Conservation of 5-HT₁A receptor-mediated autoinhibition of serotonin (5-HT) neurons in mice with altered 5-HT homeostasis

Naozumi Araragi¹*, Boris Milnar², Gilda Baccini², Lise Gutknecht³, Klaus-Peter Lesch¹ and Renato Corradetti²

¹ Division of Molecular Psychiatry, Laboratory of Translational Neuroscience, Department of Psychiatry, Psychosomatics, and Psychotherapy, University of Wuerzburg, Wuerzburg, Germany
² Department of Neurobiology, Institute of Functional Genomics, National Center for Scientific Research (UMR 5203), INSERM U661, University of Montpellier I and II, Montpellier, France
³ Department of Neurology, Institute of Functional Genomics, National Center for Scientific Research (UMR 5203), INSERM U661, University of Montpellier I and II, Montpellier, France

*Correspondence: Naozumi Araragi, Experimental Neurosurgery, Department of Neurosurgery, University of Wuerzburg, Jozef-Schneider-Strasse 11, 97080 Wuerzburg, Germany

引起的5-HT neurons in the dorsal raphe nucleus (DRN) is controlled by inhibitory somatodendritic 5-HT₁A autoreceptors. This autoinhibitory mechanism is implicated in the etiology of disorders of emotion regulation, such as anxiety disorders and depression, as well as in the mechanism of antidepressant action. Here, we investigated how persistent alterations in brain 5-HT availability affect autoinhibition in two genetically modified mouse models lacking critical mediators of serotonergic transmission: 5-HT transporter knockout (Sert−/−) and tryptophan hydroxylase-2 knockout (Tph2−/−) mice. The degree of autoinhibition was assessed by loose-seal cell-attached recording in DRN slices. First, application of the 5-HT₁A-selective agonist R(+)-8-hydroxy-2-(dipropylamino)tetralin showed mild sensitization and marked desensitization of 5-HT₁A receptors in Tph2−/− mice and Sert−/− mice, respectively. While 5-HT neurons from Tph2−/− mice did not display autoinhibition in response to L-tryptophan, autoinhibition of these neurons was unaltered in Sert−/− mice despite marked desensitization of their 5-HT₁A autoreceptors. When the Tph2-dependent 5-HT synthesis step was bypassed by application of 5-hydroxy-L-tryptophan (5-HTP), neurons from both Tph2−/− and Sert−/− mice decreased their firing rates at significantly lower concentrations of 5-HTP compared to wildtype controls. Our findings demonstrate that, as opposed to the prevalent view, sensitivity of somatodendritic 5-HT₁A receptors does not predict the magnitude of 5-HT neuron autoinhibition. Changes in 5-HT₁A receptor sensitivity may rather be seen as an adaptive mechanism to keep autoinhibition functioning in response to extremely altered levels of extracellular 5-HT resulting from targeted inactivation of mediators of serotonergic signaling.

Keywords: serotonin transporter, tryptophan hydroxylase-2, knockout, dorsal raphe nucleus, autoinhibition, 5-HT₁A receptor

INTRODUCTION

The brain serotonin (5-HT) system has been implicated in emotion regulation and related psychopathological states, including anxiety, depression, impulsivity, and aggression (reviewed in Lesch et al., 2012). The 5-HT system originates from specific neurons located in distinct nuclei of the brainstem raphe complex. Among them, the dorsal raphe nucleus (DRN) contains the majority of 5-HT neurons and sends projections to various targets in the forebrain. 5-HT neurons in the DRN are known to exhibit spontaneous regular firing activities (Trulson and Jacobs, 1979; Vandermaelen and Aghajanian, 1983). The firing rate of 5-HT neurons is a determinant of 5-HT concentration and thus function in terminal regions, together with local mechanisms (Jacobs and Azmitia, 1992). In waking states, firing of 5-HT neurons is facilitated by noradrenergic input (Levine and Jacobs, 1992). Activity of 5-HT neurons is, in turn, limited by homeostatic negative feedback control exerted by extracellular 5-HT via somatodendritic inhibitory 5-HT₁A autoreceptors (Audero et al., 2008 and references therein). The role of 5-HT₁A receptors in suppression/regulation of 5-HT neuron firing activity is considered to be relevant to the pathophysiology of disorders of emotion regulation (Pineyro and Blizer, 1999; Sharp et al., 2007). The importance of 5-HT₁A receptor function is further supported by the presumed mechanism of selective 5-HT reuptake inhibitor (SSRI) antidepressant action (Artigas et al., 1996; Pineyro and Blizer, 1999). After acute administration of SSRI, extracellular 5-HT concentrations transiently increase and activate 5-HT₁A autoreceptors, inhibiting firing of 5-HT neurons. One criterion
of antidepressants' therapeutic effects is desensitization of these 5-HT1A receptors, leading to a net increase of 5-HT levels. In this context, dysfunction of autoinhibitory 5-HT1A receptors has been proposed as a potential factor contributing to the pathogenesis of emotional disorders. However, studies on 5-HT1A receptor expression in the raphe nuclei of patients with depression measured in vivo using positron emission tomography (PET) or in post-mortem brains have yielded contradictory findings: some investigators reported decreased expression (Drevets et al., 1999; Sargent et al., 2000; Arango et al., 2001; Meltzer et al., 2004), while others found enhanced expression (Stockmeier et al., 1998) or no difference compared to controls (Parsey et al., 2006). Moreover, PET imaging data revealed reduced 5-HT1A binding in several brain regions including the raphe complex in panic disorder patients either with or without comorbid depression (Neumeister et al., 2004). To date, most studies concentrated on associations between expression levels of 5-HT1A receptors and depressive disorders and there has been no direct evidence demonstrating how altered 5-HT1A receptor availability translates into the extent of 5-HT neuron autoinhibition. The discrepancies among reports describing a relationship between 5-HT1A receptor expression and depression indicate a need for better understanding of the precise mechanisms linking autoinhibition to 5-HT1A receptor function.

