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Beyond Gene Inactivation: Evolution of Tools for Analysis of Serotonergic Circuitry

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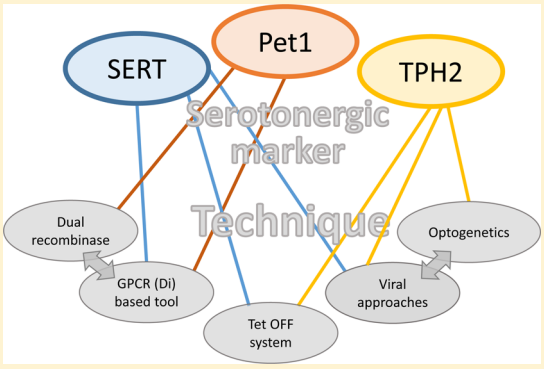
ABSTRACT: In the brain, serotonin (5-hydroxytryptamine, 5-HT) controls a multitude of physiological and behavioral functions. Serotonergic neurons in the raphe nuclei give rise to a complex and extensive network of axonal projections throughout the whole brain. A major challenge in the analysis of these circuits is to understand how the serotonergic networks are linked to the numerous functions of this neurotransmitter. In the past, many studies employed approaches to inactivate different genes involved in serotonergic neuron formation, 5-HT transmission, or 5-HT metabolism. Although these approaches have contributed significantly to our understanding of serotonergic circuits, they usually result in life-long gene inactivation. As a consequence, compensatory changes in serotonergic and other neurotransmitter systems may occur and complicate the interpretation of the observed phenotypes. To dissect the complexity of the serotonergic system with greater precision, approaches to reversibly manipulate subpopulations of serotonergic neurons are required. In this review, we summarize findings on genetic animal models that enable control of 5-HT neuronal activity or mapping of the serotonergic system. This includes a comparative analysis of several mouse and rat lines expressing Cre or Flp recombinases under *Tph2*, *Sert*, or *Pet1* promoters with a focus on specificity and recombination efficiency. We further introduce applications for Cre-mediated cell-type specific gene expression to optimize spatial and temporal precision for the manipulation of serotonergic neurons. Finally, we discuss other temporally regulated systems, such as optogenetics and designer receptors exclusively activated by designer drugs (DREADD) approaches to control 5-HT neuron activity.



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1. INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine that acts as a neurotransmitter in the central nervous system (CNS) and as an autacoid in the periphery. Serotonin synthesis in the CNS is restricted to serotonergic neurons, which originate from the raphe nuclei of the brain stem and extend vast axonal projections into different areas of the brain and spinal cord. Its synthesis is a two-step reaction. Tryptophan hydroxylase (TPH) 2 catalyzes the first, rate-limiting step in the CNS, in contrast to TPH1, which is active in the periphery.[1−3] *Tph2* is expressed exclusively in serotonergic neurons within the brain and in the enteric nervous system in the gut but not in other peripheral organs or neuronal cell types.[2−6] Serotonin packaging into vesicles is mediated by vesicular monoamine transporter (VMAT) 2, which is expressed in monoaminergic neurons.[7] Serotonin actions are mediated by its numerous receptors, which have different expression patterns within the CNS, including presynaptic locations.[8,9] For example, 5-HT1A autoreceptors, which are expressed on soma and dendrites of serotonergic neurons, mediate inhibition of cell firing.[10] Another important component of serotonergic neurons is serotonin transporter (SERT), which mediates 5-HT uptake.[11] In the brain of adult animals, SERT is mainly expressed on 5-HT producing neurons of the raphe nuclei and astrocytes.[12] During development, transient SERT expression in the brain is broader and includes nonserotonergic neurons in limbic, thalamic, and retinal areas.[13] Furthermore, SERT is located in a number of peripheral cells, such as platelets, adrenomedullary chromaffin cells, crypt cells in the gut, lung, liver, and testis.[14,15] SERT expression is therefore not restricted to serotonergic neurons.

Serotonergic neurons of the raphe nuclei are organized in groups, which are distinguished by their different anatomical

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location, axonal trajectories, and physiological properties.[16−18] The more rostrally located serotonergic cell groups, B6−B9, project to forebrain regions, whereas caudal serotonergic groups, B1−B5, project toward the spinal cord and brain stem.[19] Although anatomically clustered, groups of serotonergic neurons are not homogeneous in their electrophysiological properties and consist of different subpopulations, which are not fully characterized so far.[20,21]

During brain development, serotonergic neurons arise from distinct units of the neural tube, named rhombomeres (r1−r3 and r5−r7).[19] Developing 5-HT neuronal precursors along the rhombomeres express the PC12 ETS domain-containing transcription factor 1 (Pet1) and LIM homeobox transcription factor 1 (Lmx1b), which are involved in serotonergic neuron differentiation. Lmx1b precedes Pet1 expression and is also expressed in dopaminergic progenitors and neurons.[22] Pet1 was thought to be a specific marker of differentiated postmitotic serotonergic neurons.[16,23−25] However, more recent studies indicated that Pet1 is also active in some nonserotonergic cells in adult and developing brain and in peripheral organs such as adrenal glands, the 5-HT-synthesizing enterochromaffin cells of the intestine, developing kidney, and pancreas.[26,27]

The fact that development of serotonergic neurons and 5-HT synthesis, release, and reuptake require expression of multiple specific genes was used for the analysis of serotonergic circuits. Mouse models were engineered to express different reporters specifically in serotonergic neurons, such as the transgenic ePet-EYFP,[28] the TPH2-GFP knock-in,[29] and the TPH2-LacZ knock-in mouse lines.[30] These models have been employed in various *in vivo* and *in vitro* studies to examine electrophysiological properties and morphology of 5-HT neurons.[28−30] To manipulate the serotonergic system, various strategies for enhancement or inhibition of neuronal activity have been used. Besides numerous pharmacological interventions, a number of studies employed genetic approaches to inactivate or overexpress different genes involved in serotonergic neuron formation, 5-HT transmission, and 5-HT metabolism.[31−38] The behavior and physiological consequences of life-long alterations in serotonergic signaling in the brain in these models were recently reviewed.[39−42]

The characterization of these genetic models contributed greatly to our understanding of serotonergic circuits. However, genetic approaches that result in life-long changes in gene expression (for example, full knockouts or constitutive overexpression models) may induce compensatory mechanisms in other neurotransmitter systems, thus making it impossible to assess 5-HT function *per se*. Moreover, these models do not provide an opportunity to study functions of specific subpopulations of 5-HT neurons. In this review, we summarize new tools which provide higher specificity and temporal control to study serotonergic circuitry.

