Published in final edited form as:

Neuroscience. 2015 July 9; 298: 436–447. doi:10.1016/j.neuroscience.2015.04.032.

5-HT_{1B} autoreceptors differentially modulate the expression of conditioned fear in a circuit-specific manner

Yusha Liu, Michele A. Kelly, and Timothy J. Sexton

Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, Washington, USA

Abstract

Located in the nerve terminals of serotonergic neurons, 5-HT_{1B} autoreceptors are poised to modulate synaptic 5-HT levels with precise temporal and spatial control, and play an important role in various emotional behaviors. This study characterized two novel, complementary viral vector strategies to investigate the contribution of 5-HT_{1B} autoreceptors to fear expression, displayed as freezing, during contextual fear conditioning. Increased expression of 5-HT_{1B} autoreceptors throughout the brain significantly decreased fear expression in both wild type (WT) and 5-HT_{1B} knockout (1BKO) mice when receptor levels were increased with cell type-specific herpes simplex virus (HSV) vector injected into the dorsal raphe nucleus (DRN). Additional studies used an intersectional viral vector strategy, in which an adeno-associated virus containing a double-floxed inverted sequence for the 5-HT_{1B} receptor (AAV-DIO-1B) was combined with the retrogradely transported virus canine adenovirus-2 expressing Cre (CAV-Cre) in order to increase 5-HT_{1B} autoreceptor expression only in neurons projecting from the DRN to the amygdala. Surprisingly, selective expression of 5-HT_{1B} autoreceptors in just this circuit led to an increase in fear expression in WT, but not 1BKO, mice. These results suggest that activation of 5-HT_{1B} autoreceptors throughout the brain may have an overall effect of attenuating fear expression, but activation of subsets of 5-HT_{1B} autoreceptors in particular brain regions, reflecting distinct projections of serotonergic neurons from the DRN, may have disparate contributions to the ultimate response.

Keywords

serotonin; dorsal raphe; amygdala; viral vector; adeno-associated virus; canine adenovirus-2

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine neurotransmitter that is involved in a wide variety of emotional and cognitive behaviors; dysfunction of the serotonergic system is implicated in psychiatric illnesses such as depression, post-traumatic stress disorder, and

© 2015 IBRO. Published by Elsevier Ltd.

John F. Neumaier (corresponding author) neumaier@uw.edu, Box 359911, Harborview Medical Center, 325 Ninth Ave, Seattle, WA 98104, Phone: 206-897-5803, Fax: 206-897-5804. ykliu@uw.edu. kellymi@u.washington.edu. tsexton@uw.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

anxiety. Serotonergic neurons in the brain have cell bodies that reside in the raphe nuclei of the midbrain and brainstem but project extensively to nearly every brain region (Jacobs and Azmitia, 1992). The dorsal raphe nucleus (DRN) provides most of the serotonin fibers to the forebrain and is somatotopically organized; subpopulations of serotonergic neurons that have unique anatomical connections may also impact different elements of complex emotional behavior (Lowry et al., 2008, Gaspar and Lillesaar, 2012).

5-HT_{1B} receptors are metabotropic G_{q/i/o} receptors (Bouhelal et al., 1988, Schoeffer and Hoyer, 1989) that are primarily localized to nerve terminals (Boschert et al., 1994, Ghavami et al., 1999, Riad et al., 2000) and exist both as autoreceptors on serotonergic neurons and as heteroreceptors on nonserotonergic cells (Hen, 1992). Since 5-HT_{1B} autoreceptors are more strongly activated during bouts of intense serotonergic activity at the sites of release, they provide localized autoregulation of serotonin neurotransmission (Sari, 2004). Several lines of evidence suggest that the level of 5-HT_{1B} autoreceptor expression is a key determinant of stress reactivity (Neumaier et al., 2002a, Kaiyala et al., 2003), and several studies suggest that drugs targeting the 5-HT_{1B} receptor have promise as adjunctive therapy with SSRIs (Roberts et al., 1999, Gardier, 2009, Ruf and Bhagwagar, 2009).

Differentiating between 5-HT_{1B} autoreceptors and 5-HT_{1B} heteroreceptors is crucial in revealing their individual roles in brain circuits and behavior but poses a technical challenge. 5-HT_{1B} autoreceptors and heteroreceptors are identical in sequence and structure but are expressed in different neuron types. 5-HT_{1B} autoreceptors are diffusely distributed at nerve terminals throughout the entire brain, but are intermixed with 5-HT_{1B} heteroreceptors in most brain regions. Therefore, it is difficult to target 5-HT_{1B} autoreceptors exclusively using conventional methods like drug administration, as 5-HT_{1B} heteroreceptors will also be affected. The 5-HT_{1B} knockout mice currently available for research have a constitutive deletion of the gene, and while they have a remarkable phenotype with decreased anxiety and increased aggression (Gingrich and Hen, 2001, Groenink et al., 2003, Guilloux et al., 2011), it is unclear whether these behavioral effects relate to the loss of 5-HT_{1B} autoreceptors or heteroreceptors during early development or adulthood. One approach for manipulating 5-HT_{1B} autoreceptor function is via systemic injections of the brain-penetrant agonist CP-94,253 at low doses, which preferentially activates autoreceptors over heteroreceptors (Sarhan et al., 2000, McDevitt et al., 2011); however, it is possible that heteroreceptors may also be affected even at low doses, tending to confound any conclusions based solely on pharmacological experiments. An alternative technique utilizes viral-mediated gene transfer (Clark et al., 2002, Clark et al., 2004, McDevitt et al., 2011, Hagan et al., 2012) to target neurons residing in a particular brain region.

Novel recombinant viral vectors are continuously developed to achieve greater control over transgene expression levels and cell type-specificity (Lowenstein and Castro, 2002, Luo et al., 2008, Bouard et al., 2009, Papale et al., 2009, Zhang et al., 2010). Herpes simplex virus (HSV) has been commonly used for local manipulation of expression in specific brain targets and is advantageous in offering neuron-specific gene expression with a low immune and inflammatory response (Papale et al., 2009). In recent years, AAV viruses with a double-floxed inverted open reading frame (DIO) have gained popularity. In these constructs, the transgene is inserted in the antisense orientation and flanked by two sets of

lox sites; when Cre recombinase is present, an inversion occurs at the lox sites to flip and lock the transgene into the sense orientation (Atasoy et al., 2008). AAV-DIO viruses may be used in combination with transgenic animals expressing Cre in a cell type-specific manner or with retrogradely transported, Cre-expressing canine adenovirus (CAV-Cre) to achieve greater specificity of transgene expression (Schnutgen et al., 2003, Saunders et al., 2012, Nair et al., 2013).

Previous work in our lab has used the neuron-specific HSV to increase 5-HT_{1B} receptor expression in the rat DRN and demonstrated that viral-mediated 5-HT_{1B} receptor expression localized correctly to axon terminals throughout the brain and increased autoreceptor activity (Clark et al., 2002, Clark et al., 2004, Hagan et al., 2012). This technique has permitted in vivo studies to elucidate the specific contribution of 5-HT_{1B} autoreceptors to emotional behaviors including fear, anxiety, depression, and stress. In rats, increasing expression of 5-HT_{1B} receptors in neurons in mid-rostrocaudal DRN decreased anxiety in the open field test, as well as fear potentiation of the startle response (Clark et al., 2002, Clark et al., 2004), but when the caudal DRN was targeted instead, expression of transgenic 5-HT_{1B} receptors had no effect on anxiety (McDevitt et al., 2011). Additionally, overexpression of 5-HT_{1B} receptors decreased fear expression in contextual fear conditioning but had no effect on acquisition; similarly, context-paired administration of a low dose of CP-94,253 to preferentially activate 5-HT_{1B} autoreceptors showed the same decrease in fear. Taken together, these data suggest a protective role of 5-HT_{1B} autoreceptors in these emotional behaviors, with differences along the rostrocaudal axis. Interestingly, these anxiolytic and fear-attenuating properties of 5-HT_{1B} autoreceptors are abolished when animals were exposed to stress or received the 5-HT_{1B} antagonist SB224289 prior to behavioral testing (Clark et al., 2002, Clark et al., 2004, McDevitt and Neumaier, 2011).