Among various mediators of the brain 5-HT signaling, the 5-HT transporter (SERT, 5-HTT; SLCA6A4) plays a central role because (i) it mediates the re-uptake of 5-HT from the extracellular space/synapse and thus terminates the 5-HT signaling and (ii) it is the target of numerous antidepressant drugs which inhibit its action. Carriers of the short variant (s-allele) of the transcriptional control region of the gene encoding SERT (5-HTT gene-linked polymorphic region, 5-HTTLPR), which leads to lower expression and thus a lower amount of SERT protein, are known to convey increased risk for emotional disorders in interaction with environmental factors (reviewed in Canli and Lesch, 2007). On the other hand, tryptophan hydroxylase (TPH) is the rate-limiting enzyme of 5-HT synthesis by converting the essential amino acid L-tryptophan (Trp) into 5-hydroxy-L-tryptophan (5-HTP). 5-HTP is then transformed into 5-HT by aromatic L-amino acid decarboxylase (AADC; Carlsson et al., 1972). While the first isoform TPH1 produces 5-HT in peripheral tissues and the pineal gland, the recently discovered TPH2 isoform is responsible for 5-HT synthesis in the brain (Gutknecht et al., 2009). Variation of the gene coding for TPH2 has been associated with personality traits related to emotional regulation (Gutknecht et al., 2007). Moreover, several polymorphisms in TPH2, which had previously been linked to mood disorders, were shown to lead to reduced expression of TPH2 (reviewed in Jacobson et al., 2012a). Contribution of 5-HT to the regulation of emotion has been further verified by studies on mice with targeted inactivation of either Sert or Tph2. Indeed, Sert knockout (+/−) mice have been shown to display anxiety- and depression-like behaviors (reviewed in Murphy and Lesch, 2008). Tph2−/− mice have also been reported to have altered behaviors such as increased conditioned fear responses, aggression, depression-like behaviors, and impairment of maternal care (Savelieva et al., 2008; Aleenua et al., 2009; Mosienko et al., 2012; for review, see Lesch et al., 2012).

Here, we investigated firing activity of DRN 5-HT neurons in brain slices obtained from Sert−/− mice and Tph2−/− mice using loose- or cell-attached recording configuration. Compared to wildtype (wt) controls, Sert−/− mice were shown to have 6- to 10-fold elevated extracellular 5-HT concentrations at baseline in several brain regions including the striatum and the frontal cortex, while heterozygous Sert+−/− mice were shown to have milder increase, e.g., 3-fold in the striatum (Fabré et al., 2000; Mathews et al., 2004; Shen et al., 2004). In contrast, Tph2−/− mice were reported to display an almost complete depletion ofbrain 5-HT, while Tph2+/− mice showed lower reduction in brain 5-HT, reaching 20–25% in the rostral raphe (Gutknecht et al., 2012). Both knockout mice therefore provide useful models to investigate potential modulation of autoinhibition of 5-HT neuron firing as a function of varying degrees of 5-HT availability in the cellular environment. Moreover, since both mouse lines have extensively been investigated as models for emotional disorders, investigating 5-HT neuron autoinhibitory functions in these mice will facilitate detection of potential alterations in autoinhibition related to disorders of emotion regulation.

In order to mimic in vivo 5-HT synthesis in in vitro experimental conditions, we applied 5-HT precursors through superfusion of brain slices under recording. Prior to this, we assessed the function of autoinhibitory 5-HT1A receptors by applying their direct agonist. Feasibility of assessing autoinhibition in in vitro conditions had been established in previous studies (Li et al., 2005; Miliar et al., 2005; Evans et al., 2008; Gutknecht et al., 2012).

**MATERIALS AND METHODS**

**ANIMALS**

Animal handling followed the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC) and approved by the local committees. The generation and genotyping procedure of Tph2−/− and Sert−/− animals were described previously (Bengel et al., 1998; Gutknecht et al., 2008). Animals were housed under a 12 h light/dark cycle (lights on: 08:00–20:00) at ambient temperature of 22 ± 1°C and a relative humidity of 40–50%. Data from Tph2+− and Sert+− mice were treated together, since both mouse lines were backcrossed more than 10 generations into a C57BL/6J background and thus considered to have the same genetic background. Data from male and female mice were pooled.

**DRUGS**

SR-95531 (gabazine; GABA A receptor antagonist), D-AP5 (NMDA glutamate receptor antagonist), DNX2 (AMPA/kainate receptor antagonist) were purchased from Ascent Scientific Ltd (Bristol, UK). N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylclobexaneisobamamide maleate (WAY-10655 maleate, selective 5-HT1A receptor antagonist), CGP-55845 hydrochloride (selective GABAA receptor antagonist), and R(+)-8-hydroxy-2-(di-N-propylamino)tetratin (R(+)-8-OH-DPAT) were purchased from Tocris Bioscience (Bristol, UK). Strychnine (glycine receptor antagonist), Trp, 5-HTP, and L-phenylephrine were obtained from Sigma-Aldrich S.r.l. (Milan, Italy).
ELECTROPHYSIOLOGICAL RECORDING

