

# Optogenetics: illuminating the neural bases of rodent behavior

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**Abstract:** In vivo optogenetics has provided researchers with the ability to delve deeper into the neural basis of behavior by driving cell-type specific circuit connections within and between brain regions. The diverse toolbox available for circuit- and cell-specific manipulations is ever growing. Using these tools in conjunction with established and novel genetic and behavioral methods, neuroscience research has experienced an explosion in the understanding of the roles of specific cell subtypes in behavior. This review aims to outline recent advances in in vivo optogenetic tools for manipulation of behavior related to movement, pain and sensation, motivation, reward, emotion, learning, sleep, and epilepsy.

**Keywords:** in vivo optogenetics, behavior, neural circuits, transgenics, viral constructs

## Introduction

Optogenetics, the integration of optics and genetics to manipulate cellular functions and activity, has been in use for nearly 10 years and its utility in neuroscience research is ever increasing. Neuroscientists initially used naturally occurring microbial opsins and later mutated versions of these opsins to alter the flux of ions across membranes to drive cell activity.<sup>1-3</sup> Microbial opsins originally from archaeobacteria<sup>2,3</sup> or algae,<sup>1,3,4</sup> contain a ligand binding site that is sensitive to distinct wavelengths of light and insensitive to chemical ligands. Through a series of selective and non-selective mutations, a large library of effective, non-toxic, temporal, and wavelength specific optogenetic constructs have been generated.<sup>3,5</sup> The versatility of the available optogenetic constructs allows use in a widevariety of applications including in vivo behavioral manipulation.

The most commonly used membrane-bound optogenetic constructs fall under two major classes: 1) stimulatory/depolarizing opsins, often light-driven cation channels (channelrhodopsin; ChR2), and 2) inhibitory/hyperpolarizing opsins, light-driven chloride (halorhodopsin or *Natronomonas pharaonis*; NpHR) or proton (bacteriorhodopsin) pumps. However, other opsin constructs have emerged from the wealth of information about opsin mechanisms including light-driven constructs to regulate G-protein mediated signaling,<sup>6-8</sup> intracellular signaling molecules,<sup>6,9-13</sup> and gene expression.<sup>14,15</sup> Microbial opsins have been incorporated in combination with numerous mouse transgenic technologies and viral constructs. These tools and constructs are utilized in vivo and ex vivo to drive specific cell subtypes in a region and temporal specific manner. Optogenetic manipulation is currently used in neuroscience to understand temporal and activity specific alterations in neuronal circuits, circuit connectivity, signaling mechanisms, and gene regulation that underlie behavior. With new and

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emerging technologies, the utility of optogenetic methods is ever growing. In this review, we highlight recent advances in optogenetic tools and constructs with a particular focus on its uses in behavioral systems.

## Techniques and technological advances

A decade of optogenetic research has assembled simple, reliable, and effective means for opsin expression and light activated manipulation of neuronal activity in vivo. Opsin constructs are expressed in the central nervous system (CNS) with the use of viral vectors in wild-type or transgenic rodent models (Table 1). Specific control over particular circuits and neuronal populations within a brain region is achieved by implanting optic fibers, which are attached to a light source.<sup>16–18</sup> Additionally, researchers have developed minimally invasive and wireless technologies for

in vivo optogenetics.<sup>19,20</sup> Further, optogenetics coupled with in vivo recording or calcium imaging has the potential to create the ability to analyze temporally specific single-cell and circuit level function within awake and behaving animals.

Viral and transgenic technological advances have paved the way for innovative targeting of opsin expression. Two major strategies are used to mediate opsin expression in the CNS: 1) direct opsin transgene expression, driven by a specific promoter in rodents, or 2) indirect expression of the opsin with a secondary promoter-driven element, such as Cre recombinase. These strategies have been used with both viral constructs and transgenic<sup>3</sup> and knock-in rodent lines.<sup>21</sup> Some of the first optogenetic studies utilized transgenic animals to express ChR2 or NpHR using neuron-specific promoters.<sup>22,23</sup> Use of rodent transgenics and knock-in technology to express opsins in neurons has the selective advantage of spatial homogeneity and abundant opsin expression and this technology

**Table 1** Transgenic mouse lines used for optogenetic manipulation of behavior

	Promoter	Expression (cell type)	Opsin	Behavioral uses
Transgenic lines	<i>nNOS</i>	Molecular layer neurons (cerebellum)	ChR2(H134R)	Motor function <sup>80</sup>
	<i>Thy1</i>	Glutamatergic	ChR2(H134R)	Motor function, <sup>22,79</sup> anxiety <sup>116</sup>
Rodent lines +	<i>AGRP-Cre</i>	AGRP-expressing	ChR2(H134R)	Feeding <sup>135</sup>
Cre-inducible	<i>CamKII<math>\alpha</math>-Cre</i>	Glutamatergic	ChR2(H134R)	Anxiety <sup>118</sup>
AAVs			Arch	Anxiety <sup>118</sup>
	<i>c-fos-tTA</i>	Activated by c-fos induction	TRE-ChR2	Fear learning <sup>37,38</sup>
	<i>ChAT-Cre*</i>	Cholinergic	eNpHR3.0	Reward <sup>93</sup>
	<i>DAT-Cre</i>	Dopamine	ChR2(H134R)	Reward <sup>100</sup>
	<i>D1-Cre, D2-Cre</i>	Dopamine receptor expressing (MSN subtypes in the striatum)	ChR2(H134R)	Motor function, <sup>78</sup> motivation, <sup>91,125</sup> reward, <sup>82,84,85,87,89</sup> Feeding <sup>54</sup>
			eNpHR3.0	Reward, <sup>89</sup> motivation, <sup>125</sup> anxiety <sup>139</sup>
			ChR2(E123A)	Mood <sup>92</sup>
			ChR2(H134R)	Anxiety/motivation <sup>128</sup>
			ChR2(H134R)	Motivation, <sup>109</sup> reward <sup>111</sup>
			Vertebrate Melanopsin-5-HT <sub>2C</sub>	Anxiety <sup>7</sup>
			Arch	Anxiety <sup>119</sup>
			ChR2(H134R)	Aggression <sup>153</sup>
			ChR2(H134R)	Learning <sup>149</sup>
			Vertebrate Rhodopsin	Motor function <sup>58</sup>
			Vertebrate Melanopsin-5HT <sub>2C</sub>	Anxiety <sup>7</sup>
			ChR2(H134R)	Learning <sup>148</sup>
			eNpHR3.0	Learning <sup>148</sup>
			C1V1	Anxiety <sup>120</sup>
			ChR2(H134R)	Reward, <sup>122</sup> epilepsy <sup>73</sup>
			ChR2(H134R)	Reward/pain <sup>162</sup>
			ChR2(H134R)	Reward, <sup>30,98,99,102,113,139</sup> motivation, <sup>97</sup> mood, <sup>40,41,107</sup> sleep <sup>157</sup>
			eNpHR3.0	Mood, <sup>40,107</sup> sleep <sup>157</sup>
			ChR2(H134R)	Reward <sup>110,136</sup>
			eNpHR3.0	Reward <sup>136</sup>
			ChR2(H134R)	Anxiety/reward <sup>136</sup>

**Notes:** Transgenic lines that have been used in mouse and rat studies are denoted by an asterisk (\*). Knock-in lines are denoted by a hashtag (#) and all lines not denoted by this symbol are transgenic lines.

**Abbreviations:** AAVs, adeno-associated viruses; AGRP, agouti-related peptide; tTA, tetracycline transactivator; D1, Dopamine 1; D2, Dopamine 2; IRES, internal ribosome entry site; POMC, pro-opiomelanocortin; Rgs9, regulator of G-protein signaling 9; MSN, medium spiny neuron; nNOS, neural nitric oxide synthase; PV, parvalbumin; TH, tyrosine hydroxylase; GABA, gamma-aminobutyric acid; DG, dentate gyrus; TRE, tetracycline response element; ChR2, channelrhodopsin 2; eNpHR3.0, enhanced third generation *Natronomonas pharaonis*.

