Optogenetic activation of serotonergic neurons enhances anxiety-like behaviour in mice

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Abstract

Whether increased serotonin (5-HT) release in the forebrain attenuates or enhances anxiety has been controversial for over 25 yr. Although there is considerable indirect evidence, there is no direct evidence that indicates a relationship between acute 5-HT release and anxiety. In particular, there is no known method that can reversibly, selectively, and temporally control serotonergic activity. To address this issue, we generated transgenic animals to manipulate the firing rates of central 5-HT neurons by optogenetic methods. Activation of serotonergic neurons in the median raphe nucleus was correlated to enhanced anxiety-like behaviour in mice, whereas activation of serotonergic neurons in the dorsal raphe nucleus had no effect on anxiety-like behaviour. These results indicate that an acute increase in 5-HT release from the median raphe nucleus enhances anxiety.

Introduction

Long-term treatment with selective serotonin (5-HT) reuptake inhibitors (SSRIs) ameliorates anxiety in humans (Zohar and Westenberg, 2000). However, whether acute 5-HT release in the forebrain attenuates or enhances anxiety has been controversial for at least 25 yr since it was first determined that SSRIs are effective in treating anxiety disorders (den Boer et al., 1987). Because increased anxiety is sometimes observed during the initial phase of SSRI treatment (Harada et al., 2008), this issue must be resolved.

It has generally been thought that acute 5-HT release in the forebrain attenuates anxiety because SSRIs increase the extracellular 5-HT concentration in the forebrain (Kreiss and Lucki, 1995). However, several animal studies have shown that electrolytic and neurotoxic lesions of the median raphe nucleus (MRN), one of the origins of serotonergic projections to the forebrain, decrease anxiety-like behaviour (Andrade and Graeff, 2001; Konno et al., 2007). Furthermore, microinjection of a 5-HT₁A agonist into the MRN, which suppresses serotonergic activity, decreases anxiety-like behaviour in rats (De Almeida et al., 1998). In contrast, electrolytic lesions of the dorsal raphe nucleus (DRN), another origin of serotonergic projections to the forebrain, do not affect anxiety-like behaviour (Konno et al., 2007).

Although there is considerable indirect evidence, there is still no compelling evidence indicating a direct relationship between acute 5-HT release and anxiety. Lesion or pharmacological studies cannot rule out the possibilities that secondary, compensatory, or off-target effects contribute to behavioural changes. However, a method that reversibly, selectively, and temporally controls serotonergic activity would provide evidence for such a relationship. Moreover, on the basis of previous studies (Andrade and Graeff, 2001; Konno et al., 2007), it would be necessary to separately examine the roles of MRN and DRN in anxiety.

In the present study, we aimed to obtain direct evidence for the underlying relationship between acute 5-HT release in MRN or DRN and anxiety using recently developed optogenetic methods that can reversibly, selectively, and temporally control serotonergic activity. Therefore, we generated transgenic mice expressing mutant channelrhodopsin-2 (ChR2(C128S)) in only central serotonergic neurons. Neurons expressing this mutant channel are activated by blue light and remain activated until they are stimulated by yellow light (Berndt et al., 2009). We examined whether selective or temporal activation of serotonergic neurons in the MRN and DRN...
of transgenic mice expressing C128S affects anxiety-like behaviour.

Method

Animals

We first generated 5-HT-neuron selective tetracycline-controlled transcriptional activator (tTA) expressing line in which tTA was expressed under the control of tryptophan hydroxylase 2 (Tph2) promoter (Supplementary Methods). We created mutant channelrhodopsin-2 (ChR2(C128S)) expressing animals in central serotonergic neurons by crossing tetracycline operator (tetO)-ChR2 (C128S)–enhanced yellow fluorescent protein (EYFP) knock-in mouse line (Tanaka et al., 2012) with Tph2- tTA line. These mice were back-crossed with the C57BL/6N strain for four generations. Adult mice aged >70 days were used. The mice were housed in groups of 4–6 animals per cage and under an alternating light-dark cycle (lights on from 19:00 to 07:00 hours) at approximately 21 °C. All tests were performed during the dark period. We used an equal number of male and female mice in each experiment. There were no significant differences in the results between males and females. The treatment of animals complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Acute slice in vitro electrophysiology

Electrophysiological recordings were performed using previously described methods (Tsunematsu et al., 2011) (see Supplementary Methods).