Methods used follow those reported previously (Gutknecht et al., 2012). Mice (28–80 days old) were anesthetized with isoflurane and decapitated. The brain was immediately removed, dissected in ice-cold gassed (95% O2, 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-glucose (pH 7.4), and the brainstem was sliced coronally into 200 μm thick slices with a vibratome (DSK-1000, Dossaka Co. Ltd, Kyoto, Japan) and transferred to a multi-well incubation chamber filled with bubbled ACSF at room temperature. After at least 90 min of recovery, the slices were individually transferred into the recording chamber and superfused continuously with gassed, warmed ACSF (34–35°C) at a rate of 2 ml min⁻¹. Superfusing ACSF was supplemented with 10 μM phenylephrine to facilitate firing (Vandermaelen and Aghajanian, 1983) and with a mixture of neurotransmitter blockers for glutamate, glycine, and GABA receptors (in μM: 10 D-NQX, 20 D-AP5, 10 strychnine; 1 CGP-55845, 10 SR-95531) to functionally isolate the recorded neuron from synaptic input. Neurons were visualized by infrared differential interference contrast video microscopy with a Newcon C2400-07 camera (Hamamatsu, Hamamatsu City, Japan) mounted to an Axioskop microscope (Zeiss, Göttingen, Germany). Recordings were made using an EPC-10 amplifier (HEKA Elektronik, Lambrecht, Germany). Patch pipettes were prepared from thick-walled borosilicate glass on a P-97 Brown-Flaming electrode puller (Sutter Instruments, Novato, CA, USA) and had resistance of 3–6 MΩ when filled with solution containing (in mM): 125 NaCl, 10 HEPES, 2.75 KCl, 1.3 MgCl2, pH 7.4 with NaOH. Loose-seal cell-attached recordings (5–20 MΩ seal resistance) were acquired continuously in the 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 shapes of action current changed. Data were analyzed using Clampfit 9.2 (Molecular Devices, Sunnyvale, CA, USA).

Neurons with likely serotonergic specification were first targeted according to morphological criteria (Brown et al., 2008): 5-HT neurons are clustered along the midline of the DRN and they have a larger soma (~20–25 μm long-axis diameter) than non-serotonergic neurons (~10–15 μm). Once loose-seal cell-attached recording configuration was established, 5-HT neurons were identified according to electrophysiological criteria (Vandermaelen and Aghajanian, 1983; Allers and Sharp, 2003). Neurons were considered serotonergic if, during at least 5 min-long baseline period at the beginning of the recording displayed slow and steady firing rate (<5 Hz), asymmetric action current with long upstroke to downstroke interval (proportional to action potential half-height width, >0.85 ms). According to these criteria, 250 out of 277 recorded neurons were identified as being serotonergic. Pharmacological experiments were done on 176 presumed serotonergic neurons, whose identity was pharmacologically confirmed based on 5-HT1A receptor-mediated suppression of their firing rate. For all groups of neurons used in pharmacological experiments (Figures 2–4), the basal firing rate was matched and proved to be not different after post hoc statistical analysis (Kruskal–Wallis test, p > 0.7).

Since experiments to assess autoinhibition depend on endogenous 5-HT, recordings were made from neurons located at least 50 μm below the slice surface (Milnar et al., 2003). A single experiment was done in each slice.

For creating concentration–response curves for R(+)-8-OH-DPAT and 5-HTP application, drugs were applied for 10 min and mean firing rates were calculated from the last 1-min segment of each experimental epoch (e.g., baseline, R(+)-8-OH-DPAT 0.1 nM, 0.3 nM, etc.). Trp was applied for 15 min and mean firing rates were obtained from the last 3-min segment of baseline and Trp application.

STATISTICAL ANALYSIS

All the statistical tests were performed by GraphPad Prism version 5.04 (GraphPad Software, San Diego, CA, USA). First, normality of data distribution was tested by D’Agostino–Pearson omnibus normality test. When the data were normally distributed, genotype effects were tested by one-way ANOVA (expressed as F(letion, values) followed by Tukey’s post hoc test. If not, data were analyzed by Kruskal–Wallis test (expressed as H(calc), values) with Dunn’s post hoc test. For testing effects of Trp in comparison to respective baseline, data (% change in firing rates) were analyzed by Wilcoxon signed rank test (two-tailed). In all cases, p < 0.05 was considered statistically significant.

RESULTS

COMPARISON OF BASAL FIRING RATES ACROSS GENOTYPES

In the absence of precursor supplementation (Trp or 5-HTP), and in the presence of receptor blockers for glutamate, GABA, and glycine receptors, the basal firing of 5-HT neurons in slices is relieved from the autoinhibitory control of endogenous 5-HT (Milnar et al., 2003) and local action of major neurotransmitters. In these conditions of pharmacological isolation, the basal firing activity of 5-HT neurons reflects their intrinsic pacemaker activity, a characteristic that is difficult to study in vivo, where the firing activity is under control of both autoinhibition and synaptic input.

We compared the basal firing rates recorded before 5-HT precursor or 5-HT1A receptor agonist application, across genotypes (Figure 1). Overall, 5-HT neurons showed typical regular pacemaker activity and firing rates similar to wt controls [in Hz: TPβ2−/−, 1.61 ± 0.82 (n = 54); TPβ2+/−, 1.90 ± 0.66 (n = 45); wt, 1.97 ± 0.69 (n = 54); Sert+/−, 1.85 ± 0.74 (n = 47); Sert−/−, 2.12 ± 0.75 (n = 50); mean ± SD; n = number of recorded neurons], except for TPβ2−/− in which the firing rate was slightly, but significantly slower than in wt controls (p < 0.05, Kruskal–Wallis test followed by Dunn’s multiple comparison test). These data show that basic electrophysiological properties underlying the typical pacemaker activity of 5-HT neurons are maintained regardless of genetic inactivation of Tph2 or Sert.