2. TARGETED EXPRESSION OF Cre AND Flp RECOMBINASES IN SEROTONERGIC CELLS

To manipulate serotonergic neurons in the CNS of mice, many studies have made use of the standard Cre-LoxP approach for cell type-specific recombination. The Cre protein is a site-specific DNA recombinase, that catalyzes the recombination between two 34 bp long loxP sequences.[43,44] Cre-dependent recombination in conjunction with LoxP-flanked target genes or stop cassettes can inactivate or activate the expression of a gene of interest (GOI) respectively. Over the past decade, a number of transgenic and knock-in Cre-expressing mouse lines have been generated. To direct transgene expression with the same developmental timing and spatial expression patterns as certain endogenous genes, bacterial artificial chromosomes (BACs) have also been widely used.[45] BACs are DNA constructs, which are capable to host the DNA fragments of >350 kilobase pairs with a high degree of stability.[46,47] Therefore, they are suitable for inserting large promoter regions including distant regulatory elements to drive Cre expression in specific cell populations.[48,49] Alternatively, knock-in approaches can be applied to control expression of the recombinase using the same machinery which controls expression of cell specific genes. In these cases the expression pattern is much more accurate and does not require time-consuming isolation and characterization of the promoter.[50] Most importantly, the knocked-in transgene does not generate additional mutations at an unknown locus as classical randomly integrated transgenes do. Of note, during the knock-in approach one allele of the gene in which Cre is inserted will be disrupted leading to a heterozygous knockout of this gene unless the transgene is linked via a 2A-peptide sequence at the 3′-end of the endogenous driver gene. For some genes, such as *Tph2,* the heterozygosity does not evoke behavioral alterations,[51] whereas for other genes, *e.g., Sert,* the deletion of one allele results in changes in neurochemistry and physiology (see below 2.1.1) and should be considered as an additional factor to be controlled for. Once a specific Cre line is successfully generated, the expression of recombinase can be functionally characterized by crossbreeding with Cre-dependent reporter strains, such as Rosa26loxP-STOP-lacZ or ROSA26loxP-stop-EYFP.[52,53] which start to express β-Galactosidase (β-Gal) or EYFP, respectively, once Cre recombinase is active. Co-localization analysis of reporter expression and serotonergic markers is an important step in evaluating the specificity and confirming the efficiency of Cre-dependent recombination. In case of inducible Cre-ERT2 models, a chimeric protein consisting of a codon-optimized Cre recombinase fused to a triple mutant form of the human estrogen receptor (ERT2) is expressed in targeted cells. The mutant receptors no longer bind endogenous estradiol, but instead recognize the synthetic ligand Tamoxifen (TMX). Upon TMX administration, the recombinase gains access to the nuclear compartment and initiates recombination,[54] thereby permitting temporal control of gene manipulation.

Another enzyme, flippase (Flp), which works similar to Cre and mediates DNA recombination between 34bp-long Flp recognition target (FRT) sites, has also been used in such approaches (reviewed in[55]). Available Cre and Flp transgenic mouse lines for the serotonergic system are summarized in Table 1, and discussed below in more detail.

2.1. Cre Recombinase Expression Driven by the *Sert* Promoter. *2.1.1. Knock-in SERT-Cre.* Among the first published lines targeting specific neurotransmitter systems is the SERT-Cre mouse line, generated by knocking in Cre into the *Slc6A4* locus (gene coding for SERT) by the group of R. Hen.[50] In the raphe, 99% of cells which underwent recombination were TPH-positive, and nearly all serotonergic neurons were recombined. These results indicate a high recombination efficiency and specificity in the raphe of this SERT-Cre mouse line. This line has been widely used for different applications, including studies of heart development, effects of selective serotonin reuptake inhibitors (SSRIs), sudden infant death syndrome, depression, addiction, development, and metabolism.[56−60] For example, Narboux-Nême and

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Table 1. Available Cre and Flp Animal Models Targeting the Serotonergic System[a]

genetic model	formal designation/background	transgene	specificity of Cre expression	recombination efficiency of Cre in 5-HT neurons	ref
SERT					
Knock-in SERT-Cre	B6.129-Slc6a4tm1(cre)Xz/Cnrm[c]	knock-in into the 5′-UTR of the *Sert* gene	99% of Cre+ cells in RN are TPH2+	100% in RN	50
			During development Cre expression in thalamus, cingulate cortex, CA3 region of the hippocampus		
			recombination in 11 CNS regions, in 5 neural crest derived and 2 non-neural crest derived structures		58
			Cre+ cells are found in thyroid follicular cells (E10.5 until adulthood)		60
BAC-SERT-Cre		BAC RP24−335M24			
Slc6a4 Cre ET33[c]	B6.FVB(Cg)-Tg(Slc6a4-cre)ET33Gsat/Mmucd[c]		ET33/35: Co-labeling of TPH with EGFP reporter in DR, MnR and sparse numbers in cortex	ET33: ∼100% in DR and MnR	49[b]
Slc6a4 Cre ET35[c]	Tg(Slc6a4-cre)ET35Gsat[c]		ET33: retinal ganglion cells and dorsal-nasal retina		64
Slc6a4 Cre ET124[c]	Tg(Slc6a4-cre)ET124Gsat[c]		ET124: 5-HT neurons, corticothalamic and thalamocortical neurons		49
Slc6a4 Cre ET127[c]	Tg(Slc6a4-cre)ET127Gsat[c]		ET127: Cre+ cells in cortical and thalamic areas, principal trigeminal nucleus (Cre-expression decreases with postnatal development)		[b]
Slc6a4-CreERT2-EZ13[c]	B6.FVB(Cg)-Tg(Slc6a4-cre/Esr1)EZ13Gsat/Mmcd[c]	BAC RP24−35M24	Cre expression restricted to 5-HT neurons	ndf	[b]
Pet1					
ePet1-Cre Slc6a4-CreERT2-EZ13	B6.Cg-Tg(Fev-cre)1Esd/J[c]	40kb ePet1 enhancer	ePet-Cre: Co-localization of TPH and reporter β-Gal in B7 and B2 RN	ePet-Cre: 100% in midbrain and medullary 5-HT nuclei	28
ePet1-Cre-2			Cre expression in nonserotonergic cells during development in rostral and caudal hindbrain, enterocytes, pancreas, skin	ePet-Cre-2: ∼60% in adult RN	
BAC-Pet1-Cre	ndf	BAC	At E12.5 co-localization of 5-HT with Cre in RN with Cre+ cells found in DR, MnR and RMg	ndf	67
BAC-Pet1-CreERT2	Tg(Fev-cre/ERT2)#Yqd (FVB/N)[c]	BAC RP23−165D11	co-localization of TPH2 with β-gal reporter, no Cre+ cells found elsewhere	85% in DR and RMg	68
$Pet1_{210}$-Cre	C57BL/6J	BAC RP23−165D1, 210kb	in RN 100% of 5-HT+ cells express the YFP reporter, but some nonserotonergic cells are YFP+, with rostral to caudal decreasing ratio	100% in B1−B9 RN	26
			outside the CNS recombination reporter β-Gal found in developing pancreas and kidney		
ePet-Flpe	Mixed C57BL/6-129/SV genetic background	40kb ePet1 enhancer	β-gal reporter is detected exclusively in 5-HT neurons of ePet-Flpe; RC-Fa double transgenic mice at P7; at P21 ∼100% of TPH2+ cells are GFP+ in B1−B3 and B6−B9 of ePet-Flpe; RC-Fela double transgenic mice	∼100% in 5-HT neurons	66
			in DR of triple transgenic *(hβact-Cre, ePet-Flpe, RC-PFtox and En1-Cre, ePet-Flpe, RC-PFtox)* mice ∼100% of 5-HT cells are *GFP*+; in *En1-Cre, ePet-Flpe, RC-PFtox* mice GFP expression *pattern* in DR and MnR is similar to 5-HT cells		77
TPH2					
BAC-TPH2-CreERT2	Tg(TPH2-cre/ERT2)1.38Zi	RP24−243J21, 107 kb, CreERT2.pA inserted in the ATG of *Tph2*	recombination exclusively in 5-HT neurons of all RN low background recombination in vehicle-treated controls	91% of TPH2 cells (line 1.21, adulthood) (DR: 91%, MnR: 93%, CR: 88%)	70
			extensive colabeling for TPH2 and reporter β-Gal	66% during embryogenesis	
BAC-TPH2-CreERT2	Mixed genetic background (129sv: 12.5%; C57BL/6J: 87.5%)	BAC	recombination in CR, DR, MnR in the pattern of 5-HT cell distribution	ndf	72, 74
TPH2-icre/ERT2	C57BL/6N-Tg(TPH2-icre/ERT2)6Gloss/J	BAC RP23−151J17	recombination exclusively in raphe nuclei	ndf	100