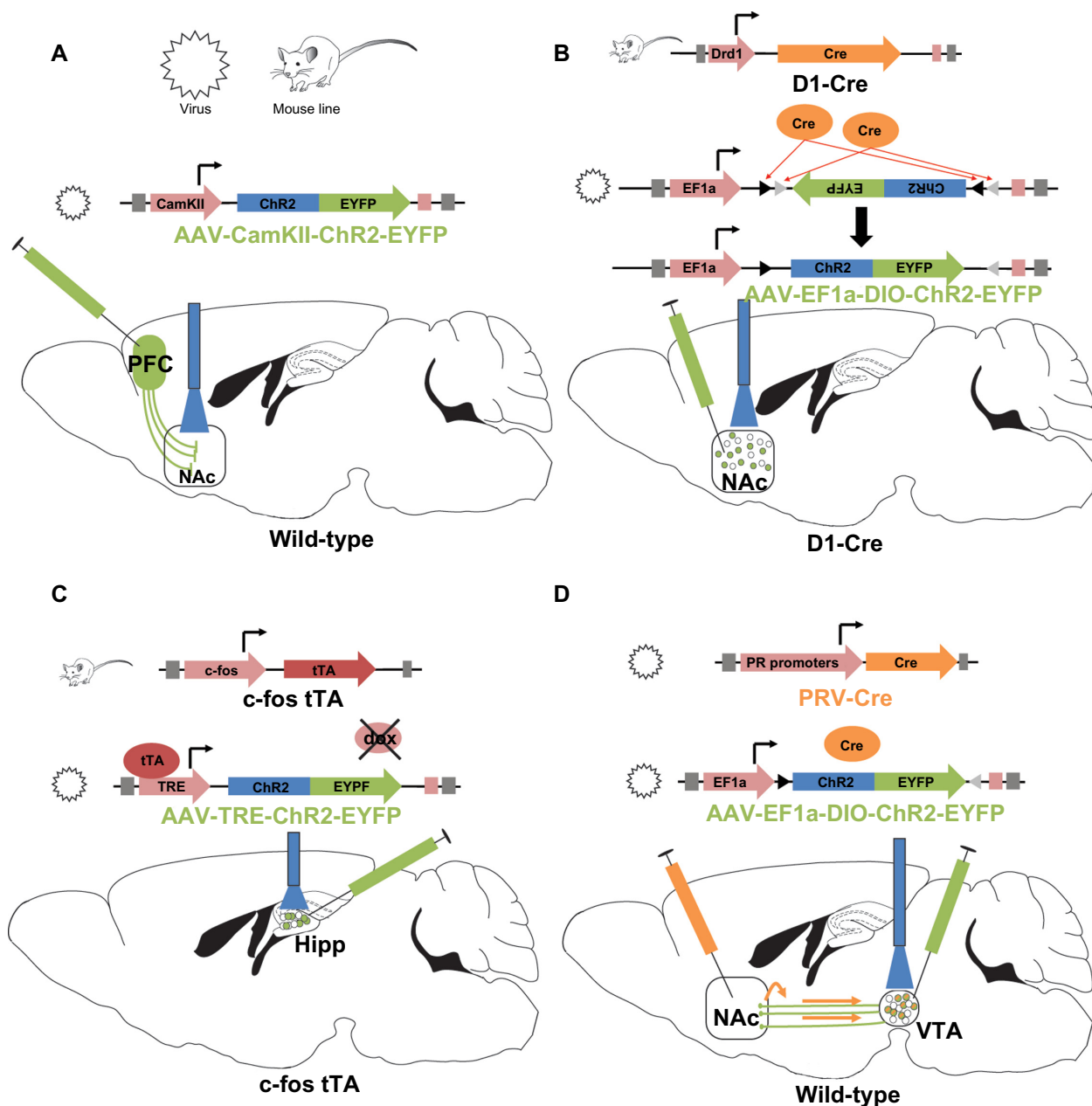
was successfully used in a number of studies in a variety of cell subtypes.<sup>24–27</sup> Knock-in mice, in particular, promote endogenous expression patterns which regulate the magnitude of opsin expression. Further, opsins expressed in genetic models have early onset expression which is useful in developmental studies.<sup>28</sup> Bacterial artificial chromosome (BAC) transgenic or knock-in lines have been widely successful for expressing Cre or opsins using numerous cell-type specific promoters. This success stems from the large genetic capacity of BAC clones (~150 kilobases), which contain many of the genetic elements necessary to drive cell-specific expression or in the case of knock-ins having the opsin driven under the endogenous promoter and regulatory elements.<sup>21,29–35</sup>

Viral systems are widely used in optogenetic studies. Adeno-associated viruses (AAVs) or lentiviruses driven by neuron-specific promoters are injected directly in the brain region of interest (Figure 1A). Since opsins infect somas, dendrites, and axons, axonal terminals can be targeted in brain regions down-stream of the injection site. Indirect, binary expression systems, such as Cre-driven systems, provide high specificity for cell subtypes and permit the use of multiple viral constructs.<sup>3</sup> One commonly used viral Cre system utilizes a double inverted open (DIO) reading frame construct to express opsins in a cell-type specific manner (Figure 1B). In a DIO system, the non-coding, opsin gene, in reverse, is flanked by two pairs of distinct lox sites specifically recognized and cleaved by Cre recombinase. In the presence of Cre, splicing occurs between one set of lox sites causing the gene to flip into the correct coding orientation. A second splicing event leads to the removal of two lox sites leaving the two incompatible lox sites, which prevents the gene from flipping back into the non-coding reverse direction, thus allowing expression of the opsin. The DIO opsin construct is often delivered via an AAV injection into a Cre expressing animal or in conjunction with a Cre expressing virus.<sup>3</sup> Other binary expression systems, such as the tetracycline regulatory system, have also successfully been used in optogenetic studies.<sup>36–38</sup> For instance, studies examining a fear memory engram, induced by fear conditioning, used a transgenic mouse line expressing a c-Fos specific promoter coupled to a gene encoding tetracycline transactivator (tTA) in the hippocampus to selectively target activated cells with ChR2 (Figure 1C).<sup>37,38</sup> Doxycycline blocks the activity of tTA and when animals no longer receive doxycycline, ChR2 is expressed in c-Fos positive neurons using a tTA-ChR2-EYFP virus.

Trans-synaptic constructs to target opsins to presynaptic axonal afferents and upstream brain regions have been

successfully used in optogenetic studies. A trans-neuronal retrograde pseudorabies virus<sup>39–41</sup> or an AAV serotype that will infect terminals is used for this purpose.<sup>42</sup> Retrograde Cre viruses are injected into the terminal site of specific projection neurons while a Cre-inducible opsin virus is targeted to the cell body region of the projection neurons (Figure 1D).<sup>40,41</sup> Additionally, a pseudorabies virus-ChR2 approach was recently used to dissect the role of lateral habenula (LHb) and laterodorsal tegmentum inputs to the ventral tegmental area (VTA) in motivational behavior.<sup>39</sup> More recently, herpes simplex virus or canine adenoviruses were utilized to deliver Cre to afferent axons and brain regions to allow for subsequent injection of opsin vectors in upstream cell bodies.<sup>24,43</sup> These viruses provide a novel way of localizing and dissecting region specific afferent activity into a brain area of interest.

Temporal precision, light response reliability, wave-length specificity, kinetics, and hyperpolarization/depolarization characteristics have all led to the development of a variety of opsins (see Mattis et al<sup>44</sup> and Yizhar et al<sup>3</sup> for a more detailed comparison of opsins). ChR2(H134R), activated with 473 nm light, is the most commonly used ChR2 variant (Tables 1 and 2). Fast kinetics, glutamate mutated opsins such as ChR2(E123A), also known as ChETA<sub>A</sub>, have been developed for high fidelity at higher frequency photo-activation,<sup>35,44,45</sup> while other constructs, such as the bistable step function opsins, were developed and selected for their slow open channel kinetics and long-lasting depolarization.<sup>3,5,46</sup> Because heating of tissue can be an issue,<sup>47</sup> step function opsins hold the selective advantage of activation with a short pulse of light, reducing the potential for tissue damage or unwanted artifacts.<sup>46</sup> Red-shifted (590–630 nm) opsins can be combined with blue light activated opsins to control different neuronal subtypes in the same animal.<sup>3</sup> Recently, a new red-shifted ChR2, ReaChR, was developed for deep transcranial stimulation of neurons without the need of skull thinning, optical windows, or implantable fibers.<sup>5,20</sup> Inhibitory opsins, (halorhodopsins) or protons, (bacteriorhodopsins), are designed to pass anions. Halorhodopsins such as NpHR, and the enhanced third generation NpHR (eNpHR3.0), pass chloride when activated with 590 nm light.<sup>3,48,49</sup> The newer generation archeorhodopsin, ArchT (activated by 575 nm green/yellow light) is a useful alternative to halorhodopsin. ArchT has the added benefit of reduced post-inhibitory spiking following inhibition in comparison to eNpHR3.0 induced inhibition.<sup>49</sup> Recently, based on structural analysis of ChR2,<sup>50</sup> chloride-conducting ChR2 was developed for equivalent temporally precise inhibition at moderate light



**Figure 1** Common schemes utilized for optogenetic manipulation of neuronal subtypes.

**Notes:** (A–D) Genetic opsin constructs are flanked by ITRs (gray box) and followed by an enhancer sequence (pink box). Promoters (pink arrow) drive cell-type specific expression. The ChR2 constructs (blue rectangle) are fused to an enhanced yellow fluorescent protein (EYFP; green arrow) for simple visualization under a microscope for electrophysiological recording or later immunohistochemical analysis. (A) An adeno-associated virus (AAV) containing ChR2 on a CamKII promoter infects excitatory neurons of prefrontal cortex (PFC) and travels down their axons, allowing for terminal stimulation in the nucleus accumbens (NAc). (B) An AAV containing a double inverted open (DIO) ChR2 construct is injected into the NAc of a mouse expressing Cre in Dopamine 1 (D1) receptor medium spiny neurons (MSNs). The opsin is double floxed by distinct flox sites (black and gray triangles). These two sites are recognized by Cre and the gene is spliced out, inverted, and re-ligated expressing ChR2 selectively in D1-MSNs. (C) A tetracycline (“Tet”) on (“Tet-off”) activated ChR2 is injected into a transgenic mouse that expresses a tet trans-activator (tTA) in response to c-fos activation. ChR2 is transcribed exclusively in the presence of c-fos-promoted tTA transcription when doxycycline (dox), an inactivator of tTA, is not present. With this strategy, previously active cells are optogenetically activated. (D) A pseudorabies virus (PRV) expressing Cre is injected in the NAc and retrogradely transports Cre up terminals in the NAc. A DIO-AAV construct containing ChR2 is injected into the ventral tegmental area (VTA) and is selectively expressed in cells that contain Cre following retrograde transport, allowing for selective optogenetic activation of VTA cells that terminate in the NAc.