COMPARISON OF 5-HT1A RECEPTOR SENSITIVITY ACROSS GENOTYPES

Since 5-HT neuron autoinhibition is mediated by 5-HT1A receptors, we investigated the functional response of 5-HT neurons to the 5-HT1A receptor agonist R(+)-8-OH-DPAT in different genotypes. Figure 2 illustrates typical experiments in which increasing
ANOV A]. Compared to Araragi et al. Autoinhibition in 5-HT system-deficient mice
sensitivity of 5-HT1A receptors shown in
( not result in relevant changes of 5-HT1A autoreceptor sensitivity
ing that limited impairment of 5-HT synthesis and re-uptake did
and the mean log value of concentrations producing an actual
dependence manner, but with different effectiveness across geno-
reduced the firing rate of 5-HT neurons in a concentration-
−Tph2
+R(−)
Sert
−Tph2
wt
−R(−)
Sert
−Tph2
Sert
−Tph2
−R(−)
Tph2−/−
wt
Sert−/−
Sert−/−
Tph2−/−
Tph2−/−
Sert−/−
Sert−/−
Tph2−/−
−8.52 ± 0.25 (n = 12), Sert+−/−/−, −8.22 ± 0.27 (n = 11); Sert−/−/−, −7.17 ± 0.42 (n = 6; Figure 2G). Differences across genotypes
were statistically significant [F(9,80) = 48.38, p < 0.0001, one-way
ANOVA]. Compared to wt controls, the response to application of
R(+)−8-OH-DPAT resulted in slightly higher effectiveness of the
agonist in Tph2−/− mice (p < 0.05) and very weak effectiveness
in Sert−/− mice (p < 0.001). Although a small decrease in the
sensitivity of 5-HT neurons was present also in Sert+/− mice, the
change was not statistically significant across genotypes. A small decrease in firing rate was significantly different from zero
(p < 0.05; Wilcoxon signed rank test). Furthermore, responses to application of Trp were not statistically different across four genotypes [H(3) = 3.336, p = 0.3427; Kruskal–Wallis test]. These data show that autoinhi-
bition of DRN 5-HT neurons by endogenous 5-HT is conserved
in all the genotypes to a similar level, irrespective of the genetic
alteration.
To quantify the extent to which each genotype conserved the
capacity to autoinhibit 5-HT neuron firing in response to
different extracellular concentrations of endogenous 5-HT, we
investigated the functional response of 5-HT neurons to 5-HTP in
different genotypes.
Figure 4 illustrates firing rate changes of 5-HT neurons in response to increasing concentrations of 5-HTP in brain
slices obtained from wt controls (Figures 4A,B), Tph2−/− (Figures 4C,D), and Sert−/− mice (Figures 4E−H). Application
of 5-HTP reduced the firing rate of 5-HT neurons in a concentra-
tion-dependent manner, but with different effectiveness across genotypes [one-way ANOVA, F(4,38) = 6.723, p = 0.0002],
as shown by the comparison of log EC50 values obtained for
each single neuron tested (log EC50 mean ± SD): Tph2−/−, −5.51 ± 0.41 (n = 15); Tph2+/−, −5.29 ± 0.30 (n = 10);
Sert−/−/−, −5.17 ± 0.20 (n = 14); Sert+−/−/−, −5.48 ± 0.36 (n = 13); Sert−/−/−, −5.70 ± 0.12 (n = 11; Figure 4I). Interestingly,
the sensitivity to the effects of endogenous 5-HT synthesized
concentrations of R(+)−8-OH-DPAT were applied in slices from
wt controls (Figures 2A,B), Tph2−/− (Figures 2C,D), and
Sert−/− mice (Figures 2E,F). Application of R(+)−8-OH-DPAT reduced the firing rate of 5-HT neurons in a concentra-
tion-dependent manner, but with different effectiveness across geno-
types, as shown by the comparison of log EC50 values obtained
for each single neuron tested (log EC50 mean ± SD): Tph2−/−/−,
−8.82 ± 0.29 (n = 16), Tph2+/−/−, −8.52 ± 0.19 (n = 11); wt,
−8.52 ± 0.25 (n = 12), Sert+−/−/−, −8.22 ± 0.27 (n = 11); Sert−/−/−,
−7.17 ± 0.42 (n = 6; Figure 2G). Differences across genotypes
were statistically significant [F(9,80) = 48.38, p < 0.0001, one-way
ANOVA]. Compared to wt controls, the response to application of
R(+)−8-OH-DPAT resulted in slightly higher effectiveness of the
agonist in Tph2−/− mice (p < 0.05) and very weak effectiveness
in Sert−/− mice (p < 0.001). Although a small decrease in the
sensitivity of 5-HT neurons was present also in Sert+/− mice, the
change was not statistically significant across genotypes. A small decrease in firing rate was significantly different from zero
(p < 0.05; Wilcoxon signed rank test). Furthermore, responses to application of Trp were not statistically different across four genotypes [H(3) = 3.336, p = 0.3427; Kruskal–Wallis test]. These data show that autoinhi-
bition of DRN 5-HT neurons by endogenous 5-HT is conserved
in all the genotypes to a similar level, irrespective of the genetic
alteration.
To quantify the extent to which each genotype conserved the
capacity to autoinhibit 5-HT neuron firing in response to
different extracellular concentrations of endogenous 5-HT, we
investigated the functional response of 5-HT neurons to 5-HTP in
different genotypes.
Figure 4 illustrates firing rate changes of 5-HT neurons in response to increasing concentrations of 5-HTP in brain
slices obtained from wt controls (Figures 4A,B), Tph2−/− (Figures 4C,D), and Sert−/− mice (Figures 4E−H). Application
of 5-HTP reduced the firing rate of 5-HT neurons in a concentra-
tion-dependent manner, but with different effectiveness across genotypes [one-way ANOVA, F(4,38) = 6.723, p = 0.0002],
as shown by the comparison of log EC50 values obtained for
each single neuron tested (log EC50 mean ± SD): Tph2−/−/−,
−5.51 ± 0.41 (n = 15); Tph2+/−/−, −5.29 ± 0.30 (n = 10);
Sert−/−/−, −5.17 ± 0.20 (n = 14); Sert+−/−/−, −5.48 ± 0.36 (n = 13); Sert−/−/−, −5.70 ± 0.12 (n = 11; Figure 4I). Interestingly,
the sensitivity to the effects of endogenous 5-HT synthesized