While it has been suggested that conditioned aversive stimuli activate the serotonergic system, which, in turn, projects to a variety of brain regions to elicit different behavioral responses (Deakin and Graeff, 1991), the specific circuits involved are still unknown. The amygdala is a brain area that receives strong serotonergic projections and is heavily implicated in fear and anxiety behaviors (Davis, 1992, Ebner et al., 2004, Lowry et al., 2005, Ciochi et al., 2010, Haubensak et al., 2010, Herry et al., 2010, Johansen et al., 2010). Although approximately 10% of serotonergic neurons in the DRN project to the amygdala, nearly all of the neurons that project from the DRN to the amygdala are serotonergic (Ma et al., 1991); within the amygdala, serotonergic innervation is densest in the basolateral amygdala and more diffuse in the central amygdala (Vertes, 1991). However, it is still unclear how the serotonergic system acts in the amygdala in fear-related responses. One hypothesis proposed that the DRN-to-amygdala circuit is important for eliciting fear and anticipatory anxiety, while the DRN-to-periaqueductal gray circuit is important for preventing inappropriate freezing or fight-or-flight responses (Deakin and Graeff, 1991, Graeff et al., 1996). Because 5-HT_{1B} autoreceptors modulate serotonergic neurotransmission locally at the site of release, it is important to understand their function in specific circuits, as this may lead to greater insight about the role of serotonin in various brain regions.

To examine the selective contribution of 5-HT_{1B} autoreceptors to emotional behaviors, we designed and utilized two novel viral vectors. First, to overexpress 5-HT_{1B} autoreceptors in nerve terminals throughout the brain, we employed an HSV viral vector using the SERT promoter to induce transgene expression only in serotonergic cells. Second, to overexpress 5-HT_{1B} autoreceptors in nerve terminals in one particular circuit, we used a conditional AAV-DIO viral vector in combination with CAV-Cre viruses or cell type-specific Cre driver mouse lines, such as Pet1-Cre mice that use the serotonergic marker Pet-1 to selectively drive Cre expression. We harnessed these complementary techniques to further elucidate the role of 5-HT_{1B} autoreceptors in contextual fear conditioning. We hypothesized that 5-HT_{1B} autoreceptors attenuate fear expression but that their contribution to contextual fear conditioning is dependent on the specific circuit in which the 5-HT_{1B} autoreceptors are expressed.

EXPERIMENTAL PROCEDURES

Vector construction

To create a serotonin-specific viral gene expression system, we utilized a plasmid containing the 1.7 kb fragment of the human SERT promoter, which was received as a generous gift from Ovie Wiborg. In rat raphe precursor cells, the 1250 bp proximal to the SERT gene shows the greatest promoter/enhancer activity (Mortensen et al., 1999), so this section, along with GFP and the SV40 polyadenylation sequence, was cloned using PCR. Two SV40 polyA fragments were ligated together, and the ligation reaction was subjected to PCR to form a double polyA site. All PCR fragments were subcloned into the TOPO-Blunt cloning vector. All three components were inserted into the a pHSV-prPUC plasmid, developed by Dr. Rachael Neve for use in a replication-defective HSV gene transfer system (Neve and Geller, 1995), to form an expression cassette. This cassette was cloned in the opposite orientation relative to the HSV promoter to avoid nonspecific induction of transgene transcription in nonserotonergic cells, and was tested in cell culture using the serotonergic-like CA-77 cell line (Greene and Tischler, 1976) and the noradrenergic-like PC-12 cell line (Clark et al., 1995). Virus produced from this construct (pSERT-GFP) was injected into rat striatum, ventral tegmental area, and dorsal raphe nucleus and was observed to express selectively in DRN (Sexton et al., 2006).

To create the AAV-DIO construct that conditionally expresses the 5-HT_{1B} receptor (AAV-DIO-1B), we constructed a DNA block using annealed synthetic oligonucleotides that was comprised of GSG, 2A skip, and a hemagglutinin (HA) tag with inclusive restriction sites. We then inserted mCitrine before the GSG sequence, and the coding sequence for the “humanized” rat 5-HT_{1B} (N357T, which confers low affinity for β -antagonists (Adham et al., 1994)) without the Kozak sequence immediately after the HA tag; thus, the final construct was mCitrine-GSG-2A-HA-5-HT_{1B}. The 2A skip was modeled on the porcine teschovirus-1 (Szymczak et al., 2004). We utilized the backbone of pAAV-FLEX-hM3D-mCherry (Krashes et al., 2011) to gain the AAV origins of replication (LITR/RITR), FLEX double lox sites (Lee and Saito, 1998), woodchuck hepatitis virus posttranscriptional response element (WPRE) (Loeb et al., 1999, Loeb et al., 2002), and antibiotic selectivity. Briefly, the pAAV-FLEX-hM3D-mCherry was cut with AscI and NheI to remove the

hM3D-mCherry portion and insert mCitrine-GSG-2A-HA-5-HT_{1B} in the reverse orientation. The final construct was packaged into AAV serotype 8 by the Vector Core at the University of North Carolina at Chapel Hill. The original CAV viral vector was a generous gift from Dr. Eric Kremer (Kremer et al., 2000) and was propagated and packaged by our lab.

The novel AAV-DIO-1B viral vector incorporated two important elements. First, because the double-floxed and inverted ORF design requires the presence of Cre recombinase to induce expression, the selection of the source of Cre gives an additional level of control; DIO viral vectors may be used in combination with transgenic mice that express Cre in a cell type-specific manner (Krashes et al., 2011), or with retrogradely transported CAV-Cre viral vectors, which may be used to study a single circuit in the brain (Nair et al., 2013, Boender et al., 2014). Second, the 2A ribosomal skip peptide allows the expression of two separate proteins from a single cassette, which offers the advantage of a small cassette size but avoids any issues associated with fusion proteins altering protein function.

Animal care

All animal procedures were approved by the University of Washington's Institutional Animal Care and Use Committee and carried out in accordance with National Institutes of Health guidelines. Care was taken to minimize animal discomfort. Adult male and female ePet1-Cre (generously provided by Dr. Evan Deneris (Scott et al., 2005)), 129S6/SvEvTac, and C57BL/6J mice were housed two to five per cage with access to food and water available ad libitum. 129S6/SvEvTac mice were either wild type (WT) or had constitutive deletions of the 5-HT_{1B} receptor (1BKO) (generously provided by Drs. Rene Hen and Suzanne Ahmari (Malleret et al., 1999)). Mice were housed on a 12/12 hour light cycle with lights on at 0600. All experiments were performed during the light cycle. Mice were 12-24 weeks of age and weighed 15-26 g at the time of surgery. Mice used in behavioral experiments were no more than 16 weeks of age at the time of testing.

Stereotaxic surgery

Mice were anesthetized with isoflurane (1-2% in oxygen) or a ketamine/xylazine cocktail (100 mg/kg ketamine and 10 mg/kg xylazine, i.p.), the scalp was shaved and cleaned with betadine and ethanol, and the mice were placed in a Stoelting stereotaxic device. The scalp was incised to reveal the skull, and small holes were drilled at the sites of injection. A 27 gauge needle was slowly inserted, and virus or fluorescent microspheres were injected using a microprocessor-controlled pump. The needle was left in position for five minutes following the completion of the injection, and then slowly withdrawn. The scalp was closed with sutures and Vetbond (3M), and mice were placed in a recovery chamber warmed by a heating pad until they recovered and moved about freely before returning to their home cage. Analgesia was provided using meloxicam (0.4 mg/kg) or ketoprofen (5 mg/kg) administered subcutaneously during surgery.

Injection of fluorescent microspheres

Fluorescent microspheres (Molecular Probes) were diluted 1:1 in sterile water and injected bilaterally, 250 nL per side at a rate of 100 nL/sec, to target the amygdala using four test coordinates: 1) AP -1.1, ML $\pm$ 2.8, DV -4.7; 2) AP -1.25, ML $\pm$ 2.8, DV -4.6; 3) AP -1.4,

ML $\pm$ 2.8, DV -4.8 ; or 4) AP -1.55 , ML $\pm$ 2.8, DV -4.6 . Animals were sacrificed 5 days after the injections and perfused with $1\times$ PBS followed by 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde for four hours before switching to $1\times$ PBS, sliced, and mounted onto slides with Prolong Gold with DAPI (Molecular Probes).

Injection of viral vectors

The DRN was targeted at an angle of 28° from the vertical with the coordinates AP -4.8 , ML $+1.5$, DV -3.4 from bregma. Bilateral injections of CAV-Cre or CAV-GFP into the amygdala were performed at the coordinates AP -1.25 , ML $\pm$ 2.8, DV -4.6 from bregma. Each site received 1 μ L of virus injected at a rate of 200 nL/min.