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Table 1. continued

genetic model	formal designation/background	transgene	specificity of Cre expression	recombination efficiency of Cre in 5-HT neurons	ref
TPH2					
BAC-TPH2-CreERT2	Sprague–Dawley rats	RP24–243J21, 107 kb	no Cre expression outside the RN, TPH2+ cells express Cre	77% of TPH2+ cells (line #15, adulthood) (DR 75%, MnR 80%, CR 74%)	71

[a]ndf, not defined; RN, raphe nuceli; MnR, medial raphe nuclei; DR, dorsal raphe nuclei; CR, caudal raphe nuceli; E, embryonic day; P, postnatal day; 5-HT, serotonin; TPH2, tryptophan hydroxylase 2; CNS, central nervous system; β-Gal, β-galactosidase. [b]A number of Sert-cre lines were generated within the GENSAT project and can be viewed at www.gensat.org/cre.jsp; however, they were not included in the current table because the specificity of Cre expression as well as efficiency of its recombination in 5-HT neurons are not published. [c]*Slc6A4* and *Fev* refer to the genes encoding SERT and Pet1, respectively.

colleagues disabled serotonergic transmission by crossing SERT-Cre to VMAT2$^{fl/fl}$ mice which led to a major (∼95%) depletion of central 5-HT, but not other monoamines, through excision of the VMAT2 in SERT-positive cells.[61]

However, this SERT-Cre line has several limitations. First, due to the disruption of the *Slc6A4* this SERT-Cre mouse represents a heterozygous knockout resulting in a mild behavioral and biochemical phenotype[50] and therefore, proper controls (optimally SERT-Cre^{+} GOI$^{fl/fl}$ versus SERT-Cre^{+} GOI$^{wt/wt}$) should be used in experiments. Another limitation of this line is Cre recombination occurring in nonserotonergic neurons of the thalamus, the cingulate cortex, and the CA3 region of the hippocampus (summarized in the Table 1) due to transient SERT expression in these regions during early development.[62] Furthermore, the SERT-Cre line is prone to germ-line transmission of the recombined allele, due to SERT-driven Cre expression in male germ cells.[59] This may lead to the generation of a total, instead of a cell-type specific, knockout. By crossing SERT-Cre and ObRb$^{fl/fl}$ mice the group of G. Karsenty inactivated the leptin receptor b (LepRb) in serotonergic neurons.[30] Based on the experiments performed in these mice they postulated that leptin regulation of appetite occurs by inhibition of 5-HT synthesis in the brain. However, a detailed analysis by another group could not confirm the expression of LepRb in serotonergic neurons and it was supposed that the phenotypes, observed by the Karsenty group were most probably resulting from total instead of conditional LepRb knockout.[59]

2.1.2. BAC-SERT-Cre. Three BAC-SERT-Cre transgenic mouse lines were generated in frames of the GENSAT project (http://www.gensat.org/index.html).[49] In two founder lines (E33/E35) Cre expression was restricted to adult serotonergic neurons, whereas in the third line (E124) Cre expression was also observed in corticothalamic and thalamocortical neurons.[49,63] Moreover, in the E33 and E124 lines of BAC-SERT-Cre mice, Cre expression was observed in the developing visual system[64] and the upper layers of the barrel cortex,[65] respectively. These findings confirm SERT expression in nonserotonergic neurons during development, as shown earlier.[13,62] Taken together, BAC-SERT-driven Cre expression targets all serotonergic neurons, however, ectopic SERT expression during development and developmental recombination in transgenic SERT-Cre lines must be considered.

2.2. Recombinase Expression Driven by the *Pet1* Promoter. *2.2.1. ePet1-Cre.* Using a 40kb-long DNA region upstream of the *Pet1* gene containing an enhancer essential for proper Pet1 expression, the laboratory of E. Deneris generated an ePet1-Cre line that enables Cre-mediated recombination restricted to all 5-HT neurons.[28] Within the CNS, no ePet activity was detected outside the serotonergic system, however the analysis of LacZ expression in the periphery of R26R$^{ePet\text{-}Cre}$ mice revealed Cre-dependent recombination in some cells of skin, gut, pancreas, and cardiac neural crest.

2.2.2. ePet-Flpe. A Flp recombinase driver line, ePet-Flpe, was generated in the laboratory of S. Dymecki[66] with the same ePet construct used by the Deneris' group previously to generate the ePet-Cre line.[28] In the raphe of ePet-Flpe mice, the activity of Flp is high and mediates recombination in most 5-HT raphe neurons.[66]

2.2.3. Pet1$_{210}$-Cre. To control somatic recombination in the Pet1 lineage more reliably, the group of M. Pasqualetti generated mice expressing Cre under control of a large 210kb-long fragment of the Pet1 genetic locus, including all