"fphar-04-00097" — 2013/8/1 — 15:40 — page 4 — #4
FIGURE 2 | Sensitivity of 5-HT neurons to R(+)-8-OH-DPAT differs across genotypes. Time courses of firing rate changes in response to increasing concentrations of R(+)-8-OH-DPAT of individual 5-HT neurons in brain slices obtained from wt (A,B), Tph2−/− (C,D), and Sert−/− mice (E,F). Traces show action current of corresponding neurons recorded. (G) Dots represent log EC50 of concentration–response from individual experiments. Red lines report mean ± SD of values. One-way ANOVA followed by Tukey’s multiple comparison test showed statistically significant differences across genotypes \(F(4,53) = 48.38, p < 0.0001\). Asterisks indicate level of statistical significance between the indicated genotypes (for Sert−−/− vs. all the other four genotypes): ***, \(p < 0.001\), *\(p < 0.05\). (H) Average concentration–response curves obtained from all the experiments. Each data point corresponds to the mean from several neurons (numbers in parentheses). For the sake of clarity, error bars are shown only for Sert−−/− mice and Tph2−/− mice in a single direction. Data are normalized on average baseline firing rates recorded before R(+)-8-OH-DPAT application. Note that, curves for Sert−−/− mice did not achieve full inhibition of firing (see E).
Araragi et al. Autoinhibition in 5-HT system-deficient mice

Figure 4I shows concentration-response curves fitted for each group on mean data obtained from the individual experiments depicted in Figure 4H.

In the present study, we have investigated the relationship between the sensitivity of 5-HT1A receptors caused by decreased 5-HT and the autoinhibitory effect of the 5-HT neurons in a panel of genetically modified mice characterized by impairment of cellular mechanisms crucial for homeostatic control of extracellular 5-HT levels (i.e., 5-HT synthesis and 5-HT re-uptake). We suggest that, due to the absence of 5-HT re-uptake, in Sert−/− mice the extracellular 5-HT concentration is lower than in wild type (wt) control mice, leading to this apparent increase in sensitivity. Collectively, these results demonstrate that the changes in sensitivity to direct activation of 5-HT1A receptors cannot directly be translated into the expected changes in autoinhibition exerted by endogenous 5-HT.

DISCUSSION

In the present study, we have investigated the relationship between the sensitivity of 5-HT1A receptors and the concomitant decrease in sensitivity of 5-HT1A receptors to agonist activation, a similar increase in sensitivity of 5-HT1A receptors produced by altered 5-HT homeostatic regulation. The consequences of genetic alterations are maintained in vivo and estimation of the functional state of 5-HT neurons in genetically modified mice proved to be similar to that of wt control mice, showing that mild change in extracellular 5-HT levels is neither a strong stimulus for 5-HT1A receptor adaptive changes in sensitivity, nor does it detectably affect autoinhibition.

In previous studies under similar recording conditions as used in this work, raphe slices showed substantial depletion of 5-HT in the absence of 5-HT precursors (Liu et al., 2005; Mlinar et al., 2005). In vitro, 5-HT content, together with 5-HT1A receptor-mediated autoinhibition, can be restored by supplementation of Tp (Liu et al., 2005; Mlinar et al., 2005; Evans et al., 2008; Gütknecht et al., 2012). This allowed electrophysiological, quantitative, assessment of the modifications in sensitivity of 5-HT1A receptors produced by altered 5-HT homeostasis in vivo and estimation of the functional state of autoinhibition when de novo synthesis of 5-HT was restored in slices.

GENETIC MANIPULATIONS DO NOT AFFECT PACEMAKER CHARACTERISTICS OF 5-HT NEURONS

The pacemaker properties of serotonergic neurons measured in slices in the virtual absence of endogenous 5-HT synthesis, hence of autoinhibition, were not substantially altered by genetic manipulation itself, as we observed similar baseline firing rates among genotypes, except for Tph2−/− mice, which had slightly lower baseline firing rates compared to the other genotypes. This shows that the basic characteristics of intrinsic pacemaker firing activity of 5-HT neurons are preserved independently from genetic manipulations that altered 5-HT homeostatic regulation. The small decrease in baseline firing rates observed in Tph2−/− mice may indicate that, in the chronic absence of 5-HT, neurons adapt their membrane properties, e.g., conductance, to compensate for absent autoinhibition and homeostatically keep pacemaker firing activity constant. The mechanism(s) underlying this adaptation is currently under investigation. It should be noted that the baseline firing rate recorded under our experimental conditions, i.e., in vitro, results from the interplay of ion conductances responsible for pacemaking activity and likely do not correspond to the “basal” firing rate recorded in vivo (e.g., Gobbi et al., 2001; Bouali et al., 2003; see below) which is under the control of 5-HT1A receptor-mediated autoinhibition in all genotypes (see Figure 3), except in Tph2−/− mice (Gütknecht et al., 2012).
FIGURE 4 | Quantification of autoinhibition capacity of 5-HT neurons across genotypes by concentration–response curves for 5-HTP. Time courses of 5-HT neuron firing rate changes in response to increasing concentrations of 5-HTP in brain slices obtained from wt controls (A,B), Tph2−/− (C,D), and Ser1−− mice (E–H). Traces show action current of corresponding neurons recorded. (I) Dots represent log EC50 of concentration–response from individual experiments. Red lines report mean ± SD of values. One-way ANOVA followed by Tukey’s multiple comparison test showed statistically significant differences [F(4,58) = 6.723, p = 0.0002] Asterisks indicate level of statistical significance between the indicated genotypes: **p < 0.01, *p < 0.05. (J) Average concentration–response curves obtained from all the experiments. Each data point corresponds to the mean from several neurons (numbers shown in parentheses). For the sake of clarity, error bars are omitted. Data are normalized on average baseline firing rates recorded before 5-HTP application.
When the level of autoinhibition restored by Tryp supplementation to R(+) 8-OH-DPAT (~40%) and a similar reduction of autoinhibitory capacity as revealed by concentration–response curves with 5-HTP. This may reflect a downregulation of 5-HT1A receptors due to lifelong exposure to increased stimulation by 5-HT or the emergence of a still-unknown adaptive mechanism directed to counteract increased autoinhibition exerted by high levels of extracellular 5-HT in vivo. In spite of the decrease, however, the remaining autoinhibition capacity of 5-HT neurons largely exceeded the magnitude of physiological autoinhibition produced by 5-HT when its synthesis was restored by Tryp (see below).