Immunohistochemistry

Animals were sedated with Beuthanasia-D (585 mg/kg pentobarbital sodium and 75 mg/kg phenytoin sodium), then intracardially perfused with $1\times$ PBS followed by 4% paraformaldehyde before removing the brains, and the brains were post-fixed in 4% paraformaldehyde for four hours before switching to $1\times$ PBS, then sliced with a vibratome into 40 μ m sections. Sections were washed once for ten minutes in $1\times$ PBS/0.5% Triton X-100, then blocked for two hours with 5% normal goat serum (NGS)/ $1\times$ PBS/0.25% Triton at room temperature with gentle shaking. Primary antibodies were diluted in 2.5% NGS/ $1\times$ PBS/0.25% Triton X-100 and incubated with brain slices overnight at 4°C with gentle shaking. The primary antibody dilutions used were: rabbit anti-GFP 1:400 (Cell Signaling), rabbit anti-HA 1:400 for single-labeling experiments and 1:1000 for double-labeling experiments (Cell Signaling), and sheep anti-tryptophan hydroxylase (Tph) 1:400 (Millipore). Sections were washed five times with $1\times$ PBS before incubation with secondary antibodies diluted in 2.5% NGS/ $1\times$ PBS for two hours at room temperature with gentle shaking. The secondary antibody dilutions used were goat anti-rabbit and goat anti-sheep at 1:400 dilutions (Molecular Probes). Brain sections were washed two more times before mounting onto slides with Prolong Gold with DAPI (Molecular Probes).

Contextual fear conditioning

Behavior was performed at an interval after viral vector infusion that allowed stabilized transgene expression (discussed later); this was after seven days for SERT and after 20 days for the Cre-dependent DIO vector system. Training and testing occurred in a mouse test cage measuring $7 \times 7 \times 12$ inches with a shock floor (Coulbourn Instruments) with a 1% acetic acid odor. Scrambled shocks were delivered manually with the Precision Animal Shocker (Coulbourn Instruments). For fear training, mice were placed in the middle of the test cage and allowed to acclimate for two minutes, then received three 2-second, 0.7 mA footshocks at two minute intervals. Following the third and final footshock, mice remained in the test cage for an additional minute before being returned to their home cage. After 24 hours, mice were placed back in the test cage for a five minute test session. Training and testing sessions were digitally recorded, and freezing was analyzed using ANY-maze software (Stoelting Co.).

Data analysis

Statistical analyses were performed with GraphPad Prism. Fear expression was measured as percent of time freezing during the five minute test session. Freezing over the course of the training session was quantified in 60 second bins. Pain sensitivity was measured as the latency from the onset of the first shock to the first episode of freezing. Two-way ANOVA was used to test for statistical significance for behavior, with virus and genotype as the between subjects variables, except for the analysis of freezing during the training session, which used two-way repeated measures ANOVA, with experimental group as the between subjects variable and time as the within subjects variable.

RESULTS

1.1 Viral mediated expression of 5-HT_{1B} autoreceptors

The first set of novel viral vectors was generated to incorporate the known sequence for the human SERT promoter to drive transgene expression (Figure 1). Using a similar strategy that we previously used to target the direct and indirect pathway neurons of striatum (Ferguson et al., 2011), we cloned the human SERT promoter and either eGFP (SERT-GFP) or hemagglutinin (HA)-tagged 5-HT_{1B} receptor (SERT-1B) into the HSV amplicon. Since the HSV IE4/5 promoter/enhancer that is intrinsic to the standard HSV amplicon is very strong, we cloned the SERT-GFP or SERT-HA1B cassette in the reverse orientation relative to the HSV promoter.

1.2 SERT promoter selectively drives transgene expression in serotonergic cells

To test the expression time course and cell type-specificity of the SERT promoter-driven viral vectors, SERT-GFP was injected into the DRN of WT mice. In contrast with conventional HSV vectors that peak around day 3 and dissipate by about day 7 (Carlezon et al., 2000, Pliakas et al., 2001, Clark et al., 2002, Ferguson et al., 2009, McDevitt et al., 2011), immunohistochemistry revealed low GFP expression at day 4, and peak expression at day 7 that persisted until at least day 14 (Figure 2a). This time course is delayed and longer lasting than that associated with HSV IE4/5 (Neve et al., 2005, Ferguson et al., 2009) but is similar to what we observed when using preprodynorphin or preproenkephalin promoters in a similar constellation (Ferguson et al., 2011). Immunoreactivity for the serotonergic marker Tph colocalized with GFP, demonstrating that transgene expression was restricted to serotonergic neurons (Figure 2b). A similar pattern of expression was observed in rat brain (data not shown).

2.1 Cre-dependent 5-HT_{1B} receptor expression using viral mediated gene transfer

As shown in Figure 3, the novel AAV-DIO-1B viral vector drives the expression of two distinct proteins: mCitrine, a fluorescent protein allowing for easy detection of viral infection, and HA-tagged 5-HT_{1B} receptor. This bicistronic construct takes advantage of the 2A ribosomal skip peptide, which allows the expression of two proteins encoded by a single messenger RNA (Donnelly et al., 2001, Luke et al., 2008, Ibrahimi et al., 2009, Sharma et al., 2012). This open reading frame (ORF) is inserted in an inverted orientation with respect to the rest of the plasmid, and is flanked by loxP and lox2272 sites as shown. In the presence

of Cre recombinase, the ORF inverts at the lox sites to allow transcription of the transgene sequences. The transgene is under the control of the human synapsin promoter, which restricts expression to neurons (Thiel et al., 1991, Kugler et al., 2003). Several AAV serotypes are readily available; we packaged these constructs into AAV8 as they infect DRN neurons well and support strong transgene expression (Klein et al., 2006).

2.2 AAV-DIO-1B viral vector expresses mCitrine and HA-tagged 5-HT_{1B} receptors in the presence of Cre recombinase

Hemizygous Pet1-Cre mice received injections of the AAV-DIO-1B viral vector into the DRN. Robust viral expression was observed in the DRN 24 days following surgery (Figure 4). As shown by costaining for HA and Tph, expression of HA-5-HT_{1B} was limited to serotonergic neurons expressing Cre recombinase under the control of the Pet-1 promoter, demonstrating the integrity of the DIO viral construct (Figure 4a). Injection of 1 μ L of AAV-DIO-1B was sufficient for robust expression (Figure 4b). Viral-mediated HA-5-HT_{1B} receptor expression correctly localized to axon terminals, as evidenced by HA immunoreactivity in beaded fibers that are characteristic of collateral axons in DRN (Figure 4c). In the DRN, mCitrine was visible readily without immunohistochemistry, and costaining with HA showed colocalization of the two viral protein products in the same cells (Figure 4d).

2.3 DRN-to-amygdala projections are targeted with intersectional viral vector approaches using AAV-DIO-1B in combination with CAV-Cre

We confirmed effective stereotaxic coordinates for retrograde transport to the caudal DRN by injecting fluorescent microspheres into the amygdala; the most effective coordinates for retrograde transport back to the caudal DRN were AP -1.25 , ML ± 2.8 , DV -4.6 from bregma (Figure 5a). The same coordinates were used for CAV-Cre injections into the amygdala combined with AAV-DIO-1B injections into the DRN. CAV-Cre was retrogradely transported from the amygdala to the DRN, where Cre was expressed and inverted the DIO transgene into the correct reading frame, allowing expression of HA-5-HT_{1B} receptors in DRN neurons that projected to the amygdala. Immunohistochemistry for HA-tagged 5-HT_{1B} receptors showed immunoreactivity at nerve terminals in the amygdala (Figure 5b), as well as in the DRN in both cell bodies and neuronal projections (Figure 5c, top), demonstrating not only expression but also correct localization of the receptor to nerve terminals as is seen with endogenous 5-HT_{1B} receptors. Colabeling with Tph (Figure 5c, middle) showed most, but not full, colocalization of HA-5-HT_{1B} with Tph (Figure 5c, bottom). The number of infected neurons detected was modest but consistent with what we would expect with viral expression, especially since only about 10% of neurons from the DRN project to the amygdala (Ma et al., 1991).

3.1 Increased levels of 5-HT_{1B} autoreceptors in nerve terminals throughout the brain reduce the expression of conditioned fear

By injecting the SERT-1B viral vector into the DRN, we tested the effect of viral-mediated expression of 5-HT_{1B} autoreceptors in WT and 1BKO mice on contextual fear conditioning. Analysis of freezing during the five minute test session revealed a significant reduction in freezing due to the 5-HT_{1B} viral vector, but no effect of genotype and no interaction

between virus and genotype (Figure 6; two-way ANOVA; $F_{1,28} = 5.553$, $p = 0.025$ for virus; $F_{1,28} = 0.825$, $p = 0.371$ for genotype; $F_{1,28} = 1.121$, $p = 0.299$ for interaction). WT and 1BKO mice that received the control SERT-GFP viral vector showed similar levels of freezing, which is consistent with previous reports suggesting that typical levels of contextual fear are observed in constitutive 1BKO mice as a result of compensatory adaptations arising from developing without 5-HT_{1B} receptors (Malleret et al., 1999). 1BKO mice receiving the SERT-1B viral vector, inducing transgenic 5-HT_{1B} autoreceptor expression while lacking 5-HT_{1B} heteroreceptors, showed a dramatic reduction in freezing, while WT mice receiving the SERT-1B viral vector, which were essentially overexpressing 5-HT_{1B} autoreceptors in caudal DRN neurons, showed an intermediate phenotype in between that of the controls and of the 1BKO mice receiving the SERT-1B viral vector.