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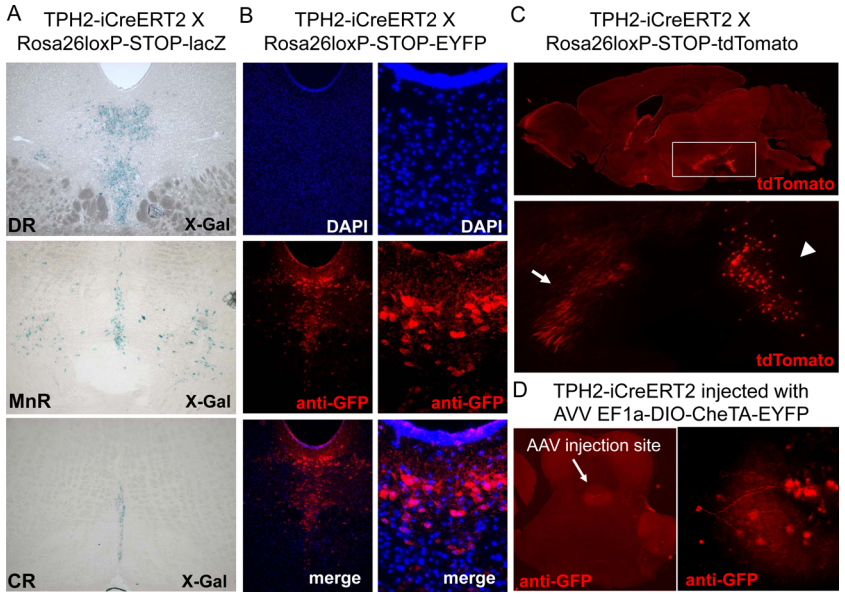

Figure 1. Cre-mediated recombination in TPH2-iCreERT2 mice. (A) LacZ expression in the raphe nuclei of TPH2-iCreERT2 x R26R double transgenic mice. DR, dorsal raphe nucleus; MnR, median raphe nuclei; CR, caudal raphe nuclei. (B) EYFP expression (immunohistochemistry with anti-GFP antibody) in the dorsal raphe nuclei of TPH2-iCreERT2 x ROSA26loxP-stop-EYFP double transgenic mice. (C) Expression of tdTomato in the raphe of TPH2-iCreERT2 x Rosa26loxP-STOP-tdTomato double transgenic mice. Arrowhead indicates neuronal cell bodies, arrow indicates neuronal processes. (D) EYFP expression (immunohistochemistry with anti-GFP antibody) in the dorsal raphe nuclei of TPH2-iCreERT2 mice injected with AAV virus (EF1a-DIO-CheTA-EYFP). In all experiments Cre expression was induced by three consecutive TMX injections (120 mg/kg for parts A, B, and C, and 100 mg/kg for part D) on days 1, 3, and 5. Mice were sacrificed on day 12−15.

putative regulatory regions.[26] $Pet1_{210}$-Cre transgenic mice revealed the existence of nonserotonergic neuronal populations, which express Pet1 within the raphe, mostly in the B8 and B9 cell groups. Cre expression was also detected in embryonic kidney and pancreas, demonstrating that $Pet1_{210}$-Cre mice are useful to study rather Pet-1 expressing lineages other than 5-HT neurons in particular (Table 1). Based on these findings, the specificity of other Pet-1 driven Cre lines and reporters, such as ePet1-EYFP,[28] should be reevaluated in these nonserotonergic neuronal populations and peripheral tissues.

2.2.4. BAC-Pet1-Cre. Another BAC-based Pet1-Cre mouse line was generated by the group of Y.Q. Ding[67] by insertion of the Cre coding sequence into the Pet1 locus. After crossing with Rosa26 reporter mice, X-Gal staining and double immunostaining for Cre and 5-HT revealed raphe nuclei-specific Cre expression.

The efficiency of Cre recombination was assessed by breeding Pet1-Cre mice with carriers of a floxed Lmx1b gene. In the resulting mice, 5-HT and SERT were almost absent in the whole brain, except for a single 5-HT-positive neuron found in the dorsal raphe (DR) (1%). It was thereby shown that adult $Lmx1b^{Pet1\text{-}Cre}$ mice lack essentially all 5-HT neurons and terminals. The HPLC measurements still revealed the presence of residual 5-HT, although at concentrations 10-fold lower than in wild type animals. It is unclear whether this is due to undetected serotonergic neurons that did not undergo recombination or the HPLC results reflect contamination by blood 5-HT due to insufficient perfusion with saline before tissue isolation. Information about nonserotonergic Cre expression was not provided for this line.

2.2.5. BAC-Pet1-CreERT2. The same group also generated a BAC-Pet1-CreERT2 transgenic mouse line that allows TMX-inducible recombination.[68] In this model, recombination was shown to be specific for serotonergic neurons in the DR and raphe magnus (RM), but only 85% of TPH2 expressing neurons coexpressed the floxed reporter gene after TMX treatment. Crossbreeding of this Pet1-CreERT2 line with $Lmx1b^{fl/fl}$ mice led to 40% reductions in brain 5-HT levels and downregulation of serotonergic markers.[68]

One limitation of this mouse line is its FVB/N background used for the generation of founder lines. Most floxed mice are generated on a C57BL/6 background, which has become the most widely used strain for developmental and behavioral studies. The FVB/N line was reported to have higher than C57BL/6 mouse line locomotor activity, anxiety, and basal body temperature and is homozygous for the *Pde6brd1* allele, causing retinal degeneration (http://jaxmice.jax.org/strain/001800.html). As a consequence, most FVB/N mice are blind[69] and thus, not suitable for behavioral experiments. This phenotype might also be inherited after crossing the Pet1-CreERT2 line to other strains and might have an unpredictable impact on experimental results.

2.3. Cre Recombinase Expression Driven by the *Tph2* Promoter. *2.3.1. BAC-TPH2-CreERT2.* After discovery of the brain specific TPH isoform[6] the *Tph2* promoter was used to direct Cre expression selectively to 5-HT neurons. In 2009, the group of D. Bartsch applied a BAC-based transgenic approach to create TMX-inducible TPH2-CreERT2 mice.[70] Besides higher specificity of the *Tph2* promoter for serotonergic neurons in comparison to SERT or Pet1, use of CreERT2 in this line allows recombination at any stage of development or adulthood. The same BAC-based construct was also later used to generate a TPH2-CreERT2 rat line.[71] High specificity of Cre expression upon TMX treatment was demonstrated in both TPH2-CreERT2 mouse and rat lines. However, rats showed a lower recombination efficacy compared with mice (77% in rats vs 90% in mice) (Table 1).

2.3.2. BAC-TPH2-CreERT2. A comparable TPH2-CreERT2 mouse line, based on a BAC construct, was also generated by Yadav and colleagues[72] to study leptin-dependent 5-HT control

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of appetite. Characterization of this line after breeding with Rosa26loxP-STOP-lacZ mice[52] and TMX treatment revealed β-Gal staining in DR, median raphe (MnR), and caudal raphe (CR) nuclei of the brainstem but not in hypothalamic or other regions. The specificity for serotonergic neurons within these areas has not been determined however.