Taken together, our data indicate that the level of 5-HT1A receptor sensitivity of 5-HT neurons is inversely correlated with extracellular levels of 5-HT in vivo, at least in extreme conditions as represented by Tph2−/− and Serotonin−/− mice.

Autoinhibition of 5-HT Neurons by Endogenous 5-HT Is Conserved in the Physiological Range, Regardless of the Sensitivity of 5-HT1A Receptors

When the level of autoinhibition restored by Tryp supplementation in slices from all the genotypes (except Tph2−/−) was measured, this resulted in being similar, irrespective of the sensitivity of 5-HT1A receptors measured in each genotype. Notably, Serotonin−/− showed greatly decreased sensitivity to the agonist but normal autoinhibition, as estimated by Tryp challenge. Accordingly, the autoinhibitory effect of endogenous 5-HT synthesized de novo from 5-HTP proved to be not decreased in all the mutants compared with wt controls, including Tph2−/− in which the absence of Tph2 was bypassed by 5-HTP. It should be noted that in Serotonin−/− mice the maximal inhibitory response was slightly decreased (~20%) in agreement with the reduced maximal response to the agonist, but the substantial residual inhibition capacity is apparently sufficient to produce a physiological level of autoinhibition as shown by Tryp experiments. In conclusion, these data indicate that the marked subsensitivity of 5-HT1A receptors observed in Serotonin−/− does not translate in the loss of normal autoinhibition capacity of 5-HT neurons.

Although counterintuitive, this notion is consistent with the observation that, in vivo, the firing rate of 5-HT neurons is not increased in Serotonin−/−, but similar to or even lower (Gobbi et al., 2001; Bouali et al., 2003) than that of wt controls, thus indicating that in vivo subsensitivity of 5-HT1A receptors in Serotonin−/− mice does not relieve 5-HT neurons from autoinhibition. Furthermore, Fox et al. (2010) reported that in these mice antagonism of 5-HT1A receptors by WAY-100635 resulted in the appearance of greater frequency of 5-HT2A receptor-mediated head twitches than in wt controls. This suggests that the relief from autoinhibition, hence the increase in 5-HT neuron firing, produces an increase in 5-HT release sufficient to produce this 5-HT1A-mediated behavioral effect (Willis and Melzer, 1997), even in the presence of partial desensitization of 5-HT1A receptors (Roux et al., 1999; Li et al., 2003; Qu et al., 2003).

Implications of the Divergence Between Sensitivity to R(+) 8-OH-DPAT and 5-HT Neuron Autoinhibition

The crucial role of somatodendritic 5-HT1A receptors in regulating the firing rate of 5-HT neurons, hence the functional state of 5-HT system, has attracted interest in the attempt to infer the degree of activity of these neurons in pathological conditions of humans and in behavioral experiments of rodents. The present work may help to better understand the limits in the interpretation of the functional state of the 5-HT system based on measurements of density/sensitivity of 5-HT1A receptors of 5-HT neurons. Furthermore, since the knockout mice used in this investigation may model different risk factors (i.e., TPH2 and SERT polymorphisms) for anxiety disorders and depression, our data showing that autoinhibition is not impaired in these mutants may provide a reference background for the interpretation of behavioral responses in these mice in the context of human psychopathology. For instance, functional autoinhibition in patients with depression were indirectly inferred from 5-HT1A receptor imaging studies in the raphe (Dreunset al., 2007; Savitz et al., 2009). Overall, however, these studies failed to clarify whether the depression-related changes in 5-HT1A receptor binding are genetically or environmentally driven during development, thus causative of the disorder, or whether they are simply an adaptation to acutely increased or decreased serotonergic transmission (Savitz et al., 2009).

Contradicting results were also gathered in the attempt to associate SERT polymorphisms with changes in the level of 5-HT1A receptor expression/density. David et al. (2005) reported that carriers of the 5-HTTLPR s-allele had lower 5-HT1A receptor binding potential in all the brain regions investigated compared to individuals homozygous for the s-allele. On the contrary, Lee et al. (2005) found that s-carriers had higher 5-HT1A binding than lll-individuals in prenatal and subgenual cingulate cortex regions while in other regions, including the DRN, no difference was detected. More recently, Borg et al. (2009) could not reveal any differences in 5-HT1A receptor density between carriers and non-carriers of the 5-HTTLPR s-allele and concluded that functional consequences of 5-HTTLPR are not likely to be mediated by differences in 5-HT1A expression. Our results showing that 5-HT system autoinhibition is not reduced in mice with impaired Sert function even in the presence of altered 5-HT1A receptor sensitivity would support this conclusion.

A second implication of our results involves the possibility to infer the degree of 5-HT system autoinhibition from functional assays using activation of 5-HT1A receptors with direct agonists, in patients or in animal models. For example, one of the most consistent findings among depressed patients is their blunted hypothalamic response to 5-HT1A receptor direct agonists (Lesch et al., 1990; Lesch, 1991; Jacobsen et al., 2012b and references therein). Such responses are usually ascribed to desensitization of somatodendritic 5-HT1A receptors (reviewed in Jacobsen et al., 2012a). Our data suggest that, whereas blunted hypothalamic response to direct agonists is likely to reflect subsensitivity of
5-HT₁A receptors in these patients, this decrease in response cannot directly be correlated to functional consequences that entail reduced autoinhibition and increase in the basal firing rate of 5-HT neurons.

On the other hand, the finding that 5-HT neurons in Tph2- and Sert-deficient mice display normal responsiveness to Tph and/or 5-HTP regarding autoinhibition of 5-HT neuron firing would support the use of Tph (or 5-HTP) as an appropriate challenge to test the functional state of 5-HT system in clinical settings and to reveal the involvement of altered autoinhibition in human psychopathology. Indeed, 5-HTP challenge has been successfully applied to reveal functional consequences dependent on 5-HTTLPR variation in humans (Maayan et al., 2004). Finally, the striking divergence between sensitivity to R(+)-8-OH-DPAT and 5-HT neuron autoinhibition in Sert−/− suggests the possibility that sustained increase in 5-HT levels by stressors or pharmacological treatments (e.g., SSRIs) may result in 5-HT₁A receptor subsensitivity, not accompanied by functional impairment of 5-HT neuron firing autoregulation. For instance, the rapid decrease in 5-HT₁A receptor sensitivity found in DRN 5-HT neurons following chronic ultramild stress and stressful uncontrolled environmental conditions is apparently not correlated with an increase in 5-HT system activity and has been suggested to be an adaptive mechanism to compensate for 5-HT fluctuations produced by stressful events (Laarini et al., 1999; Lefunemey et al., 1999).