Immunohistochemistry

Immunohistochemistry was performed as previously described (Shikanai et al., 2012). The primary antibodies used were mouse anti-GFP (1:1000; 012-20461, WAKO, Japan) and sheep anti-TPH (1:1000; AB1541, Millipore). The secondary antibodies used were Alexa 488 (Invitrogen, USA) and indocarbocyanine (Cy3; Jackson ImmunoResearch, USA).

Surgical procedure

Mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and fixed in a stereotaxic frame (Narishige, Japan). To allow light stimulation in the MRN, a stainless steel guide cannula (24 gauge) was implanted 1.3 mm above the target site at a 35° angle. To apply light stimulation to the DRN, we implanted an optic fibre (MA45, Doric) that emitted light orthogonally (Supplementary Methods, Fig. S1).

In vivo microdialysis

Microdialysis was performed as previously described (Ohmura et al., 2010) (Supplementary Methods).

Elevated plus maze test

The test apparatus consisted of two of both open and closed arms (25 × 5 cm) that extended from a central platform (5 × 5 cm). Closed arms were surrounded by 20 cm high walls. The maze was elevated 40 cm above the floor, and the room lights were turned off (<5 lux) during testing. Mice typically avoid the open arms because they innately dislike open space, and anxiolytic agents increase the time spent in open arms (Carobrez and Bertoglio, 2005). That is, a decrease in the time spent in the open arms indicates an increase in anxiety. In addition, the distance travelled in the maze was used to quantify locomotor activity. The test was initiated by placing the mouse on a central platform facing an open arm, and the recording was initiated once the mouse crossed an arm. If a mouse failed to cross an arm after 1 min, data from that mouse were excluded from the analysis (two mice were excluded). The mouse was recorded by a CCD camera over a 4 min period; the recorded data were analysed automatically using a software package (LimeLight, Actimetrics).

In vivo light illumination procedure

Blue (475 nm) or yellow (575 nm) light was generated by a SPECTRA 2-LCR-XA light engine (Lumencor). Before each experiment, a 250 μm diameter optical fibre (Lucir) was inserted through the guide cannula. An optical swivel (COME2, Lucir) was used for unrestricted in vivo illumination. The light was controlled by TTL pulses driven by a stimulator (Nihon Kohden). The light intensity at the fibre tip was approximately 1 mW/mm².

Verification of optical fibre and dialysis probe placements

After the experiments were completed, the optical fibre and dialysis probe placements were verified under a microscope. Data from mice with incorrect placements were excluded from the analysis (Supplementary Methods, Fig. S1).

Statistical analysis

The number of animals used per experiment was determined by a power analysis (α, 0.05; power, 0.8) using the effect size and variance estimated from previous studies or preliminary data. G’Power 3 was used for the power analysis (Faul et al., 2007). To assess normality, the Shapiro–Wilk test was performed. If the data failed this test, nonparametric statistical tests were performed. Most parameters were analysed using two-factor ANOVA with light colour as the within-subject factor and the genotype as between-subject factor. When there was a significant light × genotype interaction, one-factor ANOVA was performed after two-factor ANOVA. The α level was 0.05 for all comparisons. All statistical
procedures, except for the power analysis, were performed with SPSS (version 15.0).

Results

Validation of transgenic mice expressing ChR2[C128S] in central serotonergic neurons

Tph2-tTA::tetO-ChR2[C128S]–EYFP knock-in bigenic mice exhibited selective expression of EYFP in serotonergic neurons (Fig. 1a and b). Quantitative analysis in MRN and DRN showed that over 95% of TPH-positive cells expressed EYFP (n=838/858) and almost all EYFP-positive cells expressed TPH (n=838/839, see Supplementary Figs S2 and S3), indicating that serotonergic neurons per se expressed ChR2 in the raphe in bigenic mice.

In vitro electrophysiology revealed a dramatic increase in MRN neuron firing rates in response to blue light illumination, but this increase was reversed with yellow light illumination (one-way ANOVA, $F_{2,14}=4286, p<0.001$;