2.3.3. BAC-TPH2-iCreERT2. A similar mouse line (TPH2-iCreERT2) was independently generated by B. Gloss, (http://jaxmice.jax.org/strain/016584.html). However, in this case, an improved version of the Cre gene (iCre) was employed by applying mammalian codon usage and by lowering the high CpG content of the prokaryotic coding sequence, thereby reducing the chances of epigenetic silencing in mammals.[73,74]

We have confirmed iCreERT2-mediated recombination within the DR, but not in the other brain regions using crossbreeding of this mouse line to Rosa26loxP-STOP-lacZ,[52] ROSA26loxP-stop-EYFP,[53] and Rosa26loxP-STOP-tdTomato (Jackson Laboratories, B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J Stock Number: 007914) reporter mice (Figure 1A–C). Moreover, injection of an adeno associated virus (AAV), EF1a-DIO-CheTA-EYFP, into the medial DR indicated effective Cre-mediated recombination in TPH2-iCreERT2 mice (Figure 1D). However, a more detailed analysis of the specificity and efficacy of Cre expression in this line is still pending.

In summary, a wide repertoire of genetic tools to target Cre-mediated recombination to serotonergic neurons is now available (Table 1). Pet1-Cre and SERT-Cre lines are well characterized and represent useful tools to study the serotonergic system, whereby *Sert*-driven Cre lines have higher specificity and recombination efficiency than *Pet1*-driven transgenes, as recently demonstrated in the comparative study of $VMAT^{Sert\text{-}Cre}$ and $VMAT^{Pet1\text{-}Cre}$ mice.[75] However, both promoters are not exclusively selective for the serotonergic lineage, whereas *Tph2*-driven Cre expression represents a more specific tool and should be regarded as a better model to target 5-HT-producing neurons specifically in the future. Additionally, the recent availability of the *Tph2*-driven rat Cre line[71] allowed researchers to apply the more sophisticated behavioral tests for higher cognitive functions that have been already developed for use in rats, but not in mice.

3. TARGETING DEFINED SEROTONERGIC SUBPOPULATIONS

As described in the sections above, precise targeting of a transgene to most serotonergic neurons can be performed successfully using several different mouse lines, albeit with some limitations. However, the serotonergic system itself is heterogeneous and includes various subpopulations of neurons with different connectivity, functions, and developmental origins. Manipulating these subpopulations selectively requires more advanced methods.

3.1. Dual Recombinase Strategies. Making use of newly developed genetic technologies, the group of S. Dymecki, identified rhombomere (r)-defined 5-HT sublineages that differ in gene expression with respect to progenitor and precursor cells.[66] These investigators have created a novel dual recombinase-responsive indicator allele, RC-Fela, and a Flpe recombinase driver line, ePet-Flpe. While RC-Fela serves as recombination reporter, ePet-Flpe directs recombination to Pet1-expressing 5-HT precursors. Combining RC-Fela with ePet-Flpe and Cre lines, specific for particular rhombomeres, enables isolation and manipulation of specific 5-HT neuronal subpopulations arising from these rhombomeres. For example, En1-Cre mice expressing Cre under the *engrailed 1* (*En1*) promoter were used to study the subpopulation of 5-HT neurons originating from r1. In a comparable approach, the intersectional alleles RC-FrePe and RC-FPDi were used to identify 5-HT neurons mediating breathing reflex control.[76] These triple transgenic animals provide new tools for *in vivo* manipulation of specific 5-HT subpopulations and for "functional mapping" studies.

In another study using the same selective dual recombinase strategy, Kim and colleagues directed tetanus toxin light chain (tox) synthesis to serotonergic subpopulations and thereby inhibited vesicular neurotransmission in those neurons.[77] By recombinase expression under *Pet-1* and *En1* promoters in En1-Cre, ePet1-Flpe, RC-PFtox mice, they targeted the subpopulation of Pet1-descendant 5-HT neurons arising from serotonergic progenitors in r1. Tox action was thereby restricted to cells expressing both Cre and Flpe recombinases and required two recombination events. Using the described dual-recombinase technique, higher specificity (targeting of individual serotonergic subgroups) was achieved when compared with single-recombinase or conventional transgenic methodologies.

3.2. Viral Mediated Gene Transfer. An alternative to transgenic approaches is virus-mediated gene transfer into specific brain structures. Spatial control of vector delivery can be achieved by using the appropriate stereotaxic injection coordinates.

Adenoviruses were among the first viruses to be successfully used for the specific infection of DR.[78] In this approach, stereotaxic injection of a Cre-expressing adenovirus (AdCre)[79] into the DR of a Cre-dependent LacZ reporter mouse line CAG-LoxP-stop-LacZ[80] led to the expression of Cre recombinase in approximately 12% of serotonergic neurons. Within 9 days after the injection, 43% of Cre-expressing 5-HT neurons were also positive for LacZ, confirming the validity of the method to target Cre-recombination throughout the DR.

Lentiviruses have also been applied to label serotonergic neurons. To drive the expression of the EGFP exclusively in serotonergic neurons, an optimized *Tph2* promoter with a two-step transcriptional amplification design was employed.[81] Stereotactic injections of such viruses to medulla oblongata covering some of the midline and ventral raphe led to EGFP expression that was 97% colocalized with TPH expression. Of particular importance is the ability of lentiviruses to stably integrate into the genome of nondividing cells, which is useful for lineage tracing.

Meanwhile more advanced techniques combining AAV and transgenic approaches have been developed to enable highly selective transgene expression. For example, by injecting a double-floxed AAV with an inverse open reading frame for a channelrhodopsin2-mCherry fusion protein (AAV2 DIO ChR2-mCherry) into the raphe obscurus (RO) of ePet-Cre mice, DePuy and colleagues showed that roughly half of 5-HT neurons in the raphe area were infected and 97% of infected cells were serotonergic.[82] Recently, sindbis virus, canine adenovirus serotype 2 (CAV2), and rabies viruses have been developed for anterograde, retrograde, and transsynaptic retrograde transport, respectively, and used to study serotonergic circuitry.[83−90]

3.2.1. Anterograde Transport. Suzuki et al. injected a GFP-tagged sindbis virus into the DR for anterograde tracing of single serotonergic neurons to the olfactory bulb.[87] The

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advantage of using sindbis virus is its high transfection efficiency in the CNS and its capacity to carry large amounts of genetic material. However, this virus is suitable only for acute *in vivo* work as infected neurons start degenerating 7 days after viral injection.[91]

3.2.2. Retrograde Transport. For retrograde tracing studies, Wu et al. injected retrogradely transported CAV2-Cre virus into the Nucleus tractus solitarii (NTS) of conditional TPH2$^{lox/lox}$ mice.[90] Upon transport from the injection site to the cell bodies, Cre inactivates the *Tph2* gene, specifically in neurons with projections to the injected area. TPH2 staining was decreased in RO and RM serotonergic neurons by 60−80%, while DR neurons were unaffected. As a result, 5-HT levels in the NTS of injected TPH2$^{lox/lox}$ mice were decreased by about 75%. In combination with ablation of specific neuropeptide-expressing neurons, this study allowed elucidation of the serotonergic mechanisms of feeding and body weight regulation.[90]

Interestingly, many AAVs, though not all, show a robust capacity for retrograde infection and subsequent transgene expression in mice. To isolate neuromodulatory serotonergic neurons that send axons to the basolateral amygdala (BLA), Rothermel et al.[85] used recombinant, pseudotyped AAVs (rAAVs) engineered to improve transduction efficiency and tissue tropism using capsid genes from other AAV serotypes (e.g., AAV2/1). The applied virus carried a Cre-dependent expression switch (2/9.DIO.ChR2-EYFP) to allow ChR2-EYFP expression only in Cre-positive cells. Stereotaxic injection into the BLA of SERT-Cre mice resulted in ChR2-EYFP expression in a number of raphe neurons, mainly in the DR.