**Abbreviations:** ChR2, channelrhodopsin 2; TRE, tetracycline response element; ITR, inverted terminal repeats; Hipp, hippocampus; PR, .

intensities, comparable to ChR2 stimulation.<sup>51</sup> Similarly, the crystal structure of the ChR C1C2 (a chimera of ChR1 and ChR2) was resolved.<sup>50,52</sup> Using site-directed mutations of C1C2, a blue-light activated, inhibitory chloride-pump C1C2 (iC1C2) was developed with a comparable reversal potential

to eNpHR3.0.<sup>52</sup> Exciting alternatives to laser or light emitting diode illumination of opsins have been produced to avoid the use of implantable fibers. The luciferase-halorhodopsin system has been constructed for activation of ChR2 ex vivo or eNpHR3.0 by in vivo luciferase luminescence.<sup>53,54</sup>

**Table 2** Viral constructs utilized in optogenetic manipulation of behavior

Virus	Promoter	Expression	Opsin	Behavioral uses	
Adeno-associated virus	CAG	All cells	ChR2(H134R)	Reward <sup>130</sup>	
			ArchT	Reward, <sup>90</sup> learning <sup>146</sup>	
			ChR2(H134R)	Mood, <sup>115,117</sup> reward/motivation, <sup>123,127,129,131</sup> anxiety, <sup>139-142</sup> learning, <sup>145</sup> epilepsy <sup>74</sup>	
	CamKII $\alpha$	Mostly glutamatergic		eNpHR3.0	Reward/motivation, <sup>124,126,129,131</sup> anxiety, <sup>139-142</sup> feeding <sup>133</sup>
				SFO	Anxiety <sup>120</sup>
				ChR2(EI23A)	Learning <sup>146</sup>
				Vertebrate rhodopsin-5-HT <sub>1A</sub>	Anxiety <sup>8</sup>
				ChR2(H134R)	Pain, <sup>68</sup> reward <sup>113</sup>
				ReaChR	Motor function <sup>20</sup>
				eNpHR3.0	Pain, <sup>68</sup> reward <sup>54,121</sup>
Lentivirus	MCH	MCH expressing hypothalamic neurons	ChR2	Sleep <sup>159</sup>	
	TH	Dopamine	ChR2(H134R)	Reward/motivation <sup>102</sup>	
	CamKII $\alpha$	Glutamatergic	ChR2(H134R)	Motor function <sup>75,79</sup>	
			NpHR2.0	Epilepsy <sup>71</sup>	
			eNpHR3.0	Motor function <sup>79</sup>	
	HCRT	Hypocretin expressing cells	ChR2(H134R)	Sleep <sup>156-158</sup>	
	hsyn	Neuron-specific	opto- $\alpha$ 1AR, opto- $\beta$ 2AR	Reward <sup>6</sup>	
	sPRS	Predominantly catecholaminergic neurons, Phox2 expressing cells	ChR2(H134R)	Pain <sup>69</sup>	
	Herpes simplex virus	CMV	High expression in neurons	ChR2(H134R)	Pain <sup>141</sup>
		IE4/5	Neuron-specific	ChR2(H134R)	Mood <sup>114</sup>
Pseudorabies virus	RV	Presynaptic neurons	LOV-Rac1	Reward <sup>12</sup>	
	RV-Cre	Presynaptic neurons	ChR2(H134R)	Reward <sup>39</sup>	
			DIO-ChR2, DIO-eNpHR3.0	Mood <sup>40,41</sup>	

**Abbreviations:** CAG, CMV early enhancer/chicken beta actin; CMV, cytomegalovirus; MCH, melanin-concentrating hormone; TH, tyrosine hydroxylase; RV, rabies virus; SFO, step-function opsin; LOV, light-oxygen-voltage-sensing; HCRT, hypocretin; ChR2, channelrhodopsin 2; eNpHR3.0, enhanced third generation *Natronomonas pharaonis*; NpHR2.0, second generation *Natronomonas pharaonis*; DIO, double inverted open; ArchT, archeorhodopsin.

This system utilizes the luciferin-induced luminescence of luciferase to drive activation of opsins in vivo through an intraperitoneal injection of luciferin. Opsins have been used to indirectly alter the excitability of cells through activation or inhibition of ion channels.<sup>55</sup> Finally, development of novel light-sensitive tools to directly alter endogenous ion channel function provides a novel means to control cellular activity. For example, photo-switchable ion channel blockers, termed lumitoxins, allow for direct regulation of voltage dependent ion channels, particularly potassium channels, in order to regulate cell function and excitability.<sup>56</sup> Light illumination alters the structure of the lumitoxin allowing the channel to be unblocked and function normally, subsequently altering cell excitability.

There are limited but promising uses of opsins to control neuronal processes within the cell or nucleus including intracellular signaling and transcription. These include G-protein coupled receptor (GPCR) opsins, termed OptoXRs; intracellular signaling opsins; and even opsins that can alter gene transcription through epigenetic mechanisms or activation of transcription factors.<sup>6-10,12,13,48,57,58</sup> A small number of these opsins have been used in vivo and have reliably altered

signaling properties in a temporal and region specific manner leading to changes in behavioral output.<sup>6-8,12,58</sup>

## Neurobehavioral studies utilizing optogenetics

In vivo optogenetics has provided insight into the function of specific neural circuits underlying motor function and movement,<sup>59,60</sup> motivation, reward and emotion,<sup>61-65</sup> anxiety and fear learning,<sup>37,38,63</sup> sleep and circadian rhythms,<sup>66</sup> pain and sensation,<sup>67-70</sup> and epilepsy (Tables 3 and 4).<sup>71-74</sup>

## Motor function

Motor function was one the first behavioral outputs explored using in vivo optogenetics. Aravanis et al<sup>75</sup> demonstrated temporally precise whisker movement could be induced by stimulating the primary motor cortex using ChR2. Gradinaru et al<sup>22</sup> directly stimulated the motor cortex unilaterally in *Thy1* (thymus cell antigen 1)-ChR2 mice to induce motor-driven rotations in an open field.<sup>48,75</sup> These studies proved that direct in vivo optogenetic manipulation of neurons was a viable method for altering behavioral output. Subsequent studies investigated deeper brain structures and their role in

Table 3 Behavioral outcomes of in vivo optogenetic manipulation with channel opsins

Brain region	Stimulation site	Manipulation	Cell subtype	Behavior	Behavioral outcomes	References	Notes	
BLA	Cell body region	Stimulation	Glutamatergic	Learning	Increased memory retention	145,146		
		Inhibition	Non-specific	Pain	Hypersensitivity to pain	160		
	BNST	Inhibition	Non-specific	Learning	Impairs learning	146,148		
		Stimulation	Glutamatergic	Anxiety	Anxiogenic	139		
	CeA, BNST	Stimulation	Glutamatergic	Anxiety	Anxiolytic	140		
	CeA	Inhibition	Glutamatergic	Anxiety	Anxiogenic	140		
	ILmPFC	Inhibition	Non-Specific	Learning	Enhanced learning	43		
	PLmPFC	Inhibition	Non-Specific	Learning	Reduced learning	43		
	NAC	Stimulation	Glutamatergic	Reward	Promotes reward	131		
		Inhibition	Glutamatergic	Reward	Reduced responding to reward	131		
BNST	vHipp	Stimulation	Glutamatergic	Anxiety	Anxiogenic	141,142		
		Inhibition	Glutamatergic	Anxiety	Anxiolytic	141,142		
	LH	Stimulation	D1 Receptor	Anxiety	Anxiolytic	139		
		Stimulation	GABA-ergic	Feeding	Increased feeding	136		
	Cell body region	Inhibition	Glutamatergic	Feeding	Suppresses feeding	136		
		Stimulation	MLIs	Motor function	Produced fine motor movement	80		
	DSt	Inhibition	All neurons	Reward	Reduced amphetamine locomotion	54		
		Stimulation	D1-MSN	Motivation	Reduced action-value, reinforcing	82,91		
	Hippocampus	CA1	Stimulation	D1-MSN	Motor function	Increased movement	78	
			Stimulation	D2-MSN	Motivation	Enhanced action-value, aversive/promotes punishment	82,91	
Hypothalamus	PVH	Stimulation	D2-MSN	Motor function	Decreased movement	78		
		Stimulation	Glutamatergic	Epilepsy	Inhibited epileptiform activity	73		
LC	Cell body region	Inhibition	IL2b cells	Learning	Prevents learning	149		
		Stimulation	Neuron-specific	Learning	Stimulation of activated cells induces learned fear response, stimulation during conditioning blocks learning	37,38,148		
	NAC	Stimulation	PV	Epilepsy	Inhibited epileptiform activity	73	Potentiation on D1-MSNs from ventral hippocampal inputs	
		Stimulation	Glutamatergic	Reward	Promotes reward	129,130	Ventral hippocampus projections ARC cell stimulation	
	HPOA	Cell body region	Inhibition	Glutamatergic	Reward	Reduced locomotor sensitization	129	
			Stimulation	AGRP neurons	Feeding	Increased feeding	135	
	VTA	Cell body region	Stimulation	HCR1	Sleep	Induces wakefulness, fragments sleep	156-158	
			Stimulation	MCH	Sleep	Increases sleep onset and NREM and REM	159	
	Motor cortex	Cell body region	Stimulation	Gal neurons	Aggression	Increased aggression	153	
			Stimulation	DA-ergic	Sleep	Induces wakefulness	157	
NAC	Cell body region	Inhibition	DA-ergic	Sleep	Enhances sleep	157		
		Stimulation	Phox2 neurons	Pain	Altered pain response	69	LC subcompartment produced different outcomes	
LDT	VTA	Stimulation	Non-specific	Reward	Promotes reward	39		
		Stimulation	Non-specific	Reward	Blocks reward	39,112	Terminals likely synapse on DA cells	
Motor cortex	Cell body region	Stimulation	Glutamatergic	Motor function	Increased movement	22,75		
		Inhibition	Glutamatergic	Epilepsy	Inhibited epileptiform activity	71		
NAC	Cell body region	Inhibition	ChAT	Reward	Reduced drug conditioned place preference	93		
		Stimulation	D1-MSN	Reward/mood	Promotes reward, anti-depressant	84,87,89,92,100	Facilitated by NAc-VTA connection	

