mice, we compared changes in the firing rate of DRN serotonergic neurons in response to changes in PCO2 that reproduce in vivo the effects of hypercapnia (9% CO2) and hyperventilation (3% CO2). The present report is based on recordings from 31 neurons from 13 Htr1aKO mice and 64 recordings from 31 control littermates.

**Intrinsic Chemosensitive Responses of DRN Serotonergic Neurons are Markedly Decreased in Htr1aKO Mice**

To determine intrinsic chemosensitivity of serotonergic neurons in Htr1aKO and control mice we measured the responses to 9% CO2 using artificial cerebrospinal fluid (ACSF) supplemented with a mixture of drugs containing: 10 μM phencyclidine to facilitate firing; 10 μM 2,3-dimethyl-2-isoxazolyl-1(3H)-pyridazine butanoic acid (NBQX) and 20 μM 6-(2-amino-3-phosphono-propionic acid) (d-APV) to block excitatory synaptic transmission; and 10 μM 6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazine butanoic acid (SR-95531), 2 μM M-1[1-(S)-3,4-dichlorophenyl]ethyl]-amino-2-(S)-hydroxypropyl-p-benzyl-phosphonic acid (CGP-55845A) and 10 μM strychnine to block inhibitory synaptic transmission (Figure 1). In control mice, application of 9% CO2 produced an increase in firing rate of serotonergic neurons (0.260±0.041 Hz, n = 20, p = 0.0001) and application of 3% CO2 decreased their firing rate (−0.142±0.025 Hz, n = 17, p = 0.0000). In contrast, in Htr1aKO mice, firing rate of serotonergic neurons was not significantly changed by 9% CO2 (0.021±0.034 Hz, n = 16, p = 0.49) and by 3% CO2 (0.012±0.046 Hz, n = 12, p = 0.97). The baseline firing rate of serotonergic neurons in normocapnic conditions was similar in control and Htr1aKO mice (1.056±0.165 Hz, n = 22 and 1.610±0.179 Hz, n = 16, respectively, p = 0.45; Figure 1C).

The responses of individual neurons to CO2 were not correlated with their baseline firing rate both in control (9% CO2, p = 0.11; 3% CO2, p = 0.11) and Htr1aKO (9% CO2, p = 0.33; 3% CO2, p = 0.31) mice. As shown in Figure 1D, responses to CO2 in DRN serotonergic neurons from Htr1aKO mice, when compared with that of control littermates, were significantly different for both 9% CO2 (p = 0.0003) and 3% CO2 (p = 0.0123) indicating marked impairment in intrinsic chemosensitivity of DRN serotonergic neurons in Htr1aKO mice.

**Noradrenergic Drive is Required for Response to 9% CO2 in Control Mice**

It has been proposed that midbrain serotonergic neurons initiate arousal from sleep in response to hypercapnia [26]. Since facilitatory action of noradrenergic input on serotonergic neuron activity [28] is absent during sleep [29], we next investigated the response to hypercapnic challenge using ACSF supplemented with synaptic blockers, but devoid of α1-adrenoceptor agonist, phenylephrine. Under these conditions, most of recorded serotonergic neurons in slices from control mice were silent. As shown in Figure 2, in nine spontaneously active neurons, which had baseline firing rate of 0.460±0.150 Hz (range: 0.005–1.150 Hz), application of 9% CO2 did not alter their firing rate (0.020±0.018 Hz, p = 0.20). In additional six silent neurons, in which firing was transiently evoked by short (1–2 min) application of phenylephrine, subsequent hypercapnic challenge failed to induce any firing activity. These results suggest that noradrenergic input is essential for arousal response to hypercapnia in normal mice.