3.2 5-HT_{1B} receptor overexpression in the DRN-to-amygdala circuit increases fear expression in WT, but not 1BKO, mice

WT and 1BKO mice were injected with AAV-DIO-1B into the DRN in combination either with CAV-Cre into the amygdala to increase 5-HT_{1B} autoreceptors in the DRN-to-amygdala circuit, or with a CAV-GFP control vector. HA staining of cell bodies in the DRN were only observed when CAV-Cre was injected into the amygdala, indicating that recombination and expression of HA-5-HT_{1B} was Cre-dependent; no HA staining was observed in animals that received CAV-GFP injections into the amygdala. 5-HT_{1B} autoreceptor overexpression in this circuit significantly increased freezing in the test session in WT mice but not in 1BKO mice (Figure 7a; two-way ANOVA; $F_{1,39} = 4.497$, $p = 0.040$ for interaction; $F_{1,39} = 1.035$, $p = 0.315$ for virus; $F_{1,39} = 2.431$, $p = 0.127$ for genotype). This was not due to differences between the two groups in anxiety as measured by the open field test (Figure 7b; two-way ANOVA; $F_{1,19} = 0.190$, $p = 0.668$ for virus; $F_{1,19} = 0.063$, $p = 0.804$ for genotype; $F_{1,19} = 0.063$, $p = 0.804$ for interaction) or differences in pain sensitivity as measured by the latency to the first freezing episode following the first shock (Figure 7c; two-way ANOVA; $F_{1,39} = 0.029$, $p = 0.865$ for virus; $F_{1,39} = 1.386$, $p = 0.246$ for genotype; $F_{1,39} = 0.068$, $p = 0.796$ for interaction). Interestingly, while all groups showed increased freezing over the course of the seven minute training session, significant differences were found when comparing 1BKO mice receiving CAV-Cre both with WT mice receiving CAV-Cre (at 300, 360, and 420 sec) and with WT mice receiving CAV-GFP (at 420 sec) (Figure 7d; two-way repeated measures ANOVA; $F_{6,234} = 29.15$, $p < 0.0001$ for time; $F_{18, 234} = 2.063$, $p = 0.0079$ for interaction; $F_{3,39} = 1.383$, $p = 0.262$ for experimental group).

DISCUSSION

We describe two new viral vector strategies for manipulating 5-HT_{1B} expression using either phenotype-specific gene expression (with SERT promoter control of transgene expression) or intersectional transgene expression (with DIO vector and retrograde Cre expression). Both strategies transduce 5-HT_{1B} receptors predominantly in serotonergic neurons, achieving a greater degree of specificity than previous viral vector strategies allowed. Development of the serotonin-specific SERT-1B vector allows for greater control over viral expression, since a heterogeneous population of cells may be infected by an HSV injection, but only serotonergic neurons will have the necessary elements to induce

transgene expression. Additionally, viral expression is easily visualized, suggesting that the SERT promoter is sufficiently strong to drive transgene expression. These qualities are highly advantageous for manipulating 5-HT_{1B} autoreceptor levels, as the viral vector can be injected into serotonergic cell bodies located in the DRN but the receptor itself will be trafficked to the nerve terminals just as are endogenous receptors. We did observe some staining of cell bodies as well, but no staining of dendrites.

The novel AAV-DIO-1B viral vector showed strong expression in the Pet1-Cre transgenic mouse line (Figure 4) and no expression in the absence of Cre (data not shown). Both mCitrine and the HA-tagged 5-HT_{1B} receptor are clearly detectable; while we did not quantify the expression of each protein, the strong expression of both products suggests that the 2A ribosomal skip peptide may have lower failure rates compared with other polycistronic viral vector approaches such as the use of the internal ribosome entry site, which tends to cause lower expression levels of the second protein encoded in the sequence (de Felipe, 2002, Chan et al., 2011, Ho et al., 2013). This intersectional strategy allows for exquisite control of specificity and permits the targeting of subpopulations of neurons as well as of discrete neural circuits.

One caveat of viral vector use is the potential for undesired anterograde or retrograde infection. This potential has been demonstrated for both HSV (Ugolini et al., 1987, McGovern et al., 2012) and for certain serotypes of AAV (Boulis et al., 2003, Burger et al., 2004, Rothermel et al., 2013). We previously observed that injection of HSV-GFP into nucleus accumbens induced many hundreds of neurons to express GFP at the injection site, whereas only a few VTA neurons were retrogradely infected (Neumaier et al., 2002b). Therefore, we conclude that the infected neurons at the HSV injection site account for the observed results in this and previous studies.

Interestingly, we observed very different behavioral results when 5-HT_{1B} autoreceptors were manipulated in the DRN using these two different strategies. Using the SERT-1B viral vector, which increases transgene expression in serotonergic neurons projecting all over the forebrain, 5-HT_{1B} autoreceptor overexpression decreases the expression of conditioned fear, which is consistent with a previous study in our lab that used a conventional HSV injected in the DRN of rats to drive 5-HT_{1B} receptor expression (McDevitt et al., 2011). The use of the selective SERT-1B viral vector verifies that it is indeed 5-HT_{1B} autoreceptors in serotonergic neurons that are mediating the reduction in fear. Notably, injection of SERT-1B into 1BKO mice, which lack both 5-HT_{1B} auto- and heteroreceptors, also results in fear attenuation; after the viral infection these mice express 5-HT_{1B} autoreceptors only, showing that 5-HT_{1B} autoreceptors alone are sufficient for this effect.

In contrast, increasing 5-HT_{1B} autoreceptors in the DRN projections to the amygdala did not reduce freezing; rather, overexpression of 5-HT_{1B} receptors in this circuit increases the expression of conditioned fear. This observation is not a result of differences in baseline freezing, fear acquisition, or pain sensitivity between control and experimental mice. This seemingly contradictory finding in the DRN-to-amygdala circuit suggests that 5-HT_{1B} autoreceptors in the amygdala may play a role in promoting fear expression, while 5-HT_{1B} receptors in other projections may be responsible for the attenuation of fear. Ethologically,

the appropriate expression of fear responses in the face of danger offers animals a selective advantage (Blanchard et al., 1993), so it is possible that 5-HT_{1B} receptors in the DRN-to-amygdala circuit facilitate this process. This may occur through 5-HT_{1B}-mediated reductions in synaptic 5-HT levels, via increased reuptake through SERT (Daws et al., 2000, Hagan et al., 2012, Montanez et al., 2013), suppression of 5-HT synthesis (Hjorth et al., 1995), or inhibition of 5-HT release (Middlemiss and Hutson, 1990). Indeed, recent reports support this hypothesis, as depletion of 5-HT levels in the amygdala potentiates fear behaviors (Tran et al., 2013, Wellman et al., 2013), while increased 5-HT levels in the amygdala decreases fear expression (Burghardt and Bauer, 2013, Kitaichi et al., 2014). Other serotonin receptors also play a role in fear and anxiety behaviors, such as 5-HT₃ receptors, which modulate fear extinction (Kondo et al., 2014). Additionally, 5-HT_{2A} and 5-HT_{2C} receptors produce anxiety-like behaviors and enhance fear conditioning (Campbell and Merchant, 2003, Burghardt et al., 2007) while 5-HT_{1A} heteroreceptors in the amygdala decrease the expression of conditioned fear when activated (Li et al., 2006, Ferreira and Nobre, 2014). However, in contrast to these postsynaptic receptors, 5-HT_{1B} autoreceptors work through regulating synaptic 5-HT levels, which, in turn, affect signaling through all downstream 5-HT receptors.

Remarkably, manipulation of 5-HT_{1B} receptors in the DRN-to-amygdala circuit did not have the same effects on 1BKO mice as it did on WT mice. This may be due to developmental differences in 1BKO mice as a result of the constitutive absence of 5-HT_{1B} receptors. Because the amygdala receives strong serotonergic projections from the DRN, the absence of 5-HT_{1B} receptors throughout life may have led to fundamental changes in this circuit, such that reintroduction of 5-HT_{1B} autoreceptors in later life are unable to mediate the same functions as seen in WT mice. Although the virally expressed 5-HT_{1B} receptors are correctly trafficked to the axons and nerve terminals throughout the brain, it is difficult to quantify the amount of receptors that are being expressed and impossible to detect the percent change in 5-HT_{1B} autoreceptor expression as a result of viral infection.