OFC	Cell body region	Stimulation	DI-MSN	Pain/reward	Increases morphine tolerance	162
		Inhibition	D2-MSN	Reward/mood	Blocks reward, depressant	84,89,92,100
	DSt	Stimulation	Non-specific	Reward	Attenuates reinstatement	90
		Inhibition	Glutamatergic	Motivation	Increased goal-directed lever pressing	123
mPFC	Cell body region	Stimulation	Emx neurons	Mood/anxiety	Increased persistent grooming	128
		Inhibition	Glutamatergic	Reward	Alleviates compulsive grooming	127
	DSt	Stimulation	DI receptor	Anxiety	Enhanced timing to receive reward	125
		Inhibition	Glutamatergic	Motivation	Enhanced social interaction	116
STN	Cell body region	Stimulation	Glutamatergic	Motivation	Prevents habitual goal-directed actions, Disrupts fixed-interval timing task	124,125
		Inhibition	Glutamatergic	Reward	Increased social interaction	133
		Stimulation	Non-specific	Mood	Anti-depressant	114
		Inhibition	PL neurons	Reward	Decreased compulsive cocaine seeking	126
		Stimulation	PV	Anxiety	Increased social interaction	120
		Inhibition	PV	Reward	Extinction of cue-reward behavior	122
	DSt	Stimulation	DI receptor	Feeding	Increased feeding	132
		Inhibition	DI receptor	Feeding	Reduced feeding	132
		Stimulation	Glutamatergic	Mood/anxiety	Anxiolytic	115
		Inhibition	Glutamatergic	Mood/anxiety	Promotes social avoidance, anti-depressant	117,118
		Stimulation	Glutamatergic	Anxiety/mood	Decreased social aversion, prevents acquisition of social aversion	118,119
		Inhibition	Glutamatergic	Mood/anxiety	Anti-depressant	115
VTA	Cell body region	Stimulation	Neuron-specific	Reward	Inhibited cocaine reinstatement	121
		Inhibition	Non-specific	Reward	Enhanced drug seeking	130
	DSt	Stimulation	Glutamatergic	Reward	Enhanced compulsive cocaine seeking	126
		Inhibition	Neuron-specific	Pain	Enhanced pain	68
STN	Cell body region	Stimulation	Neuron-specific	Pain	Blocked pain	68
		Inhibition	Glutamatergic	Motor function	Decreased rotations, enhanced locomotion	79
	DSt	Stimulation	Glutamatergic	Epilepsy	Reduced epileptiform activity	74
		Inhibition	DA-ergic	Reward/mood	Promotes reward	19,30,98,99,101
mPFC	Cell body region	Stimulation	DA-ergic	Mood	Depressant following social defeat stress	40,41
		Inhibition	DA-ergic	Mood	Anti-depressant following chronic mild stress	107
	DSt	Stimulation	DA-ergic	Anxiety/mood	Anti-depressant following social defeat stress	40
		Inhibition	DA-ergic	Mood	Depressant during depression assays	107
VTA	Cell body region	Stimulation	GABA-ergic	Reward	Blocks reward and aversive	111
		Inhibition	DA-ergic	Reward/mood	Promotes reward, depressant following social defeat stress	40,41,100,102
	DSt	Stimulation	DA-ergic	Anxiety/mood	Anti-depressant	40
		Inhibition	GABA-ergic	Motivation	Increased discrimination of motivational stimuli	109
mPFC	Cell body region	Stimulation	DA-ergic	Anxiety/mood	Reduced social interaction	40
		Inhibition	DA-ergic	Anxiety/mood	Reduced social interaction	40

**Abbreviations:** BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; lLmPFC, infralimbic medial prefrontal cortex; PLmPFC, prelimbic medial prefrontal cortex; MLI, molecular layer interneurons; DSt, dorsal striatum; LC, locus coeruleus; HPOA, hypothalamus-precotic area; PV, parvalbumin; ARC, arcuate nucleus; REM, rapid eye movement; NREM, non-rapid eye movement; AGRP, agouti-related peptide; MCH, melanin-concentrating hormone; DA, dopamine; LDT, laterodorsal tegmental nucleus; LHb, lateral habenula; RMTg, rostromedial tegmental nucleus; OFC, orbitofrontal cortex; mPFC, medial prefrontal cortex; DRN, dorsal raphe nucleus; STN, subthalamic nucleus; VTA, ventral tegmental area; NAc, nucleus accumbens; vHipp, ventral hippocampus; DG, dentate gyrus; DI, Dopamine 1; D2, Dopamine 2; GABA, gamma-aminobutyric acid; MSN, medium spiny neuron.

**Table 4** Behavior outcomes of in vivo optogenetic manipulation with signaling opsins

Brain region	Construct	Manipulation	Cell subtype	Behavior	Behavioral outcomes	References
Cerebellum	vRh (Gi/o)	Inhibition	Purkinje cells	Motor function	Altered motor coordination	58
DRN	vMo-5HT <sub>2c</sub> (Gq)	Signal transduction	GABA-ergic	Anxiety	Anxiolytic	7
		Signal transduction	Serotonergic	Anxiety	Anxiolytic	7
	vSWO 5HT <sub>1A</sub> (Gi/o)	Inhibition	Non-specific	Anxiety	Anxiolytic	8
NAc	Alpha I-AR (Gs)	Stimulation	Non-specific	Reward	Increased drug conditioned place preference	6
	Rac1	Protein activation	Non-specific	Reward	Increased place preference, sensitization	12

**Abbreviations:** DRN, dorsal raphe nucleus; NAc, nucleus accumbens; AR, adrenergic receptor; vRh, vertebrate rhodopsin; vMo, vertebrate melanopsin; vSWO, vertebrate short-wavelength opsin.

motor movement and control. Basal ganglia (BG) circuitry, particularly the striatum, has long been implicated in motor dysfunction in a number of disorders such as Parkinson's disease.<sup>60</sup> Medium spiny neurons (MSNs), the projection neurons of the striatum, are differentiated into two major subtypes based on dopamine (DA) receptor expression, dopamine 1 (D1) or dopamine 2 (D2) receptors, and their pathway projections.<sup>76,77</sup> Optogenetic interrogation of these two parallel BG pathways demonstrated that bilateral D2-MSN stimulation in dorsal medial striatum produces a reduction in movement, as observed through freezing, and decreased ambulation, whereas unilateral stimulation yielded ipsilateral rotations.<sup>78</sup> In contrast, unilateral D1-MSN stimulation produces contralateral rotations, while bilateral stimulation increases ambulation and decreases freezing allowing for recovery from parkinsonian bradykinesia outcomes after 6-hydroxydopamine lesioning of dopaminergic innervation to striatum. In comparison, high frequency stimulation of cortical terminals in the subthalamic nucleus, using *Thy1-ChR2* mice, was able to rescue the motor deficits in the 6-hydroxydopamine lesion model.<sup>79</sup> Although the BG circuitry was known to mediate Parkinson's disease and other motor behavior, these studies were the first to specifically probe the BG sub-circuits and demonstrate their importance in motor function and dysfunction.

Optogenetic motor movement studies are not limited to the motor cortex and BG structures. One study, examining the role of cerebellar purkinje cells in fine motor movement, demonstrated that targeting stimulation to distinct subregions of the cerebellum results in distinct fine motor movements.<sup>80</sup> Using neural nitric oxide synthase (*nNOS*)-ChR2 BAC transgenic mice, interneurons in the molecular layer of the cerebellum were stimulated to suppress purkinje cell activity. Stimulation of ChR2 expressing interneurons was sufficient to induce eye blinking, among other facial movements, and kinematics of the blink was controlled by intensity and duration of photo-stimulation, suggesting interneuron activity

of the cerebellum is precisely tuned to control fine motor movement through inhibition of purkinje cells. Another study used vertebrate rhodopsin, a light sensitive G<sub>i/o</sub> linked GPCR, to examine cerebellar motor behavior.<sup>58</sup> Light-driven vertebrate rhodopsin activation of purkinje cells decreased latency to fall in a rotarod task, increased time to descend a pole in a pole test, and increased time to cross a beam in a beam walk test, indicating that G<sub>i/o</sub> signaling in purkinje cells significantly influenced coordination and motor control.