3.2.3. Transsynaptic Transport. To analyze monosynaptic inputs to DR and MnR serotonergic neurons, Ogawa and colleagues used a retrograde transsynaptic system based on a modified rabies virus that initially infects only cognate receptor (TVA protein) expressing cells and lacks a glycoprotein required for transsynaptic spread.[83] SERT-Cre mice[50] were injected with two helper viruses expressing the glycoprotein and TVA after Cre recombination into either DR or MnR.[88] This allowed the virus to spread transsynaptically to cells with direct synaptic contact to DR or MnR serotonergic neurons. Here, 95.8% of infected TVA expressing cells were shown to be serotonergic and 30.5% of 5-HT neurons were infected and expressed TVA. A small number of neighboring 5-HT-containing neurons were targeted as well.[83] A rabies virus tracing strategy in SERT-Cre mice was also used in other studies to generate a whole-brain atlas of putative monosynaptic inputs onto forebrain-projecting DR and MnR serotonergic neurons or to characterize the distinct excitatory, inhibitory, and peptidergic inputs to the DR.[84,86,89] However, released rabies virus can be taken up by adjacent terminals that may or may not be synaptically connected.[92] Classification of connectivity patterns will permit further functional dissection of the serotonergic system and enable analysis of the physiological relevance of specific input circuits.

Taken together, viral delivery entails some disadvantages in comparison to transgenic animals such as postoperative recovery, limited injection volumes, and challenges associated with injection accuracy. Moreover, adeno[93−97] and sindbis[91] viruses might evoke immune responses and inflammation. The regular serotypes (1−10) of AAV cause minimal *in vivo* immune response,[98] but some may cause gliosis[99] and neurological deficits. Lentiviruses are more conservative in this respect and pose less of a risk of CNS damage. As an advantage viruses permit much higher expression levels of transgenes and retrograde transport enabling labeling and manipulation of neuronal subpopulations defined by their axonal terminals. Furthermore, viral mediated transgene expression represents a useful tool to deliver light-sensitive proteins for optogenetic approaches (see below). Though conventional viral vectors cannot incorporate very large transcriptional control units similar to BACs, they are optimal for the delivery of small transgenes such as Cre recombinase or Cre-dependent expression cassettes. Due to the ease and low cost of viral production in comparison to transgenic animals, viral methodology allows facile implementation of new genetic tools into experimental designs.

4. TEMPORAL CONTROL OF GENE EXPRESSION WITHIN THE SEROTONERGIC SYSTEM

4.1. Inducible Cre-Expressing Mouse Lines. Performing a genetic manipulation at a selected time point during the lifetime allows the study of age-specific biochemical and behavioral effects within the serotonergic system. Several inducible Cre lines that enable excision or activation of genes at chosen time points within the serotonergic system are described in the sections above[49,68,70,71,100] and are summarized in Table 1. However, these are not without limitations. Recombination mediated by Cre or other recombinases is an irreversible and relatively slow process. Furthermore, repeated TMX injections are necessary to permit drug delivery to all serotonergic neurons and to induce appropriate recombination rates. While TMX is FDA (U.S. Food and Drug Administration) approved, administration at doses higher than 150 mg/kg has been shown to evoke toxic effects in mice.[101,102] Moreover, it should be taken into account that TMX is a strong estrogen receptor antagonist. Therefore, minimal doses of TMX should be used. Moreover, recent studies demonstrated that systemic activation of CreERT2 might elicit hematological toxicity.[101,102] Therefore, use of proper controls, such as Cre^{+}GOI$^{wt/wt}$ + TMX is essential.

4.2. Tetracycline-Sensitive Systems. Audero et al. suppressed serotonergic firing by overexpressing 5-HT1A autoreceptors reversibly in transgenic mice,[103] using the tetracycline-Off (tet-Off) system.[104,105] Double transgenic mice were generated by breeding mice carrying the *tet*-promoter (Ptet) inserted upstream of the endogenous 5-HT1A receptor gene (*5-Htr1a*^{tetO} allele) with mice encoding the tetracycline transactivator (tTA) inserted downstream the ATG codon of the endogenous *Slc6A4* (gene coding for SERT). tTA is an artificial transcription factor, that binds to Ptet, which in turn, mediates the overexpression of a GOI, in this case the *5-Htr1a.* Administration of Doxycycline (Dox, a stable tetracycline analogue) in this model leads to the prevention of transactivation of 5-HT1A receptors and, therefore, allows temporal control of their expression. Although 5-HT specificity is based on the *Sert* promoter, which might be also active in nonserotonergic cells, 5-HT1A receptor overexpression in double-transgenic animals is restricted to raphe nuclei of the mid- and hindbrain. However, it has not been determined whether the affected cells are all serotonergic.

Weber et al. designed a comparable ON/OFF system for genetic manipulations in 5-HT neurons.[106] Similar to Audero and colleagues, they generated double-transgenic mice encoding tTA under the *Tph2* promoter and a nLacZ reporter under the control of the-tet-response system (TPH2-tTA/Ptet-nLacZ mice). Dual-label fluorescence immunohistochemistry

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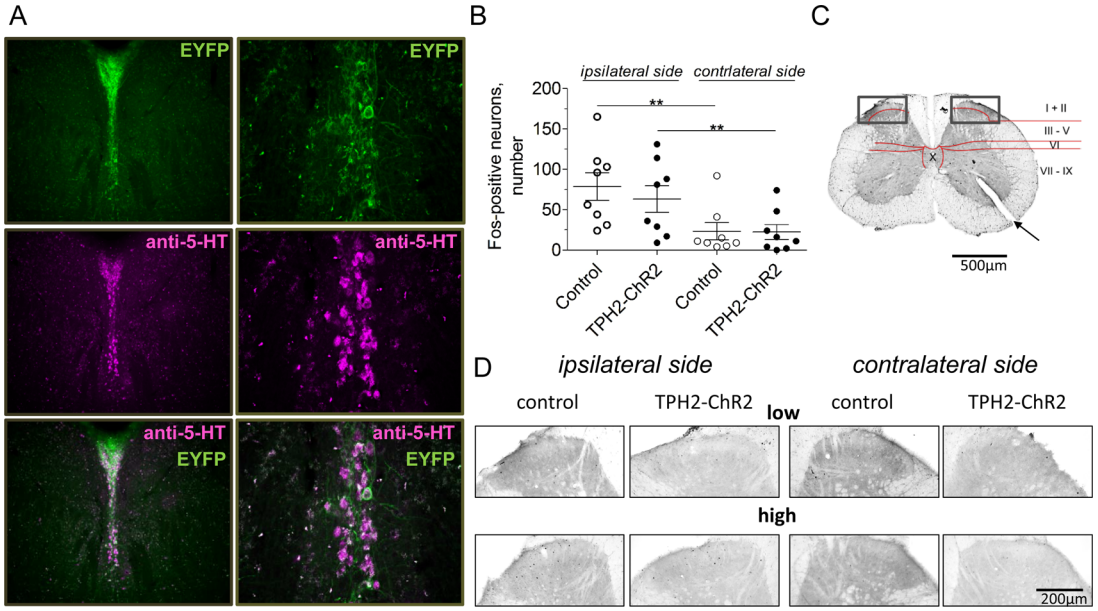