One striking observation resulting from the intersectional use of the AAV-DIO-1B and CAV-Cre viral vectors is the degree of collateralization observed in the DRN. Injections of AAV-DIO-1B into the caudal DRN combined with CAV-Cre into the amygdala were expected to increase 5-HT_{1B} autoreceptor expression in the serotonergic nerve terminals in the amygdala; however, immunohistochemical staining for HA shows that the HA-tagged 5-HT_{1B} receptor is abundant in axon collaterals in the DRN as well (Figure 5c). It is unknown whether collaterals from these amygdala-projecting neurons synapse onto other amygdala-projecting neurons to form an integrated DRN-to-amygdala circuit, or onto neurons that project to other brain regions. In the latter case, it is conceivable that serotonergic neurons with projections to the amygdala may also project to and alter the signaling of other serotonergic neurons that send efferents to different brain regions, thereby affecting multiple circuits. Another open question is whether these collaterals might have complementary or opposing roles in regulating responses to fear. In any event, the contribution of 5-HT_{1B} autoreceptors in axon collaterals within the raphe nuclei has received insufficient attention but may be an important regulatory mechanism that exists alongside somatodendritic 5-HT_{1A} autoreceptors.

One limitation of the intersectional viral vector strategy using AAV-DIO-1B in combination with CAV-Cre to target the DRN-to-amygdala circuit is the inability to target specific cell types within the DRN. The DRN is a heterogeneous structure, containing both serotonergic and nonserotonergic cells (Jacobs and Azmitia, 1992). Interestingly, nonserotonergic cells in the DRN also send efferents to the amygdala (Halberstadt and Balaban, 2008), so it is possible that 5-HT_{1B} receptor expression in nonserotonergic cells may contribute to the behavioral observations. As shown in Figure 5c, transgene expression is detected in both Tph-positive and Tph-negative cells. Recent evidence suggests that subdivisions in the DRN amongst serotonergic neurons themselves may have distinct functional roles in behavior, not only in their somatotopic organization (Paul and Lowry, 2013), but also in their expression of various genes that had previously been thought to define whether a cell was serotonergic, such as 5-HT_{1A} autoreceptors (Gaspar and Lillesaar, 2012, Kiyasova et al., 2013). A greater understanding of the organization of the DRN is necessary, as well as an investigation into the anatomical connections between subdivisions of the DRN and the different amygdaloid nuclei.

These new findings shed light on the role of both 5-HT_{1B} autoreceptors and, more broadly, the serotonergic system on fear behaviors. Further work is warranted given the complexity of the system, particularly the functionally distinct subdivisions of both the amygdala and the DRN (Pare et al., 2004, Sierra-Mercado et al., 2011, Asan et al., 2013, Paul and Lowry, 2013, Paul et al., 2014) and the collateralization of serotonergic neurons in the DRN (Steinbusch, 1981, Waselus et al., 2011).

Acknowledgments

The authors would like to acknowledge the funding sources MH63303, GM07108, MH099748, T32GM007108; Evan Deneris for providing ePet1-Cre mice; Rene Hen and Suzanne Ahmari for providing 5-HT_{1B} knockout mice; Ovie Wiborg for providing the human SERT promoter construct; Eric Kremer for the original CAV viral vectors, and Richard Palmiter for advice on propagation of the CAV viruses.

Abbreviations

1BKO	5-HT _{1B} receptor constitutive knockout
AAV	adeno-associated virus
CAV	canine adenovirus
DIO	double-floxed inverted open reading frame
DRN	dorsal raphe nucleus
HA	hemagglutinin
HSV	Herpes simplex virus
NGS	normal goat serum
ORF	open reading frame
SERT	serotonin transporter
SSRI	selective serotonin reuptake inhibitor

Tph tryptophan hydroxylase
WT wild type

References

- Adham N, Tamm JA, Salon JA, Vaysse PJ, Weinshank RL, Branchek TA. A single point mutation increases the affinity of serotonin 5-HT_{1D} alpha, 5-HT_{1D} beta, 5-HT_{1E} and 5-HT_{1F} receptors for beta-adrenergic antagonists. *Neuropharmacology*. 1994; 33:387–391. [PubMed: 7984276]
- Asan E, Steinke M, Lesch KP. Serotonergic innervation of the amygdala: targets, receptors, and implications for stress and anxiety. *Histochemistry and cell biology*. 2013; 139:785–813. [PubMed: 23494464]
- Atasoy D, Aponte Y, Su HH, Sternson SM. A FLEX switch targets Channelrhodopsin-2 to multiple cell types for imaging and long-range circuit mapping. *J Neurosci*. 2008; 28:7025–7030. [PubMed: 18614669]
- Blanchard RJ, Yudko EB, Rodgers RJ, Blanchard DC. Defense system psychopharmacology: an ethological approach to the pharmacology of fear and anxiety. *Behavioural brain research*. 1993; 58:155–165. [PubMed: 7907880]
- Boender AJ, de Jong JW, Boekhoudt L, Luijendijk MC, van der Plasse G, Adan RA. Combined use of the canine adenovirus-2 and DREADD-technology to activate specific neural pathways in vivo. *PloS one*. 2014; 9:e95392. [PubMed: 24736748]
- Boschert U, Amara DA, Segu L, Hen R. The mouse 5-hydroxytryptamine_{1B} receptor is localized predominantly on axon terminals. *Neuroscience*. 1994; 58:167–182. [PubMed: 8159291]
- Bouard D, Alazard-Dany D, Cosset FL. Viral vectors: from virology to transgene expression. *British journal of pharmacology*. 2009; 157:153–165. [PubMed: 18776913]
- Bouhelal R, Smounya L, Bockaert J. 5-HT_{1B} receptors are negatively coupled with adenylate cyclase in rat substantia nigra. *European journal of pharmacology*. 1988; 151:189–196. [PubMed: 2971554]
- Boulis NM, Willmarth NE, Song DK, Feldman EL, Imperiale MJ. Intraneural colchicine inhibition of adenoviral and adeno-associated viral vector remote spinal cord gene delivery. *Neurosurgery*. 2003; 52:381–387. discussion 387. [PubMed: 12535368]
- Burger C, Gorbatyuk OS, Velardo MJ, Peden CS, Williams P, Zolotukhin S, Reier PJ, Mandel RJ, Muzyczka N. Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2004; 10:302–317. [PubMed: 15294177]
- Burghardt NS, Bauer EP. Acute and chronic effects of selective serotonin reuptake inhibitor treatment on fear conditioning: implications for underlying fear circuits. *Neuroscience*. 2013; 247:253–272. [PubMed: 23732229]
- Burghardt NS, Bush DE, McEwen BS, LeDoux JE. Acute selective serotonin reuptake inhibitors increase conditioned fear expression: blockade with a 5-HT_{2C} receptor antagonist. *Biol Psychiatry*. 2007; 62:1111–1118. [PubMed: 17524369]
- Campbell BM, Merchant KM. Serotonin 2C receptors within the basolateral amygdala induce acute fear-like responses in an open-field environment. *Brain Res*. 2003; 993:1–9. [PubMed: 14642825]
- Carlezon WA Jr, Nestler EJ, Neve RL. Herpes simplex virus-mediated gene transfer as a tool for neuropsychiatric research. *Critical reviews in neurobiology*. 2000; 14:47–67. [PubMed: 11253955]
- Chan HY, V S, Xing X, Kraus P, Yap SP, Ng P, Lim SL, Lufkin T. Comparison of IRES and F2A-based locus-specific multicistronic expression in stable mouse lines. *PloS one*. 2011; 6:e28885. [PubMed: 22216134]
- Ciocchi S, Herry C, Grenier F, Wolff S, Letzkus J, Vlachos I, Ehrlich I, Sprengel R, Deisseroth K, Stadler M, Muller C, Luthi A. Encoding of conditioned fear in central amygdala inhibitory circuits. *Nature*. 2010; 468:277–282. [PubMed: 21068837]
- Clark M, Sexton T, McClain M, Root D, Kohen R, Neumaier J. Overexpression of 5-HT_{1B} receptor in dorsal raphe nucleus using Herpes Simplex Virus gene transfer increases anxiety behavior after

inescapable stress. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2002; 22:4550–4562. [PubMed: 12040062]

Clark M, Vincow E, Sexton T, Neumaier J. Increased expression of 5-HT_{1B} receptor in dorsal raphe nucleus decreases fear-potentiated startle in a stress dependent manner. *Brain Research*. 2004; 1007:86–97. [PubMed: 15064139]