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**Fig. 1.** Validity of Tph2-tTA::tetO-ChR2[C128S]–EYFP knock-in bigenic mice. (a) High-magnification view of channelrhodopsin-2 (ChR2[C128S])–enhanced yellow fluorescent protein (EYFP) expression and tryptophan hydroxylase (TPH) staining in median raphe nucleus (MRN) cell bodies. White scale bars indicate 50 μm. Higher magnification view is presented in Supplementary Fig. S3. (b) TPH staining and ChR2[C128S]–EYFP expression in coronal slices display co-localization in cell bodies of the dorsal raphe nucleus (DRN) and MRN. (c) In the current clamp recording (Fig. 1c), blue light illumination (500 ms) was followed by yellow light illumination (500 ms) with a 4 s interval between illuminations. This protocol was repeated 5 times with 10 s intervals. Blue light pulses depolarized the neuron, whereas yellow light pulses cancelled the depolarization. (d) In the loose cell-attached recording (n=4) (Fig. 1d), blue (500 ms) and yellow (500 ms) illumination were applied every min for 10 min. This protocol was the same as those used in the *in vivo* behavioural experiments (Supplementary Figs S9 and S12). (e) Fig. 1e summarizes the data shown in Fig. 1d. ‘Pre’ indicates the average of firing frequency for 30 s before each blue light illumination. ‘Blue’ is the average of firing frequency for 60 s after blue light illumination. ‘Post’ is the average of firing frequency for 30 s after each 30 s yellow light illumination (30–60 s). (f) Schematic representation of microdialysis recording in combination with MRN-serotonin neuron illumination. (g) Time course of changes in extracellular serotonin levels in the ventral hippocampus of bigenic mice (n=8). Mice received both blue (once per min, 5 ms duration) and yellow (once per min, 500 ms duration) light application to the MRN; light application was conducted with intervals of at least 1.5 h. The data are presented as the mean±S.E.M. *p<0.05.
Fig. 1c, d, and e). Blue light activation/deactivation rates of the C128S mutant were slower than those for wild-type ChR2 (ChR2(C128S): τ on = 20 ms, τ off = 108 s; wild-type ChR2: τ on = 1.7 ms, τ off = 10 ms) (Berndt et al., 2009). Neurons expressing ChR2(C128S)-EYFP were identified as EYFP fluorescent and patch-clamped for recording. In the loose cell-attached recording, the average firing rate was 4.4 ± 0.5 Hz (n = 4). In the whole cell recording, the average resting membrane potential and cell capacitance were −54.8 ± 2.4 mV (n = 8), and 23.7 ± 1.9 pF (n = 8), respectively. These averages are similar to those reported from previous studies in which serotonergic neurons were recorded in vivo or in vitro (Liu et al., 2002; Tsunematsu et al., 2011). All EYFP fluorescent neurons were activated with blue light illumination.

Furthermore, we used in vivo microdialysis in freely moving mice. Three-factor ANOVA with Greenhouse–Geisser correction revealed a significant interaction for light × genotype × time (F4.46, 57.946 = 2.71, p = 0.03). Two-factor ANOVA revealed a significant interaction between the light colour (blue or yellow) and time (F2.97, 20.81 = 4.64, p = 0.01); one-way ANOVA revealed that blue light illumination of the MRN in bigenic mice increased 5-HT release in the ventral hippocampus 10 min after (F1.7 = 14.74, p = 0.006, Fig. 1f, g and Supplementary Fig. S1) and 30 min after (F1.7 = 10.29, p = 0.015) the illumination was initiated. These effects were not observed in monogenic or wild-type mice (light × time, F3.57, 21.39 = 2.09, p = 0.12). In fact, a significant EYFP expression was observed in the ventral hippocampus (Supplementary Fig. S4). We observed similar results when we illuminated the DRN and measured 5-HT levels in the dorsal striatum. Blue light illumination of the DRN increased 5-HT release but not dopamine release in the dorsal striatum (Supplementary Figs S1 and S5). We did not observe a significant increase in 5-HT levels in the dorsal striatum with MRN illumination (Supplementary Fig. S6), which indicates our illumination targeted only MRN and did not affect DRN activity. In contrast, we found that DRN and MRN illumination increased 5-HT levels in the ventral hippocampus (Supplementary Fig. S7). Thus, we could not determine whether illumination targeting DRN was restricted to DRN.