Figure 2. TPH2-ChR2-EYFP mouse as a model for the study of serotonergic circuitry. (A) Specificity of EYFP expression controlled by the *Tph2* promoter: immunohistochemical analysis with anti-5-HT antibodies in dorsal raphe nuclei in TPH2-ChR2 mouse (10× (left panel) and 40× (right panel) magnification). Neuronal activation in lamina I/II of the dorsal horn of the spinal cord was investigated through analysis of c-Fos expression (as previously described in ref 112) following application of a series of noxious pinch stimuli (pneumatic pincher, 2.5 N × 3) of the left hindpaw in control and TPH2-ChR2-EYFP mice during optogenetic stimulation (10 ms ON, 10 ms OFF, 20 s, 20 mV) in the dorsolateral dorsal raphe nucleus (coordinates from lambda, 0.3 mm ML, −1 mm AP, −3.4 mm DV). (B) In both TPH2-ChR2 and control mice, higher numbers of c-Fos positive neurons were observed ipsilateral to the pinch stimulus compared with the contralateral side. No significant difference was observed between control and stimulated animals; however a large variance in the number of c-Fos positive neurons was observed in both groups of animals. $n = 8$ per group. $^{**}p < 0.01$ compared with contralateral side of the animals of the same genotype, Wilcoxon matched-pairs signed rank test. (C) Transverse spinal cord section with lamina boundaries indicated. Black arrow indicates an incision made prior to sectioning to mark the contralateral side of the cord. Boxed areas indicate regions displayed at higher magnification in part D. (D) Representative images of spinal cord sections from control and TPH2-ChR2-EYFP animals that displayed high or low counts of c-Fos positive dorsal horn neurons.

identified LacZ expressing cells as serotonergic. Extraserotonergic brain regions were largely devoid of X-Gal staining, except for sparse β-Gal activity in the hypothalamus and beneath the aqueduct. Additionally, in the raphe, a few TPH2-negative cells were β-Gal positive, revealing some nonspecific gene targeting. Dox administration completely suppressed Ptet-mediated reporter expression and subsequent Dox withdrawal resulted in reactivation.

When 5-HT-specific tTA mice are mated with Ptet-Cre/loxP-flanked target gene animals, spatially controlled, inducible gene deletion (TPH2-tTA/Ptet-Cre/loxP) can be achieved, comparable to TMX-inducible CreERT2 systems. In addition to the spatial control by the chosen promoter, temporal control of gene expression is possible with the described tet-Off methodologies. Unlike the Cre/loxP system, gene manipulations are reversible. However, Ptet-controlled gene expression after Dox withdrawal occurs with a delay, depending on the animal's age, Dox concentration, duration of treatment, and many other factors that have to be controlled for. In contrast to i.p. administration of TMX, Dox treatment is more suitable for behavior studies as it can be added to the drinking water diminishing effect of stress. Since Dox has a bitter taste, sugar or sweeteners are often added to Dox solutions that may lead to an increase in drinking volume, additional body weight gain, or other unexpected behavioral outcomes.

4.3. DREADDs. A different method for efficient modulation of signal transduction in selected neurons is the introduction of designer receptors exclusively activated by designer drugs (DREADD), engineered G-protein-coupled receptors that are activated by otherwise inert synthetic ligands. During recent years, several modifications of this technique have been used for *in vitro* and *in vivo* experiments.[107−109] The example below made use of a Gi-DREADD (Di), a synthetic Gi/o-protein−coupled receptor that activates inwardly rectifying potassium channels upon stimulation with the compound clozapine-N-oxide (CNO) resulting in hyperpolarization and transient neuronal silencing.[110]

To study the influence of serotonergic activity on homeostatic control, Di was expressed via previously described dual-recombinase methodology with cell-subtype precision.[66,77,109] A combination of the single recombinase-responsive allele RC-PDi, responsive to Cre, with SERT-Cre was used to express Di in nearly all serotonergic neurons. These cells responded to CNO with hyperpolarization that led to the suppression of cell excitability. Furthermore, incorporation of a Cre-inducible reporter allele, RC-rePe, allowed visualization of Di expressing cells by GFP. Beyond serotonergic neurons, a population of extraserotonergic neurons in the thalamus was shown to express Di as well, due to presence of SERT-positive cells in this brain region. Current-clamp membrane potential recordings from cultured serotonergic neurons of the lower brainstem revealed a ∼40% reversible reduction in firing rates in Di expressing neurons after CNO exposure. Administration of CNO to transgenic animals resulted in rapid attenuation of chemoreflex, as well as a decrease in core body temperature representing vital physiological functions of 5-HT.[109] DREADDs enable ligand-inducible inhibition of SERT-expressing neurons that is reversible within hours, and serves

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as model for functional mapping of neurons. CNO-mediated inhibition happens within a shorter time frame as compared with Dox- or TMX-inducible systems. At the same time, inertness of CNO has been questioned by others due to its redox partners and their biological effects.[111] If CNO is not effectively eliminated by the kidneys, it can be reduced to the tertiary amine clozapine *in vivo*.[112,113] Various tertiary amine reduction routes generate compounds that efficiently act as agonists for DREADDs and a number of other receptors. In turn, receptor-mediated intracellular signaling pathways may be activated and impact on animal physiology.[114] These other possible actions of CNO metabolites are capable of reducing designer receptor−based cell and effect specificity and should be excluded by the use of appropriate controls. As another word of caution, all G-protein coupled receptors when artificially expressed at high levels are able to produce downstream coupling events without ligand presence. Various groups, including ourselves have observed this with DREADDs (S. Kasparov, unpublished observations). Therefore, it is critical to control for changes in cellular biochemistry or physiology in animals expressing DREADDs before CNO application.