## Motivation, reward, and emotion – striatal neurons

The limbic system and the BG circuit are critical mediators of reward, motivation, reinforcement, and emotion. Along with motor dysfunction observed in dorsal striatal MSN circuits, the balance of MSN subtype tone is predicted to control motivational behavioral outcomes.<sup>76,81–83</sup> Optogenetic activation of nucleus accumbens (NAc) D1-MSNs drives conditioned place preference (CPP) to cocaine and morphine,<sup>83–85</sup> indicating that D1-MSN stimulation promotes reward. These results are likely due to the connection of D1-MSNs to VTA gamma-aminobutyric acid (GABA) interneurons, which, in part, disinhibit VTA-DA neurons.<sup>86–88</sup> This disinhibition hypothesis was further investigated with optogenetic methods. High frequency stimulation of the D1-MSN terminals disinhibits VTA-DA neurons and high frequency stimulation optical stimulation of D1-MSN terminals prior to cocaine treatment subsequently results in an increase in cocaine locomotor sensitization and an occlusion of CPP.<sup>87</sup> Conversely, we recently observed that D1-MSN inhibition using eNpHR3.0 decreases cocaine-induced locomotion.<sup>89</sup> Together these results indicate that heightened activity of D1-MSNs on VTA inhibitory interneurons enhances reward. In contrast, cocaine<sup>84</sup> and morphine<sup>85</sup> place preference and reward are blunted by optogenetic stimulation of D2-MSNs. Further, ArchT inhibition of NAc core terminals in the ventral pallidum, which includes terminals of both MSN subtypes, attenuates



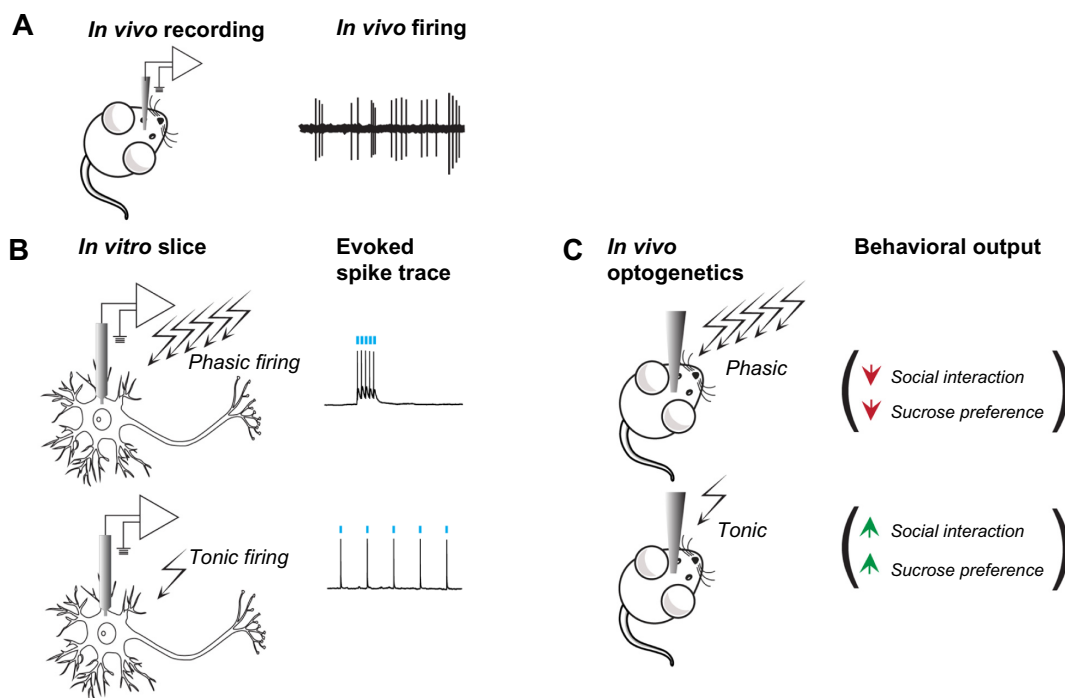
reinstatement for cocaine seeking.<sup>90</sup> In a non-drug paired paradigm, self-stimulation of D1-MSNs in the dorsomedial striatum causes persistent reinforcement whereas stimulation of D2-MSNs results in transient avoidance or punishment.<sup>82</sup> A similar effect was observed in a goal-directed selection task, which demonstrated that optogenetic stimulation of distinct MSN subtypes oppositely mediates changes in action value.<sup>91</sup> In line with these studies, we found positive effects of driving D1-MSN activity. High frequency optogenetic stimulation of NAc D1-MSNs with a ChETA<sub>A</sub> ChR2 promotes anti-depressant responses after chronic social defeat stress, while optogenetic activation of NAc D2-MSNs promotes depression-like behavior to subthreshold defeat stress.<sup>92</sup> Collectively, optogenetic interrogation of striatal circuits demonstrates opposing behavioral functions for D1-MSNs and D2-MSNs, where heightened D1-MSN activity drives reward and reinforcement, while D2-MSN activity blocks reward and reinforcement or promotes negative outcomes. Optogenetic manipulation of striatal neurons is not limited to MSNs. Despite the low percentage of cholinergic interneurons in the striatum (~1%), direct inhibition of NAc cholinergic interneurons diminishes cocaine CPP.<sup>93</sup> This effect may be mediated by diminished DA within the striatum, since increased DA release from VTA neurons occurs after synchronous stimulation of cholinergic interneurons.<sup>94–96</sup>

Optogenetic signaling tools are to date sparsely used in behavioral neuroscience. OptoXR proteins, GPCR chimeras with photo-activatable domains that allow for light-induced G-protein coupled signaling have been developed for the G<sub>q</sub>-coupled  $\alpha 1$  adrenergic receptor ( $\alpha 1$ AR-Gq).<sup>6</sup> Light stimulation of this receptor induced calcium influx, activated G<sub>q</sub> signaling including mitogen-activated protein (MAP) kinase pathway activation, and increased firing rates in NAc neurons.<sup>6</sup> Photo-activation of this receptor was sufficient to produce enhanced place preference for a chamber associated with  $\alpha 1$ AR-G<sub>q</sub> signaling. Optogenetic-induced signaling methods are not strictly limited to membrane-bound receptors. For instance, a constitutively active rho-GTPase (Rac1) fused to a photo-activatable light oxygen voltage construct was used to transiently activate Rac1 in the NAc. This decreased cocaine-induced CPP by increasing phosphorylation of the Rac1 target cofilin leading to decreased cocaine-induced structural plasticity in NAc MSNs.<sup>12</sup>

## Motivation, reward, and mood – VTA

The VTA is a critical mediator of motivational behavior. With the advent of optogenetics, researchers can carefully unravel the complex mechanisms by which the VTA and its associated

circuits modulate such behavior. Distinct behavioral outcomes observed from VTA manipulation are based on a number of factors: 1) cellsubtypes of the VTA and their projections, 2) location of the cells in the VTA, 3) firing frequency (Figure 2), and 4) temporally sensitive firing synchrony. One study conducted early on in the course of optogenetic research demonstrated that optogenetic induction of phasic, but not tonic firing of VTA DA neurons induced CPP.<sup>30</sup> In fact, optical stimulation of VTA-DA neurons in TH-Cre rats or mice promotes intracranial self-stimulation.<sup>19,97</sup> Furthermore, TH-Cre mice, with selective expression of ChR2 in VTA-DA neurons, were shown to rapidly learn to lever press in order to receive phasic photo self-stimulation.<sup>98</sup> This stimulation requires temporally specific VTA-DA activation in reward learning. Cue-elicited responding to a reward is driven by temporally precise VTA-DA neuron activation at the time a reward is delivered or expected.<sup>99</sup> Similarly, phasic VTA-DA and substantia nigra DA optogenetic stimulation produces operant place preference whereas eNpHR3.0 inhibition produces aversion and avoidance of the paired chamber.<sup>98</sup> To add to this mounting evidence, phasic stimulation of VTA terminals in NAc reverses the diminished place preference for morphine caused by brain-derived neurotrophic factor (BDNF) in VTA.<sup>100</sup> Additionally, phasic optogenetic stimulation of VTA-DA neurons facilitates food-seeking behavior and reinitiates lever pressing for food following extinction of the trained behavior.<sup>101</sup> Intriguingly, tonic but not phasic VTA-DA signaling attenuates ethanol self-administration.<sup>102</sup> Taken together, these findings support the hypothesis that phasic firing of DA neurons promotes positive rewarding outcomes while inhibition of phasic firing attenuates reward. However, this may be oversimplified as DA neurons have been demonstrated to encode aversive outcomes.<sup>103,104</sup> An earlier observation noted that phasic firing of VTA-DA neurons was important in encoding depression-like behavior in mice that had previously undergone chronic social defeat stress.<sup>105,106</sup> Optogenetic induction of phasic activity of VTA-DA neurons in mice during a subthreshold form of the social defeat paradigm rapidly induces a depressive phenotype.<sup>40</sup> These studies directly confirmed the functional importance of VTA-DA neurons in encoding for the depressive phenotype. However, a concurrent study reported that induction of phasic firing in VTA-DA neurons reverses the expression of the depressive phenotype in mice that underwent chronic unpredictable stress.<sup>107</sup> These two findings coincide with a series of studies showing that exposing rats to either strong or weak stress paradigm induces increased or decreased firing respectively in VTA-DA neurons.<sup>103</sup> It is feasible that the magnitude of



**Figure 2** Optogenetically mimicking naturalistic neuronal firing differentially regulates cellular and behavioral outcomes.