**Discussion**

In the present study, we successfully established transgenic mice expressing mutant (ChR2(C128S)) in central serotonergic neurons. Our findings indicate that serotonergic activity in MRN enhances anxiety. This finding is consistent with previous studies that reported acute SSRI administration increased anxiety-like behaviour in rodents (Birkett et al., 2011); (Silva and Brandao, 2000) but contradicts the generally accepted theory that 5-HT release in the forebrain attenuates anxiety. Previous pharmacological and lesion studies could not exclude the possibilities of secondary, compensatory, or off-target effects. However, optogenetic control of serotonergic neurons provides selective and temporally specific activation. Using optogenetic stimulation, we demonstrated that activation of serotonergic neurons in MRN consistently increased anxiety-like behaviour in the elevated plus and dark/bright maze tests. Furthermore, our results indicate that an acute increase in 5-HT release from the MRN enhances anxiety, which may explain why increased anxiety is sometimes observed during the initial phase of SSRI treatment (Harada et al., 2008). Finally, our findings would provide an important clue to end a 25 yr dispute on this issue; however, further studies are required to extrapolate our findings in animals to humans. It is important to note that we focused on the acute effects of 5-HT release but not the chronic effects. Therefore, our results should be interpreted separately from the therapeutic effects of chronic SSRI treatments (Zohar and Westenberg, 2000).

Our findings also indicate that the MRN, but not the DRN, plays a pivotal role in anxiety. Our findings, however, do not necessarily indicate the DRN is unimportant for anxiety. For example, a previous study demonstrated that pharmacological inactivation of the DRN blocks the learned fear response (Maier et al., 1995), and a recent...
study showed that the DRN is involved in social avoidance (Challis et al., 2013). Thus, the DRN may be involved in social anxiety and/or learned fear, whereas the MRN is involved in innate fear or anxiety.

Blue light illumination of MRN increased 5-HT release in the ventral hippocampus (Fig. 1g), but did not increase 5-HT levels in the dorsal striatum (Supplementary Fig. S6). This indicates that illumination targeting MRN did not affect DRN activity. However, we found that either DRN or MRN illumination increased 5-HT levels in the ventral hippocampus (Fig. 1g, Supplementary Fig. S7). With respect to DRN-targeting, there are two interpretations for our findings. One interpretation is our findings are consistent with previous findings: serotonergic neurons in MRN heavily project to the hippocampus, but not to the striatum, whereas those in DRN heavily project to the striatum and moderately to the hippocampus (Azmitia and Segal, 1978). The other interpretation is our DRN-targeting method affected MRN activity. Therefore, our findings would confirm the specificity of the intervention to MRN, but not to DRN.

It is difficult to determine the extent to which DRN and MRN were separately activated by illumination. When we illuminated DRN, we observed a significant increase in extracellular 5-HT levels in both the dorsal striatum and ventral hippocampus. Because the hippocampus receives projections from the caudal DRN, but not from the rostral DRN (Vertes, 1991), it is likely that we illuminated at least the caudal part of the DRN. We speculate that we illuminated most of the DRN because most of the implanted optical fibres were placed in the rostral-mid DRN (Supplementary Fig. S1). Given that the DRN can be divided into subregions (Fox and Lowry, 2013), it is necessary to develop techniques for controlling each subregion separately. Regarding MRN, we cannot speculate on the range of activation because the optical fibre placements differed among mice, and all mice showed increased 5-HT levels in the ventral hippocampus following blue light illumination of MRN.
Although we observed that blue light illumination of MRN enhanced anxiety-like behaviour in a time-locked manner (Supplemental Fig. S8), we cannot exclude the possibility that the blue light-induced increase in 5-HT levels continued after yellow light illumination reversed the serotonergic neuron activation because we did not measure 5-HT release in real time. Future development of techniques that can measure neurotransmitter release in real time will resolve this issue.

It also should be noted that we used animals with normal anxiety levels instead of animal models of anxiety disorders. Although our results indicate that acute serotonergic activation in MRN enhances anxiety in healthy subjects, it does not necessarily mean that serotonergic activation enhances anxiety in patients with anxiety disorders. For example, nicotine suppresses impulsivity in patients with attention deficit hyperactivity disorder (Potter and Newhouse, 2004) but increases impulsivity in normal healthy subjects (Ohmura et al., 2005). The effect of 5-HT receptor stimulation on anxiety depends on the receptor subtypes (Setem et al., 1999). If the functions of some 5-HT receptors are altered or impaired in patients with anxiety disorders, the net effect of central 5-HT release also could be altered. Increased anxiety is only occasionally observed during the initial phase of treatment of SSRIs. Thus, it is possible that serotonergic activation rarely enhances anxiety in patients with anxiety disorders.

Although future studies are required to further address these issues, our results are an important first step towards elucidating the relationship between central serotonergic systems and anxiety.

Supplementary material
For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145714000637.

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Conflict of Interest
None

References