**Responses of DRN Serotonergic Neurons to Hypercapnic Challenge Persist in the Absence of Synaptic Blockade**

We next examined serotonergic neuron chemosensitivity in conditions of preserved local network functioning, in which local mechanisms regulating serotonergic neuron activity were maintained. These experiments were done in the absence of synaptic blockade, using normal, phenylephrine-supplemented ACSF (Figure 3). In control mice, firing rate of serotonergic neurons was significantly increased by application of 9% CO2 (0.192±0.042 Hz, n = 27, p <0.0001) and significantly decreased by application of 3% CO2 (−0.093±0.021 Hz, n = 22, p = 0.0011). In contrast, firing rate of Htr1aKO serotonergic neurons did not significantly change in response to 9% CO2 (0.044±0.025 Hz, n = 15, p = 0.12) and 3% CO2 (−0.054±0.028, n = 13, p = 0.13). When compared to control littermates, responses to hypercapnic challenge in Htr1aKO mice were significantly decreased (p = 0.0097), but responses to 3% CO2 did not reach statistical significance (p = 0.24), likely due to small response in controls in this set of experiments. Taken together, the data obtained in both experimental conditions, i.e. with and without synaptic blockade, showed a marked impairment of response to hypercapnia in serotonergic neurons from Htr1aKO mice (p<0.0001 vs. control littermates).
Distribution of Chemosensitive Responses within the DRN

Since impairment of response to hypercapnia in Htr1aRO mice may contribute to death phenotype we further examined responses to 9% CO2 (Figure 4). To increase population size for post-hoc analysis, data obtained in the presence of synaptic blockers and normal ACSF were pooled. There was no significant difference in responses to hypercapnic challenge between experimental conditions (p = 0.15 for control, p = 0.74 for Htr1aRO). Analysis of pooled data revealed that responses in control mice did not follow a normal distribution (D’Agostino-Pearson omnibus test, p = 0.0049) and were well fitted with a double Gaussian function (Figure 4A, up). On the contrary, responses in the Htr1aRO group followed a normal distribution (p = 0.80) and were well fitted with a single Gaussian function (Figure 4A, bottom). We next analyzed responses to hypercapnic challenge in respect to anatomical location of recorded neurons and to postnatal age of mice. In control mice, Spearman’s test revealed no correlation of responses (n = 47) with the rostrocaudal location of neurons (p = 0.47), with their lateral distance from the midline (p = 0.89), and with vertical distance from the aqueduct (p = 0.19). In Htr1aRO mice, Spearman’s test revealed no correlation of responses (n = 31) with distances from the midline (p = 0.66) and the aqueduct (p = 0.25), but there was a significant correlation with the rostrocaudal location of neurons (p = 0.0199). An increase in firing rate in response to 9% CO2 was recorded in six serotonergic neurons located in the rostral margin of DRN, but not in more caudal neurons (Figure 4C). Finally, in both genotypes, responses to hypercapnic challenge did not correlate with postnatal age of mice (control, p = 0.63; Htr1aRO, p = 0.83, Figure 4D).

Discussion

We recently reported that Htr1aRO mice, in which overexpression of Htr1a autoreceptors produces excessive serotonin autoinhibition, exhibit sporadic autonomic crises that frequently progress to death [1]. The present study demonstrates that DRN serotonergic neurons in brainstem slices from Htr1aRO mice have dramatically attenuated responses to hypercapnic challenge. In control mice, firing activity of DRN serotonergic neurons was proportional to P_{CO2} both in the presence and the absence of synaptic blockers, indicating that they are intrinsically chemosensitive. The magnitude of responses was moderate, comparable to that observed by others in response to change in PCO2 or pH in DRN of rat [25,26] and mice [30]. Previous evidence indicated that chemosensitivity is mild on average since it is not a property shared by all serotonergic neurons. Thus, in awake cats, only a subgroup (8 of 36) of serotonergic neurons in DRN increased their activity in response to inhalation of CO2 [31] and in rat brainstem slices only 16 out of 100 serotonergic neurons increased...
firing activity in response to hypercapnic challenge [26]. In our study, hypercapnic responses did not follow a normal distribution and were well fitted with double Gaussian function, supporting this notion. However, due to the limited sample size, the existence of a specific highly-chemoresponsive subpopulation cannot be conclusively demonstrated. If such a subpopulation exists, it is likely to be dispersed throughout DRN since analysis of hypercapnic responses in respect to anatomical location of recorded neurons revealed no evidence of a localized subgroup within the margins of dorsal and ventromedian subnuclei of DRN.