**Notes:** (A–C) Example of stimulation paradigms to mimic naturalistic firing patterns of ventral tegmental area (VTA) dopamine (DA) neurons. (A) *In vivo* recording is used to determine naturalistic firing patterns of DA neurons. The example cartoon trace shows phasic bursts of VTA-DA neurons following social defeat stress. (B) *In vitro* slice recordings validate expression of the opsin and functional firing frequency during optogenetic stimulation. (C) *In vivo* phasic optogenetic stimulation of VTA-DA neurons promotes depression-like outcomes to social defeat stress, while tonic optogenetic stimulation reverses susceptibility.

the stress used induces differing firing dynamics in the VTA neurons<sup>103</sup> which could be mediated by time-dependent activation and synchronization of specific cell types and projection regions.<sup>108</sup> Furthermore, differential VTA projections and neuronal subpopulations may mediate differing outcomes to stress. For instance, activity of VTA neurons projecting to NAc versus medial prefrontal cortex (mPFC) differentially encodes for susceptibility to depression-related behavior.<sup>40</sup> These factors demonstrate the overall complexity of an integrative brain region and demonstrate the utility of optogenetics in the dissection of complex circuit function.

As noted before, VTA-GABA neuronal function plays an important role in reward behavior and optogenetics provides the means to dissect local and global VTA circuits. ChR2 activation of VTA-GABA neurons projecting to NAc cholinergic interneurons causes enhanced discrimination between non-motivational and motivational stimuli.<sup>109</sup> Optical activation of VTA-GABA interneurons reduces reward consummatory behavior but not conditioned anticipatory behavior following reward-predictive cues.<sup>110</sup> Taken together, these results implicate that activation of VTA-GABA interneurons is essential for driving adaptive responses to stimuli. Using a combination of glutamic acid decarboxylase (*GAD*)-Cre

mice, in order to express and specifically stimulate GABA-ergic VTA interneurons with ChR2 and TH-Cre animals to inhibit VTA-DA neurons with eNpHR3.0, Tan et al<sup>111</sup> discovered that inhibition of VTA-DA neurons through activation of inhibitory VTA-GABA interneurons produces an aversion to a conditioned chamber. This finding and previously discussed findings indicate that bidirectional modulation of VTA-DA neuron activity can promote opposite reward outcomes.<sup>101</sup>

Circuit specific modulation of different inputs to the VTA further highlights the complexity of VTA circuits in differential behavioral outcomes. For example, optogenetic induction of phasic activity in Lhb cells specifically projecting to the VTA was shown to induce conditioned place aversion, while induction of phasic activity of laterodorsal tegmentum neurons projecting to VTA induced CPP.<sup>39</sup> The same study reported that the VTA sends putative GABA-ergic projections to the Lhb and that optical activation of the VTA-Lhb circuit decreases post-synaptic Lhb activity resulting in downstream increased activity of VTA-DA neuron activity. Furthermore, at the behavioral level, optical activation of the VTA-Lhb circuit induces reward-related behavior as evidenced by increased CPP.<sup>112</sup> Similarly, activation of Lhb inputs to another midbrain, VTA-projecting region, the rostromedial

tegmental nucleus, produces avoidance to a light-paired stimulation chamber and disrupts positive reinforcement.<sup>113</sup> Collectively, these midbrain optogenetic studies demonstrate a more complex role, than previously thought, for midbrain neuronal populations and circuits in mediating both reward and aversion.

## Mood, reward, and reinforcement – mPFC, ventral hippocampus, amygdala, and beyond

Similar to VTA manipulation, driving activity in distinct neuronal subpopulations of glutamatergic brain regions produces unique behavioral outcomes. High frequency ChR2 activation of PFC neurons produces anti-depressant responses after social defeat stress, as shown by increased social interaction and sucrose preference.<sup>114</sup> Similarly, negative behavioral outcomes to social defeat stress, following administration of cholecystokinin to the mPFC, can be reversed by optical activation of mPFC terminals. At a circuit specific level, social avoidance is reversed by high frequency optogenetic stimulation of mPFC terminals in the NAc, while anxiety responses are blocked by activation of cortical terminals in basolateral amygdala (BLA), indicating the circuit specificity of these behavior.<sup>115</sup> Using *Thy1-ChR2* mice to optically activate PFC projection neurons, Kumar et al<sup>116</sup> were able to reverse anxiety responses produced by chronic social stress and cause an anti-depressant response in forced swim test. The authors further showed that optical activation of PFC projection neurons entrains neural oscillatory activity and drives synchrony across limbic brain areas that regulate affect.<sup>116</sup> Another study demonstrated that 20 Hz optogenetic stimulation of mPFC inputs into the dorsal raphe nucleus (DRN) increased kick frequency in the forced swim test.<sup>117</sup> These studies promote the hypothesis that global activation of PFC circuits drive anxiolytic and anti-depressant outcomes which is in part mediated by the amygdala, NAc, and DRN. In contrast, driving mPFC terminals to the DRN with ChR2 activation during a sensory interaction period between social defeat episodes increases aversive behavior while inhibition with ArchT decreases aversive behavior.<sup>118</sup> In addition, inhibition of DRN GABA-ergic interneurons, which receive direct glutamatergic connections from the mPFC, prevents acquisition but not expression of social avoidance behavior.<sup>119</sup> These differing effects may be due to 1) the frequency of stimulation, 2) time-dependent stimulation effects or 3) activity alterations of differing mPFC sub-circuits and projections. Finally, optogenetic evidence

suggests that imbalance of excitatory and inhibitory function in the mPFC mediates social behavior disruption. Using a step-function opsin to depolarize pyramidal cells in the mPFC to increase the excitation to inhibition ratio, social interaction was diminished in a three chambered social interaction test.<sup>120</sup> Step-function opsin stimulation, with a redshifted ChR (C1V1) in parvalbumin interneurons, in order to restore the excitatory inhibitory balance through increased inhibition, partially restored social interaction in the same test.<sup>120</sup>

Though the frontal cortex is divided into many regions that have a diverse set of functions, optogenetic investigation into frontal cortex function in reward and motivation supports the idea that indiscriminate enhancement of frontal cortex activity promotes rewarding and positive outcomes, while inhibition blocks these effects. Inhibition of the prelimbic cortex of the mPFC, the NAc core itself, and prelimbic fibers to the NAc core inhibited cocaine reinstatement.<sup>121</sup> Inhibition of mPFC pyramidal neurons through photo-stimulation of mPFC parvalbumin interneurons promotes extinction of cue-reward behavior.<sup>122</sup> Frontal cortex stimulation is associated with positive rewards. Optogenetic orbitofrontal cortex (OFC) activation increases goal-directed lever pressing.<sup>123</sup> In contrast, optogenetic inhibition of the infralimbic cortex during overtraining prevents learning necessary to develop habitual goal-directed actions.<sup>124</sup> Optical inhibition of D1 expressing mPFC neurons, which are associated with heightened DA release in and enhanced activation of the mPFC, disrupts a fixed-interval timing task while temporally precise stimulation of these neurons during the interval enhances timed responses to receive rewards in the same task,<sup>125</sup> suggesting that temporally specific stimulation is an important factor in frontal cortex mediated rewarding behavior.

Harmful and negative outcomes produced by compulsions are prevented by driving the frontal cortex. Optogenetic stimulation of the prelimbic cortex suppresses compulsive cocaine seeking when paired with a noxious stimulus (shock) while inhibition enhances compulsive seeking behavior even when the aversive shock stimulus is present.<sup>126</sup> These outcomes suggest that enhancement of frontal cortex activity drives adaptive responses. Further evidence from optogenetic manipulation of OFC circuits supports this point. Acute 10 Hz optogenetic stimulation of OFC terminals in the striatum or of OFC projection neurons in a mouse model of obsessive grooming, alleviates compulsive grooming.<sup>127</sup> However, in one study, 5 days of repeated 10 Hz stimulation of the OFC-striatal pathway increased persistent grooming behavior in mice, which was reversed by chronic fluoxetine

treatment.<sup>128</sup> Together, these two OFC studies indicate the behavioral importance of stimulation timing and stimulation frequency.

Various glutamatergic inputs into NAc can influence motivational behavior. Optogenetic stimulation of ventral hippocampal (vHipp), mPFC, and BLA glutamatergic terminals in the NAc shell produce rewarding effects and promote place preference.<sup>129</sup> Cocaine selectively potentiates vHipp inputs to the NAc shell and ChR2 activation of this pathway enhances cocaine induced locomotor sensitization while NpHR inhibition reduces sensitization.<sup>129</sup> Cocaine seeking is driven by potentiation of vHipp synapses on D1-MSNs.<sup>130</sup> In contrast, depressing mPFC synapses on D1-MSNs attenuates cocaine seeking,<sup>130</sup> suggesting cocaine can differentially modify glutamatergic synapses on MSN subtypes. Another study examined distinct BLA glutamatergic projections specifically innervating the NAc. Terminal BLA, but not mPFC ChR2 stimulation in the NAc promotes self-stimulation.<sup>131</sup> Furthermore, NpHR inhibition of BLA terminals reduces behavioral responding to sucrose.<sup>131</sup> Optogenetic inhibition of BLA to NAc core by the ArchT opsin prevents cocaine reinstatement and seeking.<sup>121</sup> These findings indicate that glutamatergic signaling to the NAc facilitates reward and reinforcement in a projection specific manner.