Clark MS, Lanigan TM, Page NM, Russo AF. Induction of a serotonergic and neuronal phenotype in thyroid C-cells. *J Neurosci*. 1995; 15:6167–6178. [PubMed: 7666199]

Davis M. The role of the amygdala in fear and anxiety. *Annu Rev Neurosci*. 1992; 15:353–375. [PubMed: 1575447]

Daws LC, Gould GG, Teicher SD, Gerhardt GA, Frazer A. 5-HT_{1B} receptor-mediated regulation of serotonin clearance in rat hippocampus in vivo. *Journal of neurochemistry*. 2000; 75:2113–2122. [PubMed: 11032901]

de Felipe P. Polycistronic viral vectors. *Current gene therapy*. 2002; 2:355–378. [PubMed: 12189721]

Deakin JF, Graeff FG. 5-HT and mechanisms of defence. *Journal of psychopharmacology*. 1991; 5:305–315. [PubMed: 22282829]

Donnelly ML, Luke G, Mehrotra A, Li X, Hughes LE, Gani D, Ryan MD. Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'. *The Journal of general virology*. 2001; 82:1013–1025. [PubMed: 11297676]

Ebner K, Rupniak NM, Saria A, Singewald N. Substance P in the medial amygdala: emotional stress-sensitive release and modulation of anxiety-related behavior in rats. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:4280–4285. [PubMed: 15024126]

Ferguson S, Eskenazi D, Ishikawa M, Wanat M, Phillips P, Dong Y, Roth B, Neumaier J. Transient neuronal inhibition reveals opposing roles of indirect and direct pathways in sensitization. *Nature Neuroscience*. 2011; 14:22–24.

Ferguson SM, Sandygren NA, Neumaier JF. Pairing mild stress with increased serotonin-1B receptor expression in the nucleus accumbens increases susceptibility to amphetamine. *Eur J Neurosci*. 2009; 30:1576–1584. [PubMed: 19817843]

Ferreira R, Nobre MJ. Conditioned fear in low- and high-anxious rats is differentially regulated by cortical subcortical and midbrain 5-HT_{1A} receptors. *Neuroscience*. 2014; 268:159–168. [PubMed: 24657773]

Gardier A. Mutant mouse models and antidepressant drug research: focus on serotonin and brain-derived neurotrophic factor. *Behavioural pharmacology*. 2009; 20:18–32. [PubMed: 19179848]

Gaspar P, Lillesaar C. Probing the diversity of serotonin neurons. *Philosophical transactions of the Royal Society of London Series B, Biological sciences*. 2012; 367:2382–2394.

Ghavami A, Stark KL, Jareb M, Ramboz S, Segu L, Hen R. Differential addressing of 5-HT_{1A} and 5-HT_{1B} receptors in epithelial cells and neurons. *Journal of cell science*. 1999; 112:967–976. Pt 6. [PubMed: 10036246]

Gingrich JA, Hen R. Dissecting the role of the serotonin system in neuropsychiatric disorders using knockout mice. *Psychopharmacology*. 2001; 155:1–10. [PubMed: 11374326]

Graeff FG, Guimaraes FS, De Andrade TG, Deakin JF. Role of 5-HT in stress, anxiety, and depression. *Pharmacol Biochem Behav*. 1996; 54:129–141. [PubMed: 8728550]

Greene LA, Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proceedings of the National Academy of Sciences of the United States of America*. 1976; 73:2424–2428. [PubMed: 1065897]

Groenink L, van Bogaert MJ, van der Gugten J, Oosting RS, Olivier B. 5-HT_{1A} receptor and 5-HT_{1B} receptor knockout mice in stress and anxiety paradigms. *Behavioural pharmacology*. 2003; 14:369–383. [PubMed: 14501251]

Guilloux J-P, David D, Xia L, Nguyen HT, Rainer Q, Guiard B, Repérant C, Deltheil T, Toth M, Hen R, Gardier A. Characterization of 5-HT_{1A/1B}–/– mice: An animal model sensitive to anxiolytic treatments. *Neuropharmacology*. 2011

- Hagan CE, McDevitt RA, Liu Y, Furay AR, Neumaier JF. 5-HT(1B) autoreceptor regulation of serotonin transporter activity in synaptosomes. *Synapse*. 2012; 66:1024–1034. [PubMed: 22961814]
- Halberstadt AL, Balaban CD. Selective anterograde tracing of nonserotonergic projections from dorsal raphe nucleus to the basal forebrain and extended amygdala. *J Chem Neuroanat*. 2008; 35:317–325. [PubMed: 18434087]
- Haubensak W, Kunwar P, Cai H, Cioocchi S, Wall N, Ponnusamy R, Biag J, Dong H-W, Deisseroth K, Callaway E, Fanselow M, Luthi A, Anderson D. Genetic dissection of an amygdala microcircuit that gates conditioned fear. *Nature*. 2010; 468:270–276. [PubMed: 21068836]
- Hen R. Of mice and flies: commonalities among 5-HT receptors. *Trends in pharmacological sciences*. 1992; 13:160–165. [PubMed: 1589910]
- Herry C, Ferraguti F, Singewald N, Letzkus JJ, Ehrlich I, Luthi A. Neuronal circuits of fear extinction. *Eur J Neurosci*. 2010; 31:599–612. [PubMed: 20384807]
- Hjorth S, Suchowski CS, Galloway MP. Evidence for 5-HT autoreceptor-mediated, nerve impulse-independent, control of 5-HT synthesis in the rat brain. *Synapse*. 1995; 19:170–176. [PubMed: 7784957]
- Ho SC, Bardor M, Li B, Lee JJ, Song Z, Tong YW, Goh LT, Yang Y. Comparison of internal ribosome entry site (IRES) and Furin-2A (F2A) for monoclonal antibody expression level and quality in CHO cells. *PloS one*. 2013; 8:e63247. [PubMed: 23704898]
- Ibrahimi A, Vande Velde G, Reumers V, Toelen J, Thiry I, Vandeputte C, Vets S, Deroose C, Bormans G, Baekelandt V, Debyser Z, Gijssbers R. Highly efficient multicistronic lentiviral vectors with peptide 2A sequences. *Human gene therapy*. 2009; 20:845–860. [PubMed: 19419274]
- Jacobs BL, Azmitia EC. Structure and function of the brain serotonin system. *Physiological reviews*. 1992; 72:165–229. [PubMed: 1731370]
- Johansen J, Hamanaka H, Monfils M, Behnia R, Deisseroth K, Blair H, LeDoux J. Optical activation of lateral amygdala pyramidal cells instructs associative fear learning. *Proceedings of the National Academy of Sciences*. 2010; 107:12692–12697.
- Kaiyala K, Vincow E, Sexton T, Neumaier J. 5-HT1B receptor mRNA levels in dorsal raphe nucleus: inverse association with anxiety behavior in the elevated plus maze. *Pharmacology, biochemistry, and behavior*. 2003; 75:769–776.
- Kitaichi Y, Inoue T, Nakagawa S, Omiya Y, Song N, An Y, Chen C, Kusumi I, Koyama T. Local infusion of citalopram into the basolateral amygdala decreased conditioned fear of rats through increasing extracellular serotonin levels. *Progress in neuropsychopharmacology & biological psychiatry*. 2014
- Kiyasova V, Bonnavion P, Scotto-Lomassese S, Fabre V, Sahly I, Tronche F, Deneris E, Gaspar P, Fernandez SP. A subpopulation of serotonergic neurons that do not express the 5-HT1A autoreceptor. *ACS chemical neuroscience*. 2013; 4:89–95. [PubMed: 23336048]
- Klein RL, Dayton RD, Leidenheimer NJ, Jansen K, Golde TE, Zweig RM. Efficient neuronal gene transfer with AAV8 leads to neurotoxic levels of tau or green fluorescent proteins. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2006; 13:517–527. [PubMed: 16325474]
- Kondo M, Nakamura Y, Ishida Y, Yamada T, Shimada S. The 5-HT3A receptor is essential for fear extinction. *Learning & memory*. 2014; 21:1–4. [PubMed: 24344177]
- Krashes MJ, Koda S, Ye C, Rogan SC, Adams AC, Cusher DS, Maratos-Flier E, Roth BL, Lowell BB. Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. *The Journal of clinical investigation*. 2011; 121:1424–1428. [PubMed: 21364278]
- Kremer EJ, Boutin S, Chillon M, Danos O. Canine adenovirus vectors: an alternative for adenovirus-mediated gene transfer. *Journal of virology*. 2000; 74:505–512. [PubMed: 10590140]
- Kugler S, Kilic E, Bahr M. Human synapsin 1 gene promoter confers highly neuron-specific long-term transgene expression from an adenoviral vector in the adult rat brain depending on the transduced area. *Gene therapy*. 2003; 10:337–347. [PubMed: 12595892]
- Lee G, Saito I. Role of nucleotide sequences of loxP spacer region in Cre-mediated recombination. *Gene*. 1998; 216:55–65. [PubMed: 9714735]