During wakefulness, serotonergic neuron activity is facilitated by noradrenergic afferents via full activation of α1-adrenoceptors [28] while in sleep the noradrenergic input is decreased producing disinhibition of serotonergic neuron firing [29,32]. In the absence of the α1-adrenoceptor agonist phenylephrine, serotonergic neurons in brainstem slices from control mice lacked the response to hypercapnic challenge, suggesting that the presence of noradrenergic drive is required for the functional response of serotonergic system to hypercapnia. This implies that during sleep, when noradrenergic input is absent, serotonergic neurons are not able to respond to hypercapnia. Beside noradrenaline, several other arousal systems, such as orexin and histamine systems, converge to excite serotonergic neurons, and we hypothesize that serotonergic system per se is insufficient to generate arousal in response to hypercapnia, and that its activity as well as chemosensitivity have to be facilitated by concurrent activity of other arousal systems.

In brainstem slices from Htr1αRO mice, responses of serotonergic neurons to hypercapnic challenge were essentially abolished, except in neurons located in the most rostral margins of DRN which exhibited some residual sensitivity. Since in control mice rostral responses were similar to those in the rest of DRN there is the possibility that rostral serotonergic neurons are less sensitive to the effects of excessive autoinhibition. A more comprehensive study is needed to clarify this issue. In Htr1αRO mice, death was most frequent between 25 and 80 days of life [1]. In brainstem slices from Htr1αRO mice, responses of DRN serotonergic neurons to hypercapnic challenge were greatly reduced over the same time period suggesting a link between reduced chemosensitivity of serotonergic neurons and the death phenotype. It should be mentioned that susceptible period for SIDS (from birth to one year of age) appears more restricted than that for the death phenotype in Htr1αRO mice. This discrepancy could derive from the fact that excessive serotonin autoinhibition in genetically engineered Htr1αRO mice represents relatively persistent vulnerability factor to sudden death. Indeed, when overexpression of Htr1α was induced with the beginning at 40 or 60 days of age, significantly fewer mice died, suggesting that older mice are less vulnerable to excessive serotonin autoinhibition [1]. It is conceivable that in humans a similar vulnerability becomes crucial during susceptible period for SIDS and is later overcome by adaptive mechanisms.

The mechanism of serotonergic neuron CO2/pH sensitivity is still unknown. There is convincing evidence that chemosensitivity of serotonergic neurons derives predominantly from TASK-1 and TASK-3 channels, which are intrinsically pH chemosensitive in the physiological range [25,30]. As in Htr1αRO mice, in TASK-1 and TASK-3 double knock-out (TASK−/−) mice there is a marked reduction in chemosensitivity of serotonergic neurons. However, different from Htr1αRO mice, TASK−/− mice are viable and apparently healthy, suggesting that at least in some serotonergic neurons chemosensitivity is mediated by different ion channel types or mechanisms, or alternatively altered chemosensitivity is not involved in the death phenotype of Htr1αRO mice. This discrepancy may also be due to methodological differences since the conclusion that TASK-1 and TASK-3 channels mediate serotonergic neuron chemosensitivity is based on whole-cell recordings, in which cytoplasm dialysis can cause loss of chemosensitivity, and in the absence of noradrenergic drive which our data suggests is critical for chemosensitivity of serotonergic neurons. Although the relationship between excessive autoinhibi-
Figure 4. Distribution of pooled responses to hypercapnic challenge. A, Bar graph showing the distribution of responses in control and Htr1aKO mice. Curves represent best fit of data to a double (Control, \( R^2 = 0.901 \)) and single Gaussian function (Htr1aKO, \( R^2 = 0.952 \)). B, Schematic diagram of frontal sections at various rostrocaudal levels of the DRN mouse raphe in which positions of the recorded serotonergic neurons in control (open circles) and Htr1aKO mice (filled circles) are reported. Numbers correspond to plates in [39]. C, Individual responses to 9% CO2 application in slices from control (open circles) and Htr1aKO mice (filled circles) plotted against the rostrocaudal position of the corresponding recorded neuron indicated by the plate number. Continuous and broken lines are best linear regressions of data in control and Htr1aKO, respectively. D, Individual responses to 9% CO2 application in slices from control (open circles) and Htr1aKO (filled circles) plotted against the postnatal age of the mouse at time of recording. Continuous and broken lines are best linear regressions of data in control and Htr1aKO, respectively.