Feeding behavior has been examined with optogenetics in a number of studies.<sup>27</sup> Heterogeneous effects on motivation, food consumption, and feeding behavior are observed with PFC lesions<sup>132</sup> and optogenetics provides a means of dissecting feeding behavior through bidirectional alteration of neuronal activity. D1 receptor expressing mPFC projection neurons are activated during feeding behavior, an effect that was mimicked by optogenetic activation of these neurons,<sup>132</sup> while inhibition of D1 mPFC neurons reduced feeding. These optogenetic-driven behavioral effects are localized to medial BLA neurons as stimulation of D1 mPFC terminals in the medial BLA enhances feeding while inhibition of medial BLA cell somas during terminal stimulation restores normal feeding.<sup>132</sup> Similarly, studies in female rats demonstrate that eNpHR3.0 mediated inhibition of mPFC blocks stress-induced reinstatement to food seeking.<sup>133</sup> Currently, there is a lack of optogenetic studies in female rodents, but given the role of sex differences in behavior and CNS disease,<sup>134</sup> we will likely see more sex specific studies in the future. Sex differences are likely to be more apparent in well-studied sexually dimorphic regions such as the hypothalamus. The hypothalamus is heavily involved in basal states and behavior including feeding. Activation of agouti-related peptide (AGRP) positive neurons and their axon terminals in the paraventricular hypothalamus

(PVH) evokes feeding behavior, which is mediated by AGRP inhibition of PVH cells. This effect is mimicked by chemogenetic inhibition of PVH cells.<sup>135</sup> These experiments identified that AGRP neurons from the arcuate nucleus (ARC) suppresses PVH oxytocin neurons to evoke feeding. However, it has been found that acute stimulation of AGRP axons in multiple brain regions was sufficient to enhance feeding,<sup>26</sup> including areas that are not normally associated with a rapid enhancement of feeding behavior. Dissection of specific circuits arising from the lateral hypothalamus has distinguished the bed nucleus of the stria terminalis (BNST), a region that integrates motivational information and interacts with the VTA and the lateral hypothalamus, as a particularly important region in feeding behavior.<sup>136</sup> Optogenetic activation of BNST GABA-ergic projections to the lateral hypothalamus enhance feeding while inhibition is aversive and suppresses feeding. In conjunction with these findings, stimulation of glutamatergic lateral hypothalamic neurons suppresses feeding, indicating the BNST in part controls lateral hypothalamic control of feeding behavior,<sup>136</sup> distinguishing the complexity of circuits involved in natural, primal behavior.

## Anxiety, fear, and aggression

The amygdala is well-known for its role in emotion, as lesions of this structure produce profound deficits in emotion-related behavior.<sup>137–139</sup> Tye et al examined the bidirectional effects of stimulating and inhibiting the amygdala in anxiety behavioral tasks.<sup>140</sup> Activity of glutamatergic projection neurons from the BLA, expressing either ChR2 or eNpHR3.0, was bidirectionally manipulated during the elevated plus maze test and open field test. Somatic stimulation of the BLA produced a robust anxiolytic effect. This result was mediated through BLA to central amygdala (CeA) synapses since stimulation of BLA fibers in the CeA produced an anxiolytic effect while inhibition of these fibers was anxiogenic.<sup>140</sup> Further interrogation of anxiety-related circuits demonstrated that eNpHR3.0 inhibition of BLA inputs to the vHipp decreases anxiety-related behavior in the elevated plus maze and open field while stimulation produces anxiety.<sup>141</sup> Stimulation of this pathway is also sufficient to drive avoidance to a resident intruder while inhibition reversed this effect.<sup>142</sup> These studies suggest BLA to vHipp connections drive an adaptive form of anxiety, allowing for avoidance of possible harmful stimuli. The extended amygdala including the BNST is also involved in fear and anxiety. Optogenetic mapping and behavioral analysis of the BNST and BLA circuit indicates BLA inputs in part aid in distinguishing locations that promote anxiety.<sup>136,139</sup> Stimulation of BNST glutamatergic projections

to VTA-GABA-ergic cells promoted anxiogenic and aversive effects while activation of BNST GABA-ergic projections to these same VTA neurons produced anxiolytic and rewarding effects.<sup>136</sup> These results shed light on non-reward related functions of VTA circuits. However, other midbrain nuclei involved in reward and mood such as the DRN are also involved in anxiety-like behavior. Using light sensitive 5-HT<sub>1A</sub> G<sub>iv</sub> serotonin receptor in the DRN, Maseck et al<sup>8</sup> examined anxiety behavior in the open field and in novelty suppressed feeding.<sup>8</sup> Indirect photo-inhibition of DRN neurons, with this light sensitive 5-HT<sub>1A</sub> variant, induced anxiogenic behavior. A subsequent study from this same group used a vertebrate melanopsin coupled with a 5-HT<sub>2C</sub> receptor, a G<sub>q</sub> linked receptor, in GABA-ergic neurons of the DRN.<sup>7</sup> They found that activation of the light sensitive 5-HT<sub>2C</sub> receptor in GABA-ergic neurons decreased activity in serotonergic neurons, and produced an anxiolytic response. The same response was reproduced with ArchT inhibition of DRN serotonergic neurons. These outcomes demonstrate that differential modes of GPCR signaling can produce similar effects on neuronal activity and ultimately behavior.

Well-established fear learning paradigms allow for simple characterization of fear-related behavior with optogenetics in order to better define the circuitry that mediates fear. Particularly interesting are optogenetic studies examining mPFC, vHipp, and amygdala circuits in fear conditioning and extinction. It was observed that specific connections from both the mPFC and the vHipp monosynaptically innervate both principle excitatory neurons and inhibitory neurons in the BLA.<sup>143</sup> These connections recruit feed-forward inhibition of excitatory neurons in the amygdala. mPFC to BLA connections are associated with retrieval of fear extinction memory. Using optogenetic and electrophysiological methods, the strength of mPFC to BLA connections was observed to be depressed following extinction of conditioned fear.<sup>144</sup> However, extinction is not associated with feed-forward inhibition of excitatory amygdala mPFC to BLA circuits.<sup>144</sup> These results implicate a role for mPFC in controlling BLA output in relation to fear memories. However, reciprocal connections to the mPFC also control fear behavior. Connections from the basal amygdala to subregions of the mPFC, specifically the prelimbic and infralimbic subdivision, are oppositely involved in retrieval of a fear memory and direct inhibition produces opposite freezing responses in conjunction with a conditioned stimulus.<sup>43</sup> Local manipulation of the amygdala also produces similar learning outcomes. Optogenetic activation of the lateral amygdala during a conditioned auditory stimulus is capable of enhancing fear stimulus learning.<sup>145</sup>

In conjunction with these findings, gamma-frequency stimulation (40 Hz) of BLA glutamatergic pyramidal cells with a ChETA<sub>A</sub> ChR2 enhanced retention of an inhibitory avoidance cue while inhibition using ArchT impairs retention of the learned avoidance.<sup>146</sup> These results demonstrate that driving excitatory cells of lateral subregions of the amygdala promote fear learning.

To understand how contextual fear memory is encoded and stored, optogenetics has been used in the hippocampus of rodents.<sup>147</sup> Inhibition of dentate gyrus (DG) cells during encoding of a contextual fear memory blocks learning as shown by poor retrieval.<sup>148</sup> Interestingly, stimulation of the same cells produces a similar effect, suggesting any alteration of DG cell firing during learning prevents encoding of a fear memory. Furthermore, inhibition of DG cells during training in a spatially driven, active avoidance place task attenuates learning of the avoidance zone.<sup>148</sup> Inhibition of DG GABA-ergic neurons prevents acquisition in a morris water maze task.<sup>149</sup> Taken together, these results demonstrate that alteration of naturalistic neuronal firing in the DG prevents normal learning. A recent study used a complex genetically-driven optogenetic paradigm to selectively activate neuronal ensembles important for memory engrams. A c-fos induced ChR2 was used to control activated cells (see Techniques and technological advances section and Figure 1C) and reactivate cell populations of the DG that were previously activated during fear memories in a fear conditioning paradigm.<sup>37,38</sup> Stimulating DG neurons involved in the memory engram recapitulated freezing behavior that occur during this conditioning. This optogenetic system is underutilized in rodent behavior and has yet to be used in other brain regions. However, similar, non-optogenetic methodologies to inactivate neuronal ensembles using other methodologies have also provided insight into context-induced behavior.<sup>150,151</sup>

Aggression studies involving optogenetic manipulations have provided insights into overlapping circuitry mediating attacking and mating behavior. Neuron-specific optogenetic stimulation of the ventrolateral subdivision of the ventromedial hypothalamus (VMHvl) enhances male mice aggression and leads to indiscriminate attacking behavior.<sup>152</sup> Interestingly, activity of these aggression neurons are suppressed when a distinct population of VMHvl neurons involved in mating are active. Inhibition of VMHvl firing can attenuate aggressive behavior.<sup>152</sup> The medial preoptic area (MPOA) has overlapping functions in both mating and aggression similar to that of the VMHvl. Two subsets of galanin-expressing neurons of the MPOA mediate female parental responses and mating.<sup>153</sup> Activating the MPOA optogenetically reduces

male aggression behavior to other males and pups. Further, activation of this region can induce pup grooming. These results provide evidence that sex, aggression, and parenting behavior are produced in overlapping brain regions, but are distinguished neurobiologically by cell type and region specific neuronal activity.