- Li X, Inoue T, Abekawa T, Weng S, Nakagawa S, Izumi T, Koyama T. 5-HT_{1A} receptor agonist affects fear conditioning through stimulations of the postsynaptic 5-HT_{1A} receptors in the hippocampus and amygdala. *European journal of pharmacology*. 2006; 532:74–80. [PubMed: 16460727]
- Loeb JE, Cordier WS, Harris ME, Weitzman MD, Hope TJ. Enhanced expression of transgenes from adeno-associated virus vectors with the woodchuck hepatitis virus posttranscriptional regulatory element: implications for gene therapy. *Human gene therapy*. 1999; 10:2295–2305. [PubMed: 10515449]
- Loeb JE, Weitzman MD, Hope TJ. Enhancement of green fluorescent protein expression in adeno-associated virus with the woodchuck hepatitis virus post-transcriptional regulatory element. *Methods in molecular biology*. 2002; 183:331–340. [PubMed: 12136769]
- Lowenstein PR, Castro MG. Progress and challenges in viral vector-mediated gene transfer to the brain. *Current opinion in molecular therapeutics*. 2002; 4:359–371. [PubMed: 12222874]
- Lowry C, Hale M, Evans A, Heerkens J, Staub D, Gasser P, Shekhar A. Serotonergic systems, anxiety, and affective disorder: focus on the dorsomedial part of the dorsal raphe nucleus. *Annals of the New York Academy of Sciences*. 2008; 1148:86–94. [PubMed: 19120094]
- Lowry CA, Johnson PL, Hay-Schmidt A, Mikkelsen J, Shekhar A. Modulation of anxiety circuits by serotonergic systems. *Stress*. 2005; 8:233–246. [PubMed: 16423712]
- Luke GA, de Felipe P, Lukashev A, Kallioinen SE, Bruno EA, Ryan MD. Occurrence, function and evolutionary origins of '2A-like' sequences in virus genomes. *The Journal of general virology*. 2008; 89:1036–1042. [PubMed: 18343847]
- Luo L, Callaway EM, Svoboda K. Genetic dissection of neural circuits. *Neuron*. 2008; 57:634–660. [PubMed: 18341986]
- Ma QP, Yin GF, Ai MK, Han JS. Serotonergic projections from the nucleus raphe dorsalis to the amygdala in the rat. *Neuroscience letters*. 1991; 134:21–24. [PubMed: 1815148]
- Malleret G, Hen R, Guillou JL, Segu L, Buhot MC. 5-HT_{1B} receptor knock-out mice exhibit increased exploratory activity and enhanced spatial memory performance in the Morris water maze. *J Neurosci*. 1999; 19:6157–6168. [PubMed: 10407051]
- McDevitt R, Hiroi R, Mackenzie S, Robin N, Cohn A, Kim J, Neumaier J. Serotonin 1B autoreceptors originating in the caudal dorsal raphe nucleus reduce expression of fear and depression-like behavior. *Biological Psychiatry*. 2011
- McDevitt RA, Neumaier JF. Regulation of dorsal raphe nucleus function by serotonin autoreceptors: a behavioral perspective. *J Chem Neuroanat*. 2011; 41:234–246. [PubMed: 21620956]
- McGovern AE, Davis-Poynter N, Rakoczy J, Phipps S, Simmons DG, Mazzone SB. Anterograde neuronal circuit tracing using a genetically modified herpes simplex virus expressing EGFP. *J Neurosci Methods*. 2012; 209:158–167. [PubMed: 22687938]
- Middlemiss DN, Hutson PH. The 5-HT_{1B} receptors. *Ann N Y Acad Sci*. 1990; 600:132–147. discussion 347-148. [PubMed: 2252306]
- Montanez S, Munn JL, Owens WA, Horton RE, Daws LC. 5-HT receptor modulation of the serotonin transporter in vivo: Studies using KO mice. *Neurochemistry international*. 2013
- Mortensen OV, Thomassen M, Larsen MB, Whittemore SR, Wiborg O. Functional analysis of a novel human serotonin transporter gene promoter in immortalized raphe cells. *Brain research Molecular brain research*. 1999; 68:141–148. [PubMed: 10320791]
- Nair SG, Strand NS, Neumaier JF. DREADDing the lateral habenula: a review of methodological approaches for studying lateral habenula function. *Brain Res*. 2013; 1511:93–101. [PubMed: 23085473]
- Neumaier J, Edwards E, Plotsky P. 5-HT(1B) mRNA regulation in two animal models of altered stress reactivity. *Biological Psychiatry*. 2002a; 51:902–908. [PubMed: 12022964]
- Neumaier JF, Vincow ES, Arvanitogiannis A, Wise RA, Carlezon WA Jr. Elevated expression of 5-HT_{1B} receptors in nucleus accumbens efferents sensitizes animals to cocaine. *J Neurosci*. 2002b; 22:10856–10863. [PubMed: 12486179]
- Neve RL, Geller AI. A defective herpes simplex virus vector system for gene delivery into the brain: comparison with alternative gene delivery systems and usefulness for gene therapy. *Clinical neuroscience*. 1995; 3:262–267. [PubMed: 8914792]

- Neve RL, Neve KA, Nestler EJ, Carlezon WA Jr. Use of herpes virus amplicon vectors to study brain disorders. *BioTechniques*. 2005; 39:381–391. [PubMed: 16206910]
- Papale A, Cerovic M, Brambilla R. Viral vector approaches to modify gene expression in the brain. *J Neurosci Methods*. 2009; 185:1–14. [PubMed: 19699233]
- Pare D, Quirk GJ, Ledoux JE. New vistas on amygdala networks in conditioned fear. *Journal of neurophysiology*. 2004; 92:1–9. [PubMed: 15212433]
- Paul ED, Johnson PL, Shekhar A, Lowry CA. The Deakin/Graeff hypothesis: Focus on serotonergic inhibition of panic. *Neurosci Biobehav Rev*. 2014
- Paul ED, Lowry CA. Functional topography of serotonergic systems supports the Deakin/Graeff hypothesis of anxiety and affective disorders. *Journal of psychopharmacology*. 2013; 27:1090–1106. [PubMed: 23704363]
- Pliakas AM, Carlson RR, Neve RL, Konradi C, Nestler EJ, Carlezon WA Jr. Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. *J Neurosci*. 2001; 21:7397–7403. [PubMed: 11549750]
- Riad M, Garcia S, Watkins KC, Jodoin N, Doucet E, Langlois X, el Mestikawy S, Hamon M, Descarries L. Somatodendritic localization of 5-HT1A and preterminal axonal localization of 5-HT1B serotonin receptors in adult rat brain. *The Journal of comparative neurology*. 2000; 417:181–194. [PubMed: 10660896]
- Roberts C, Boyd DF, Middlemiss DN, Routledge C. Enhancement of 5-HT1B and 5-HT1D receptor antagonist effects on extracellular 5-HT levels in the guinea-pig brain following concurrent 5-HT1A or 5-HT re-uptake site blockade. *Neuropharmacology*. 1999; 38:1409–1419. [PubMed: 10471095]
- Rothermel M, Brunert D, Zabawa C, Diaz-Quesada M, Wachowiak M. Transgene expression in target-defined neuron populations mediated by retrograde infection with adeno-associated viral vectors. *J Neurosci*. 2013; 33:15195–15206. [PubMed: 24048849]
- Ruf BM, Bhagwagar Z. The 5-HT1B receptor: a novel target for the pathophysiology of depression. *Current drug targets*. 2009; 10:1118–1138. [PubMed: 19702551]
- Sarhan H, Grimaldi B, Hen R, Fillion G. 5-HT1B receptors modulate release of [3H]dopamine from rat striatal synaptosomes: further evidence using 5-HT moduline, polyclonal 5-HT1B receptor antibodies and 5-HT1B receptor knock-out mice. *Naunyn-Schmiedeberg's archives of pharmacology*. 2000; 361:12–18.
- Sari Y. Serotonin1B receptors: from protein to physiological function and behavior. *Neuroscience and biobehavioral reviews*. 2004; 28:565–582. [PubMed: 15527863]
- Saunders A, Johnson CA, Sabatini BL. Novel recombinant adeno-associated viruses for Cre activated and inactivated transgene expression in neurons. *Frontiers in neural circuits*. 2012; 6:47. [PubMed: 22866029]
- Schnutgen F, Doerflinger N, Calleja C, Wendling O, Chambon P, Ghyselinck NB. A directional strategy for monitoring Cre-mediated recombination at the cellular level in the mouse. *Nature biotechnology*. 2003; 21:562–565.
- Schoeffter P, Hoyer D. 5-Hydroxytryptamine 5-HT1B and 5-HT1D receptors mediating inhibition of adenylate cyclase activity. Pharmacological comparison with special reference to the effects of yohimbine, rauwolscine and some beta-adrenoceptor antagonists. *Naunyn-Schmiedeberg's archives of pharmacology*. 1989; 340:285–292.
- Scott MM, Wylie CJ, Lerch JK, Murphy R, Lobur K, Herlitze S, Jiang W, Conlon RA, Strowbridge BW, Deneris ES. A genetic approach to access serotonin neurons for in vivo and in vitro studies. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102:16472–16477. [PubMed: 16251278]
- Sexton, TJ.; Clark, MS.; Neumaier, JF. Construction of a serotonin specific HSV-based virally mediated gene transfer vector. *Society for Neuroscience; Atlanta, GA: 2006. Program No. 199.17*
- Sharma P, Yan F, Doronina VA, Escuin-Ordinas H, Ryan MD, Brown JD. 2A peptides provide distinct solutions to driving stop-carry on translational recoding. *Nucleic acids research*. 2012; 40:3143–3151. [PubMed: 22140113]