Interestingly, in vivo recording after chronic unpredictable stress in rats showed that the reduced ability of 8-OH-DPAT to inhibit 5-HT neuron firing was accompanied by a decrease in firing rate of DRN 5-HT neurons (Bambico et al., 2009), indicating that functional autoinhibition may be preserved in spite of 5-HT₁A receptor desensitization. Furthermore, desensitization of autoinhibitory 5-HT₁A receptors occurring with chronic SSRI administration (Le Poul et al., 2000; Heinsler, 2002; Castro et al., 2003) has been proposed as a mechanism for 5-HT neurons to escape the sustained autoinhibition produced by the increase in 5-HT in raphe nuclei by blockade of Sert and to represent an important step to achieve enhanced therapeutic effects of SSRIs (Artigas et al., 1996). On the other hand, Richardson-Jones et al. (2010) showed that desensitization of 5-HT₁A autoreceptors is not sufficient for antidepressants to convey their efficacy, indicating dissociation between desensitization of 5-HT₁A autoreceptors and behavioral effects of chronic SSRI treatment. Thus, desensitization of 5-HT₁A autoreceptors appears rather to be an adaptive mechanism to neutralize elevated extracellular 5-HT levels, and not a primary factor leading to behavioral alteration.

Under a functional perspective, however, dynamic changes in the sensitivity/expression of 5-HT₁A receptors appear to be crucial to fulfill the requirements for physiological homeostasis of 5-HT system functioning. Thus, any impairment of adaptive mechanisms of 5-HT₁A receptors in response to sustained changes in 5-HT levels, or constitutive alteration of their expression even in the absence of altered 5-HT levels in vivo, becomes a potential source of pathological consequences. In fact, genetically induced overexpression of somatodendritic 5-HT₁A receptors in mice has been shown to produce autonomic dysregulation (Audero et al., 2008), behavioral alterations, and decreased response to antidepressant drugs (Richardson-Jones et al., 2010). In humans, the C(-1019)G 5-HT₁A promoter polymorphism leading to 5-HT₁A receptor overexpression is proposed to represent a risk factor for depression (Lemone et al., 2003; Strobel et al., 2003; Rothe et al., 2004; reviewed in Albert and Fransson, 2010) and response to antidepressant drugs (reviewed in Albert, 2012).

In conclusion, our data reveal that 5-HT neuron autoinhibition is similar in all Tph2 and Sert genotypes studied, regardless of the different sensitivity of their somatodendritic 5-HT₁A receptors to R(+)-8-OH-DPAT. This suggests that adaptive changes in receptor sensitivity occur to compensate for variable extracellular 5-HT levels in different genotypes to homestatically conserve autoinhibition in a physiological range. Thus, it appears that response to 5-HT₁A agonists per se is not always sufficient for evaluating the functional state of the 5-HT system, for which Tph and/or 5-HTP challenges may provide more informative data, both in clinical and animal experimental settings.

ACKNOWLEDGMENTS

Supported by the DFG (KFO 125, SFB TRR 58/A1 and A5), BMBF (IZK Wuerzburg, 01KS9603), Compagnia di San Paolo (Programma Neuroscienze-2008.2265), and the EC (NEWMOOD LSHM-CT-2004-503474). Naozumi Araragi was supported by a grant of the German Excellence Initiative to the Graduate School of Life Sciences (GSLS), University of Wuerzburg. The article publishing fee was funded by the DFG and the University of Wuerzburg in the funding program Open Access Publishing.

REFERENCES

Albert, P. R. (2012). Transcriptional regulation of the 5-HT1A receptor: implications for mental illness. Philos. Trans. R. Soc. Lond. B Biol. Sci. 367, 2012–2015. doi: 10.1098/rstb.2011.0576

Albert, P. R., and Fransson, K. L. (2010). Modulating 5-HT1A receptor gene expression as a novel target for antidepressant therapy. Front. Neuosci. 4:35. doi: 10.3389/fnins.2010.00035

Alkema, N., Kike, T., Todreas, M., Mouardou, V., Qadir, F., Pilkins, R., et al. (2009). Growth retardation and altered autonomic control in mice lacking brain serotonin. Proc. Natl Acad. Sci. U.S.A. 106, 10325–10330. doi: 10.1073/pnas.0810793106

Allen, K. A., and Sharp, T. (2003). Modifying 5-HT1A receptor mRNA expression in the brainstem of mice lacking brain serotonin. J. Neurosci. 23, 2236(96)10037-0

Audero, E., Coppi, E., Mlinar, B., Rosci, G. (2009). Decline in somatodendritic firing activity and desensitization of 5-HT1A autoreceptors after chronic unpredictable stress. Eur. Neuropsychopharmacol. 19, 215–228. doi: 10.1016/j.euroneuro.2008.11.005

Bengel, D., Murphy, D. L., Andrews, A. M., Wichems, C. H., Feltner, D., Heils, A., et al. (1998). Altered brain serotonin homeostasis and locomotor immotility in 5, 7-methylenedioxytryptamine (“Ecstasy”) in serotonin transporter-deficient mice. Mol. Pharmacol. 55, 649–655.