## Circadian, sleep, alertness

The circadian and sleep-wake regulator cycles are independent, but closely linked systems. The suprachiasmatic nucleus, a pair of nuclei in the hypothalamus, governs the rhythms of the sleep-wake cycle. The suprachiasmatic nucleus in-turn coordinates the activity of other self-sustained clocks and brain regions implicated in mood disorders such as the hippocampus, VTA, mPFC, LHb, and NAc.<sup>154</sup> Since these neurons exhibit daily rhythms in firing, inhibition of a circuit at the period of time an animal is most active may yield more robust behavioral effects compared to inhibition when the neural circuit is the least active. Furthermore, since circadian rhythms exhibit rhythms that last from minutes to hours within a 24 hour cycle, it would be beneficial to use chronic optogenetic stimulation paradigms lasting minutes to hours in order to better elucidate naturalistic diurnal and circadian rhythmicity of neural circuits and the subsequent changes in animal behavior.

There is a great prevalence of published studies using optogenetics in the modulation of the sleep-wake cycle. The circuitry of arousal involves various neurotransmitter systems from differing nuclei such as the hypocretin (HCRT), noradrenergic (NOR), serotonergic, cholinergic neurons from the lateral hypothalamus, locus coeruleus (LC), DRN, and pedunculopontine/laterodorsal tegmental nuclei, respectively. Previous pharmacological evidence suggested that HCRT and NOR circuitry are important in promoting arousal in the animal.<sup>155</sup> However, with optogenetics, it is possible to selectively, reversibly, and rapidly modulate these circuits in order to study the effects on arousal and determine the causal link between circuit activity and sleep or arousal states. Optogenetically mimicking naturalistic activity of HCRT neurons increases behavioral transitions from non-rapid eye movement (NREM) sleep or REM sleep to wakefulness.<sup>156</sup> Furthermore, optogenetic stimulation of NOR, LC neurons induces rapid wakefulness from both NREM and REM sleep.<sup>157</sup> It should be noted that both HCRT and LC neurons are also implicated in a wide variety of behavior such as depression, stress, attention, learning and memory, and addiction. For example, in vivo optogenetic manipulation of HCRT neurons, resulting in sleep fragmentation, impairs memory consolidation.<sup>158</sup>

A compelling hypothesis posits that differing firing dynamics of HCRT and LC neurons may result in differing states of arousal. Future experiments involving optogenetic induction of distinct firing patterns in HCRT and LC neurons may help elucidate the subtle role of differing firing dynamics in behavioral output. Melanin concentrating hormone neurons located in the hypothalamus project widely throughout the brain, including to arousal neurons, and have been proposed to have a role in promoting sleep. Recent optogenetic studies show that specific activation of melanin concentrating hormone neurons increases both sleep onset and subsequent time animals spent in NREM and REM sleep.<sup>159</sup> Elucidating the roles of specific neural circuits in sleep regulation prove potentially useful for the future development of treatments for insomnia. Another intriguing possibility is that optogenetics will help elucidate the circuitry involved in, the interesting yet counterintuitive observations that, sleep deprivation induces rapid reversal of depression.

## Pain

Very few studies use optogenetics to directly study pain behavior.<sup>67</sup> One study used non-specific opsins injected into the sciatic nerve to trans-dermally produce pain responses through stimulation, while preventing pain responses through inhibition.<sup>68</sup> This technique holds the potential for relatively non-invasive and direct therapeutic strategies to treat peripheral pain. Other optogenetic studies, focused on the CNS, led to insights about subcortical and brain stem regions that mediate pain. The NOR system mediates in part analgesic outcomes to peripheral pain and alters spinal nociception.<sup>67</sup> Optogenetic ChR2 excitation of the LC enhanced the withdrawal threshold for a normally noxious heat stimulus.<sup>69</sup> Similarly excitation in dorsal LC regions reduced the withdrawal threshold, while stimulation in ventral LC regions enhanced withdrawal threshold, demonstrating that different subpopulations within the LC mediate opposing nociception outcomes. Optogenetic studies have explored atypical pain-related regions of the brain such as the CeA. Hypersensitivity to bladder distention can increase visceromotor responses to painful stimuli when the CeA is optogenetically stimulated.<sup>160</sup> Further, optogenetic investigation of central brain regions may be important to assess mechanisms of pain-associated tolerance and addiction. Analgesic tolerance to opioid medications for a number of diseases, such as chronic pain, has produced problems for long-term treatment strategies. One study addressed morphine analgesia and tolerance by activating NAc MSNs expressing regulator of G-protein signaling 9 (Rgs9) complexes. Rgs9 complexes, known to modulate

opioid GPCR responsiveness and desensitization<sup>161,162</sup> are important in morphine analgesia and tolerance in the NAc. This study demonstrated that optogenetic activation of Rgs9 expressing cells of the NAc, which should include all MSNs, enhanced the development of morphine tolerance, an effect that was mediated through D1-MSNs.<sup>162</sup>

## Epilepsy

Epilepsy is characterized by abnormal neuronal activity promoted by enhanced connectivity, synaptic transmission, and intrinsic excitability, which can lead to spontaneous generation of seizures.<sup>71</sup> Due to a wide array of inhibitory tools, optogenetics holds great potential in preventing seizure generation or silencing seizures before their onset. It was first observed that stimulation train induced bursting can reliably be inhibited by NpHR in organotypic culture, indicating that halorhodopsin held the potential to stifle seizure induced activity.<sup>72</sup> Later, in vivo studies supported this approach as a valid and reliable model for the treatment of epilepsy. NpHR reduced seizure activity produced by tetanus toxin-mediated generation of spontaneous seizures.<sup>71</sup> Halorhodopsin inhibition of seizures was observed to halt seizure activity induced by kainic acid in the hippocampus.<sup>73</sup> This strategy has been further extended to a more naturalistic model of seizures in which a spontaneous seizure disorder was produced by cortical injury.<sup>74</sup> In this study, thalamic seizures were interrupted by light stimulation of eNpHR3.0 containing cells. However, inhibition of seizure activity has not been limited to direct inhibition of primary excitatory cells. Stimulation of parvalbumin interneurons and thus an enhancement in inhibitory GABA-ergic signaling has been observed to attenuate kainic acid induced seizure activity and behavioral seizures.<sup>73</sup>

## The future of optogenetics

In the past decade optogenetics has rapidly advanced, leading to a large toolbox of available technologies. These tools continue to progress and improve researchers' ability to dissect complex brain circuitry. Viral and transgenic optogenetic constructs may be used for mapping large parts of the neural connectome with both ex vivo slice and in vivo electrophysiology techniques. Coupling these methodologies with microscopy techniques including in vivo calcium imaging, optogenetics could provide cell-specific investigation of neuronal firing in awake, behaving animals while maintaining optical control over specific cell subtypes.<sup>163</sup> The ability to exert fine temporal control of cell firing using optogenetics, will make it possible to elucidate the functional role of subtle changes in both firing frequency and patterns

in specific cell types and neural circuits and their effects on behavior. Using structural information and mutagenesis, development of alternative, wavelength specific opsins would allow for various wavelengths of light to be used in one region for simultaneous optical control of different cell subtypes, to dissect discrete connections and firing patterns in specific circuits through combinatorial experiments with multiple light-driven constructs. Future studies will likely use activity-dependent promoters allowing for manipulation of previously activated neuronal ensembles that mediate behavioral outcomes. Activity-dependent activation of opsins in combination with luminescent proteins (eg, luciferase) could potentially prove a useful therapeutic tool to prevent negative outcomes of neural hyperactivity through inhibition (eg, seizure activity) or hypoactivity through stimulation, while bypassing invasive optic fiber implants. Lastly, many more promising applications will be developed for optogenetics in higher animals considering opsins and optogenetics are now currently being used in primates.<sup>164</sup>

## Conclusion

In vivo stimulation and inhibition of specific circuits is essential for understanding the emergence of behavioral phenotypes and region specific functions, particularly in neurological and psychological diseases and disorders. As treatments for many of these diseases are few and often ineffective, optogenetics holds the potential to unearth new therapeutic strategies. Optogenetics is currently used for circuit mapping, temporal specific activation or inhibition of cell subtypes, signaling alterations, and functional discovery of brain circuits and their behavioral correlates. Using optogenetics, under the control of cell-type specific promoters or Cre rodent lines, has led to a wealth of information about the connectivity and activity among brain structures and has thus provided researchers with the knowledge of microcircuit level dysfunction in rodent behavior. Such findings have enlightened potential treatment strategies for many CNS diseases.

## Disclosure

The authors have no conflicts of interest to disclose.

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