- Sierra-Mercado D, Padilla-Coreano N, Quirk GJ. Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 2011; 36:529–538. [PubMed: 20962768]
- Steinbusch HW. Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. *Neuroscience*. 1981; 6:557–618. [PubMed: 7017455]
- Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S, Vanin EF, Vignali DA. Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. *Nature biotechnology*. 2004; 22:589–594.
- Thiel G, Greengard P, Sudhof TC. Characterization of tissue-specific transcription by the human synapsin I gene promoter. *Proceedings of the National Academy of Sciences of the United States of America*. 1991; 88:3431–3435. [PubMed: 1849657]
- Tran L, Lasher BK, Young KA, Keele NB. Depletion of serotonin in the basolateral amygdala elevates glutamate receptors and facilitates fear-potentiated startle. *Translational psychiatry*. 2013; 3:e298. [PubMed: 24002084]
- Ugolini G, Kuypers HG, Simmons A. Retrograde transneuronal transfer of herpes simplex virus type 1 (HSV 1) from motoneurons. *Brain Res*. 1987; 422:242–256. [PubMed: 2445438]
- Vertes RP. A PHA-L analysis of ascending projections of the dorsal raphe nucleus in the rat. *The Journal of comparative neurology*. 1991; 313:643–668. [PubMed: 1783685]
- Waselus M, Valentino RJ, Van Bockstaele EJ. Collateralized dorsal raphe nucleus projections: a mechanism for the integration of diverse functions during stress. *J Chem Neuroanat*. 2011; 41:266–280. [PubMed: 21658442]
- Wellman CL, Camp M, Jones VM, MacPherson KP, Ihne J, Fitzgerald P, Maroun M, Drabant E, Bogdan R, Hariri AR, Holmes A. Convergent effects of mouse Pet-1 deletion and human PET-1 variation on amygdala fear and threat processing. *Experimental neurology*. 2013; 250:260–269. [PubMed: 24100022]
- Zhang F, Gradinaru V, Adamantidis AR, Durand R, Airan RD, de Lecea L, Deisseroth K. Optogenetic interrogation of neural circuits: technology for probing mammalian brain structures. *Nature protocols*. 2010; 5:439–456.

Highlights

- Two novel viral vectors express 5-HT_{1B} receptors in a cell type-specific manner.
- The effect of 5-HT_{1B} autoreceptors on fear depends on the circuitry involved.
- Increased levels of 5-HT_{1B} autoreceptors in the brain decrease fear expression.
- Increased 5-HT_{1B} receptors in raphe neurons projecting to amygdala increase fear.

Figure 1. HSV viral vectors utilize the SERT promoter to drive transgene expression

Plasmid maps for the SERT-GFP (left) and SERT-1B (right) vectors are shown. The human SERT promoter sequence is located directly upstream of the transgene to be expressed. HSV viral elements are the origin of DNA replication (oriS), the constitutive IE4/5 promoter, and the HSV packaging signal.

Figure 2. HSV viral vectors utilize the SERT promoter to express transgenes selectively in serotonergic neurons, with expression peaking at day 7

SERT-GFP was injected into the DRN of WT mice. (a) Viral expression was assessed at days 4, 7, and 14, with peak expression at day 7. (b) Viral GFP expression colocalized with Tph immunoreactivity, demonstrating the selectivity of using the SERT promoter.

Figure 3. AAV viral vector utilizes a DIO design and the ribosomal 2A skip to express mCitrine and HA-5-HT_{1B} in the presence of Cre recombinase

Plasmid map for the AAV-DIO-1B vector is shown. The ORF, encoding mCitrine and an HA-tagged 5-HT_{1B} receptor separated by a 2A ribosomal skip peptide, is in an inverted orientation flanked by two sets of lox sites. Cre recombinase flips the ORF into the correct orientation, with transcription driven by the human synapsin (hSyn) promoter, restricting expression of the transgenes to neurons.

Figure 4. Injections of AAV-DIO-1B into Pet1-Cre mice produce robust mCitrine and HA-5-HT_{1B} expression in the DRN

Viral expression was observed in the DRN 24 days following surgery. (a) Dual labeling of HA-5-HT_{1B} (green) and Tph (red) showed colocalization only in serotonergic neurons, demonstrating that the DIO viral vector was only expressed in the presence of Cre recombinase. Staining for HA-5-HT_{1B} was prominent throughout the DRN (b), in both cell bodies as well as in axon collaterals (c). (d) Both mCitrine (unstained) and HA (red) were readily detectable, and expression of the two separate proteins were perfectly colocalized.

Figure 5. Intersectional use of the AAV-DIO-1B viral vector in combination with CAV-Cre can selectively target specific brain circuits

DRN-to-amygdala projections are validated with immunohistochemistry. (a) When red fluorescent microspheres were injected into several targets within the amygdala, the coordinates AP -1.25 , ML ± 2.8 , DV -4.6 from bregma showed the greatest level of retrograde transport to the caudal DRN. Injection of AAV-DIO-1B into the DRN combined with injection of CAV-Cre into the amygdala resulted in visible transgene expression at nerve terminals in the amygdala (b; anti-HA staining in green) and in the DRN (c; anti-HA staining in green, anti-Tph staining in red), with HA-5-HT_{1B} receptors in both cell bodies and axon collaterals; HA-5-HT_{1B} colocalizes with Tph immunoreactivity in most, but not all, cells.

Figure 6. Increased levels of 5-HT_{1B} autoreceptors reduce the expression of conditioned fear WT and 1BKO animals receiving the SERT-1B viral vector showed significantly less freezing in the test session following contextual fear conditioning compared to animals receiving the SERT-GFP control vector, with no effect of genotype on freezing or interaction between virus and genotype (two-way ANOVA, $p = 0.025$ for virus, $p = 0.371$ for genotype, $p = 0.299$ for interaction).

Figure 7. Increased levels of 5-HT_{1B} receptors in the DRN-to-amygdala circuit increases fear expression in WT mice

WT and 1BKO mice received injections of AAV-DIO-1B into the DRN, and either CAV-Cre for 5-HT_{1B} receptor overexpression or CAV-GFP as a control. (a) 5-HT_{1B} receptor overexpression in the DRN-to-amygdala circuit significantly increased freezing following contextual fear conditioning in WT but not 1BKO mice (two-way ANOVA, $p = 0.04$ for interaction, $p = 0.315$ for virus, $p = 0.127$ for genotype). Viral-mediated expression of 5-HT_{1B} receptors in the DRN-to-amygdala circuit had no effect on (b) anxiety in the open field test (two-way ANOVA, $p = 0.668$ for virus, $p = 0.804$ for genotype, $p = 0.804$ for interaction) or on (c) pain sensitivity, as measured by latency to the first freezing episode following the first shock (two-way ANOVA, $p = 0.865$ for virus, $p = 0.246$ for genotype, $p = 0.796$ for interaction). (d) Freezing during the training session increased over time, and freezing in 1BKO mice receiving CAV-Cre was significantly different from WT mice receiving CAV-Cre at 300, 360, and 420 sec and significantly different from WT mice receiving CAV-GFP at 420 sec (two-way repeated measures ANOVA, $p < 0.0001$ for time, $p = 0.0079$ for interaction, $p = 0.262$ for experimental group).