Bambico, F. R., Nguyen, N. T., and Gould, G. (2009). Decline in somatodendritic firing activity and desensitization of 5-HT1A autoreceptors. Eur. J. Pharmacol. 569, 80–93. doi: 10.1016/j.ejphar.2009.01.046

Bennett, K. G., and Loewy, A. S. (1991). Serotonin neurotransmission. Annu. Rev. Neurosci. 14, 1–36. doi: 10.1146/annurev.ne.14.030191.000215

Bennett, K. G., and Loewy, A. S. (1995). Serotonin neurotransmission. Annu. Rev. Neurosci. 18, 73–108. doi: 10.1146/annurev.neuro.18.1.73

Bhattacharya, S., Hinds, S. B., Goudsmit, J., and Hawley, R. A. (2003). Serotonin M(type) receptors, serotonin transporter binding and serotonin transporter mRNA expression in the brainstem of depressed suicide victims. Anurophy-}
Araragi et al. Autoinhibition in 5-HT system-deficient mice

Bouali, S., Evrard, A., Chastanet, M., Borg, J., Henningsson, S., Saijo, T., David, S. P., Murthy, N. V., Rabiner, E. A., Munafo, M. R., Johnstone, E. C., Basheer, R., Yanagawa, H., Jacob, R., et al. (2005). A functional genetic variation of the serotonin (5-HT) transporter affects 5-HT1A receptor binding in depression. J. Neurosci. 25, 1103–1109. doi: 10.1523/JNEUROSCI.3769-04.2005

Gutknecht, L., Krüger, C., Hofmann, B., Rief, A., et al. (2008). Deficiency of brain 5-HT synthesis but serotonergic neuron formation in Tph2 knockout mice. J. Neurosci. 31, 1127–1132. doi: 10.1523/JNEUROSCI.0899-08.2008

Hendler, J. G. (2002). Differential regulation of 5-HT1A receptor-G protein interactions in brain following chronic antidepressant administration. Neuropsychopharmacology 26, 565–573. doi: 10.1016/S0893-8913(01)00199-5

Jacobs, B. L., and Ammirati, E. C. (1991). Structure and function of the brain serotonin system. Physiol. Rev. 71, 265–229.

J. (2003). Sex hormone-dependent serotonin receptor binding in humans. Nucl. Med. Biol. 30, 309–320. doi: 10.1016/S0969-8010(03)00315-7

Lee, M., Bailer, U. F., Frank, G., Gutknecht, L., Jacob, C., Strother, W., et al. (1998). Antagonist properties of 5-HT1A receptor knockout mice. Br. J. Pharmacol. 125, 1189–1197. doi: 10.1038/sj.bjp.0701860

Lemonde, S., Tarakci, G., Baksh, D., Du, L., Hildibrand, P. D., Brown, C. D., et al. (2001). Impaired expression at a 5-Hydroxytryptamine 1A receptor gene polymorphism associated with major depression and suicide. J. Neuropsychopharmacology 23, 879–8790.

Le Poul, E., Bon, C., Hansen, N., Laporte, A. M., Laurin, N., Chauvaux, J., et al. (2009). Differential adaptation of brain 5-HT1A and 5-HT1B receptors in 5-HT transporter in rats treated chronically with fluoxetine. Neuropsychopharmacology 34, 110–122. doi: 10.1038/npp.2008.98

Lesh, K. P. (1991). 5-HT1A receptor responsivity in anxiety disorders and depression. Prog. Neuropsychopharmacol. Biol. Psychiatry 15, 723–733. doi: 10.1016/0278-5846(91)90085-J

Lesh, K. P., Aragaki, N., Waidler, J., Van Den Hove, D., and Gutknecht, E. (2012). Targeting brain serotonin synthetic insights into neuropsychiatric disorders with long-term outcomes related to negative emotionality, aggression and antisocial behaviour. Philos. Trans. R. Soc. Lond. B Biol. Sci. 367, 2424–2444. doi: 10.1098/rstb.2012.0109

Lowry, C. A. (2008). Evidence for serotonin dysregulation in a non-clinical 5-HT deficiency model, the tryptophan hydroxylase-2 (Tph2) knock down mouse. Philos. Trans. R. Soc. Lond. B Biol. Sci. 363, 2046–2055. doi: 10.1098/rstb.2008.0069

M., et al. (1998). Antagonist properties of 5-HT1A receptor knockout mice. Br. J. Pharmacol. 125, 1189–1197. doi: 10.1038/sj.bjp.0701860

M., et al. (1998). Antagonist properties of 5-HT1A receptor knockout mice. Br. J. Pharmacol. 125, 1189–1197. doi: 10.1038/sj.bjp.0701860
Araragi et al. Autoinhibition in 5-HT system-deficient mice

August 2013 | Volume 4 | Article 97 |

Mathews, T. A., Fedele, D. E., Coppelli, F., Meltzer, C. C., Price, J. C., Mathis, A., Neumeister, A., Bain, E., Nugent, A. C., Murphy, D. L., and Lesch, K. P. (2004). Serotonin 1A receptor binding in mice with reduced serotonin translocation, and implications for human neurobiology. J. Neurosci. 24, 4090–4098. doi: 10.1523/JNEUROSCI.7217-04.2004

Mlinar, B., Tatin, F., Ballini, R., Aghajanian, G., Gutknecht, L., and Corradetti, R. (2005). Electrophysiological and pharmacological characterization of serotonin dorsal raphe neurons recorded extracellularly and intracellularly in rat brain slices. Brain Res. 1082, 109–119. doi: 10.1016/j.brainres.2005.06.027

Morrison, B. E., Naquin, A. C., Caron, R. E., Bonne, O., Luckenburger, L., Kung, H. F., Parsey, R. V., Oquendo, M. A., Ogden, L., and Blier, P. (1999). Direct injection of 5-HT2A receptor agonists into the medullu prefrontal cortex produces a head-twitch response in rats. J. Pharmacol. Exp. Ther. 282, 698–706.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

Received: 06 March 2013, paper pending publication 12 June 2013, accepted 17 July 2013, published online 02 August 2013.

Copyright: © 2013 Araragi, Mlinar, Baccini, Gutknecht, Lesch and Corradetti. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

www.frontiern.org

August 2013 | Volume 4 | Article 321 | 11