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rapidly removed, dissected in ice-cold gassed (95% O₂ and 5% CO₂) artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 2.75 KCl, 1.25 NaH₂PO₄, 1.3 MgCl₂, 2 CaCl₂, 26 NaHCO₃, 11 D-glucose, and the brainstem sliced coronally into 250 μm thick slices with a DSK-T1000 vibratome (Dosaka). After recovery for 2–6 h at room temperature, the slices were individually transferred into the recording chamber and superfused continuously with warmed ACSF (34–35 °C) at a rate of 2 ml min⁻¹.

**Electrophysiology** Neurons were visualized by infrared differential interference contrast video microscopy with a Newcon C2400-07 camera (Hamamatsu) mounted to an Axioskop microscope (Zeiss). Recordings were made using an EPC-10 amplifier (HEKA). Patch pipettes were prepared from thick-walled borosilicate glass on a P-97 puller (Sutter) and had resistance of 3–6 MΩ when filled with solution containing (in mM): 125 NaCl, 10 HEPES, 2.75 KCl, 2 CaCl₂, 1.3 MgCl₂ (pH 7.4 with NaOH). Loose-seal cell-attached recordings (5–20 MΩ seal resistance) were acquired continuously in voltage-clamp mode. Signals were filtered at 3 kHz and digitized at 10 kHz. Pipette potential was maintained at 0 mV. Recordings were aborted if firing rate was sensitive to changes in pipette holding potential or if shape of action current changed. Data were analyzed using Clampfit 9.2 (Molecular Devices). Unless otherwise stated, extracellular saline was supplemented with 10 μM phenylephrine to facilitate firing [38]. Neurons were presumed serotonergic when displayed firing rate of less than 4 Hz and asymmetric action current with peak-to-peak interval (proportional to action potential half-height width) greater than 1 ms. At the end of the recording, the response to the Htr1a agonist R(+)-8-hydroxy-2-(di-n-propylamino)tetralin (R-8-OH-DPAT; 30 nM) was routinely tested and neurons in which firing was not abolished (n = 2) were deemed unhealthy or non-serotonergic and excluded from analyses. One experiment was done in each slice. To test the effects of acid/base changes, normocapnic superfusing solution (5% CO₂; pH 7.3) was replaced by hypercapnic solution containing 9% CO₂ (pH 7.10) or hypocapnic solution containing 3% CO₂ (pH 7.50). Solutions with different PₐCO₂ were applied for 10–15 min and 90% of change in pH in the recording chamber was reached in ~3 min. Steady-state values were calculated as average firing rate over the last 3–5 minutes of application and responses to 9 and 3% CO₂ were calculated respective to firing rates measured in normocapnic solution immediately before and 10–15 min after application of CO₂-modified solutions. Responses are expressed as difference in firing rate rather than as percent change to permit accurate quantification of the effect in slowly firing neurons, which would otherwise contribute disproportionately to average values in respect to faster firing neurons. There was no significant correlation between baseline firing rate and the change in firing rate produced by CO₂-modified solutions (see results).

**Drugs** CDP-55845A was purchased from Tocris Bioscience. NBQX, d-APV and SR-95531 were from Ascent Scientific. All other substances were from Sigma-Aldrich.

**Statistical Analysis** Data are presented as mean and SEMs. Statistical analysis was conducted using Prism 5 (GraphPad). For assessment of significance two-tailed non-parametric tests were used: Wilcoxon signed rank test for significance of response in single groups, Mann-Whitney test for comparison between groups, and Spearman’s test for correlation between parameters. A p<0.05 was considered significant.

**Author Contributions** Conceived and designed the experiments: BM RC. Performed the experiments: GB BM EA. Analyzed the data: GB BM. Contributed reagents/materials/analysis tools: EA CTG. Wrote the paper: BM RC CTG.

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