4.4. Optogenetics. Optogenetics is another current technique to modulate neuronal function that does not involve chemical ligands and enables much higher temporal resolution.[115] Optogenetics enables researchers to manipulate firing rates of specific neuronal populations and to measure the outcome using physiological and behavioral readouts *in vivo*. For tight optical control of neuronal excitability, light-sensitive channels or light-driven ion pumps are expressed in genetically defined neurons. The most commonly used optogenetic actuator is the nonselective cation channel channelrhodopsin, ChR2, and its derivatives. Using BAC technology, Zhao and colleagues generated a mouse line expressing ChR2 driven by the *Tph2* promoter, TPH2-ChR2-EYFP.[116] Co-localization of the reporter EYFP with 5-HT was shown for serotonergic neurons in the DR (ref 116 and Figure 2A). Transgene expression was moderate in MnR and DR as approximately 15% of TPH2-positive cells were EYFP-negative.[116] Electrophysiological recordings from the EYFP-positive neurons showed responsiveness to blue light. It is important to stress that ChR2 and similar proteins have to be expressed in the target cells at fairly high levels to enable efficient control of excitability. We employed this mouse model to study the impact of descending 5-HT-ergic control on sensory transmission in the spinal cord, based on previous studies that revealed a dual role for 5-HT in inhibition and facilitation of nociceptive input.[117−120] These earlier studies relied mainly on gross lesions, broadly acting pharmacological manipulations, and electrical stimulation of the DR, techniques with obvious limitations such as compensatory rewiring of the pathways, impact on the release of other neuromodulators,[121,122] and coactivation of non-5-HT neurons and fibers of passage.[123] In theory, optogenetic stimulation of DR 5-HT neurons should help to overcome these limitations.[124] In anaesthetized TPH2-ChR-EYFP mice, optogenetic stimulation of 5-HT neurons in dorsolateral DR (coordinates from lambda: 0.3 mm ML, −1 mm AP, −3.4 mm DV; blue light stimulation: 10 ms ON, 10 ms OFF, 20 s, 20 mV) did not significantly alter the expression of c-Fos, a marker of neuronal excitability,[125] in the dorsal horn of the spinal cord (Figure 2B), which serves as an index of the nociceptive input.[126] Despite the refinement of the targeting techniques to specifically excite DR 5-HT neurons, the impact of serotonergic innervation of the spinal cord on nociceptive transmission remains ambiguous. Our negative finding might also indicate that different 5-HT subpopulations (that we could have stimulated simultaneously) have opposing effects on the nociceptive control so as to cancel each other out.

To refine the spatial specificity, mouse lines expressing Cre recombinase under cell-specific promoters, in combination with viral delivery can also be used for optogenetic experiments. Such an approach was used in the study by Dugue et al., where viral vectors were injected into transgenic SERT-Cre mice (described above).[127] In this study, response thresholds in awake behaving SERT-Cre mice following injection of viruses carrying Cre-dependent ChR2-EYFP into the DR (coordinates from bregma: 0 mm ML, 4.4 to −4.7 mm AP, −2.8 to −2.9 mm DV, with a 34° caudal angle) were higher compared with control mice in the von Frey assay, a simple test of mechanosensitivity.[128] The work of Dugue et al.[127] and our studies (Figure 2B-D) show that the response might differ between anesthetized and conscious animals, and that the roles of different serotonergic subpopulations may not be identical.

Delivery of light-sensitive channels is possible by combining the above-described mouse lines expressing Cre under cell-type specific promoters with recently developed mouse lines expressing various opsins in a Cre-dependent manner.[129] However, due to the heterogeneity of the serotonergic system, this will not allow precise targeting of particular subpopulations of 5-HT neurons.

Finally, since rat transgenics are much less developed, injection of viral vectors with optogenetic actuators (either direct targeting with *Tph2* promoter[130] or a combination of TPH2-Cre with a Cre-dependent vector) may be used in this species.

In any case, using blue light for activation of optogenetic constructs requires careful controls for the effects of temperature and blue light itself. For example, we recently reported that blue light induces activation-like signals in functional magnetic resonance imaging experiments probably due to the local heating.[131] Control for the effects of blue light has become a particular issue since the recent demonstration of the vasomotor effects mediated by melanopsin in peripheral blood vessels.[132] Development of more sensitive optogenetic tools that can be activated by application of low intensity light[133−136] will help to overcome these concerns.

The techniques discussed in this section allow manipulations of specific neuronal populations in freely behaving animals with improved spatial and temporal precision. Optogenetics and DREADDs both involve the introduction of non-native proteins into neurons that thereby respond to defined stimuli (light or CNO) with altered excitability. For the analysis of temporal dynamics of specific circuits, optogenetics must be used, whereby an optical fiber needs to be implanted and manipulations can only affect the illuminated area in the brain. In contrast, by a simple systemic CNO administration all DREADDs distributed within large tissue volumes can be stimulated simultaneously. Likewise, the tet-Off system allows gene expression control in all tissues expressing the chosen promotor. In all cases, unknown side effects of chemical agent administration or blue light have to be considered.[101,102,131,132,137−139]

5. SUMMARY

Serotonergic neurons in the raphe nuclei give rise to a complex and extensive network of axonal projections throughout the whole brain. A major challenge in the analysis of these circuits

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is to understand how the serotonergic networks are linked to the numerous functions of this neurotransmitter. Several new methodologies to manipulate subpopulations of serotonergic neurons and to control 5-HT neuron activity have been developed during recent years, including the generation of different serotonergic-neuron-specific Cre lines, the invention of a dual recombination strategy, effective tools for temporal control of gene expression, DREADDs, and optogenetics. The nature of the experimental question dictates which option should be chosen as each has different merits and limitations. Importantly, since none of the described systems is totally free of nonspecific effects. For the most accurate and informative interpretation of results, adequate controls should be incorporated into experimental designs. Nonetheless, these new tools and methods, alone and in combination, enable powerful new routes to investigate the serotonin system.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

5-HT, 5-hydroxytryptamine; AAV, adeno-associated viral vector; BAC, bacterial artificial chromosome; BLA, basolateral amygdala; CAV2, canine adenovirus serotype 2; ChR2, channelrhodopsin 2; CNO, clozapine-*N*-oxide; CNS, central nervous system; CR, caudal raphe; Di, synthetic Gi/o-protein coupled receptor (DREADD); Dox, doxycycline; DR, dorsal raphe; DREADD, designer receptors exclusively activated by designer drugs; En1, engrailed 1; Flp, flippase; GOI, gene of interest; IHC, immunohistochemistry; *LacZ*, gene coding for β-galactosidase; Lmx1b, LIM homeobox transcription factor 1; NTS, nucleus tractus solitarii; MnR, median raphe; ObRb, leptin receptor b; LepRb, leptin receptor b; Pet1, PC12 ETS domain-containing transcription factor 1; *Ptet*, Tet-promoter; r, rhombomere; RM, raphe magnus; RO, raphe obscurus; SERT, serotonin transporter; SSRI, serotonin reuptake inhibitor; Tet, tetracyclin; TMX, tamoxifen; Tox, tetanus toxin light chain; TPH2, tryptophan hydroxylase 2; tTA, tetracycline trans-activator; VMAT, vesicular monoamine transporter; β-Gal, β-galactosidase; DT, diphteria toxin; TVA protein, cognate receptor

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