Controlling the Elements: An Optogenetic Approach to Understanding the Neural Circuits of Fear

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Neural circuits underlie our ability to interact in the world and to learn adaptively from experience. Understanding neural circuits and how circuit structure gives rise to neural firing patterns or computations is fundamental to our understanding of human experience and behavior. Fear conditioning is a powerful model system in which to study neural circuits and information processing and relate them to learning and behavior. Until recently, technological limitations have made it difficult to study the causal role of specific circuit elements during fear conditioning. However, newly developed optogenetic tools allow researchers to manipulate individual circuit components such as anatomically or molecularly defined cell populations, with high temporal precision. Applying these tools to the study of fear conditioning to control specific neural subpopulations in the fear circuit will facilitate a causal analysis of the role of these circuit elements in fear learning and memory. By combining this approach with in vivo electrophysiological recordings in awake, behaving animals, it will also be possible to determine the functional contribution of specific cell populations to neural processing in the fear circuit. As a result, the application of optogenetics to fear conditioning could shed light on how specific circuit elements contribute to neural coding and to fear learning and memory. Furthermore, this approach may reveal general rules for how circuit structure and neural coding within circuits gives rise to sensory experience and behavior.

Key Words: Electrophysiology, fear conditioning, learning and memory, neural circuits, neural plasticity, optogenetics

Neural circuits are anatomically and functionally interconnected networks of neurons that mediate specific aspects of experience and behavior. Many neural circuits control behavior by integrating sensory signals from the environment, memories acquired from previous experience, and information about the current state of the organism. Specific circuits mediate a range of adaptive functions, from feeding and mating, to visual and other forms of sensory processing, to emotional learning, to working memory, attention, and other higher cognitive functions. A central goal in neuroscience research is to define the functional anatomy and the neural computations occurring within these circuits.

Fear conditioning is a powerful system in which to study neural circuits, neural coding in these circuits and the influence of learning, memory and plasticity on circuit processes (1–8); as well as being an important model for studying fear and anxiety (4,9,10). Fear conditioning occurs when a sensory conditioned stimulus (CS, usually an auditory tone) is paired with an aversive unconditioned stimulus (US, usually a mild electric shock) during a training phase. Following training, the presentation of the CS alone produces behavioral and visceral fear conditioned responses (CRs), demonstrating that a long-term memory has been formed (1). One major advantage in using fear conditioning to study neural circuits is that it is a relatively simple procedure in which easily quantifiable behaviors are elicited by stimuli that are under the control of the experimenter (1–8). This relative simplicity facilitates the mapping of functional circuits and the identification of sites of neural plasticity in these circuits.

Over the past 30 years, studies using lesion, electrophysiological, pharmacological, and biochemical/molecular techniques have revealed a great deal about the neural mechanisms of fear learning (1–7,11–13). Despite this progress, much remains to be understood about the fundamental principles by which fear conditioning is implemented at the level of defined neural circuits. In addition, information processing by neurons in these brain regions and particularly how circuit mechanisms give rise to these computations is largely unknown. Although traditional techniques have been valuable in defining the fear circuit, they lack the temporal and spatial specificity needed to make further progress on many of these issues. To address these questions, techniques for manipulating specific circuit elements (i.e., subpopulations of neurons and specific axonal projections) with high temporal precision are needed.

The recent development of optogenetics—the combined use of optical and genetic technologies to control cells and measure their activity in intact neural circuits (14)—provides a tool to ask important and previously unaddressable questions. This is because the optogenetic approach offers the ability to modulate specific circuit elements with high temporal precision (see below for a detailed discussion of some of these questions in fear conditioning) (14–17). An important step in the development of optogenetics was the discovery of the algael light activated Channelrhodopsin-2 (ChR2) and the functional expression of ChR2 in neurons to control neural activity (18–20). ChR2 is a blue light activated, nonspecific cation channel that can be expressed heterologously in neurons and used to depolarize and excite cells using light (see Figure 1A; see Luo *et al.* [21] and Zhang *et al.* for review [22]). Other ion channels and pumps activated by different wavelengths of light have since been developed, including two which inhibit neural activity, Halorhodopsin (Figure 1B) and Archaerhodopsin (Figure 1C) (23–26). Throughout the rest of this review, ChR2 (and the other modified ChR2 variants) will be referred to as "excitatory opsins," and Halo- and Archaerhodopsin (and their variants) will be referred to as "inhibitory opsins." Opsins can be expressed globally or in specific subpopulations of neurons in distinct brain regions using transgenic animals, local viral infection, or combinations of Cre-recombinase (Cre) expressing mouse lines with Cre-dependent viral mediated opsin expression (see Figure 2 for description of these different approaches and

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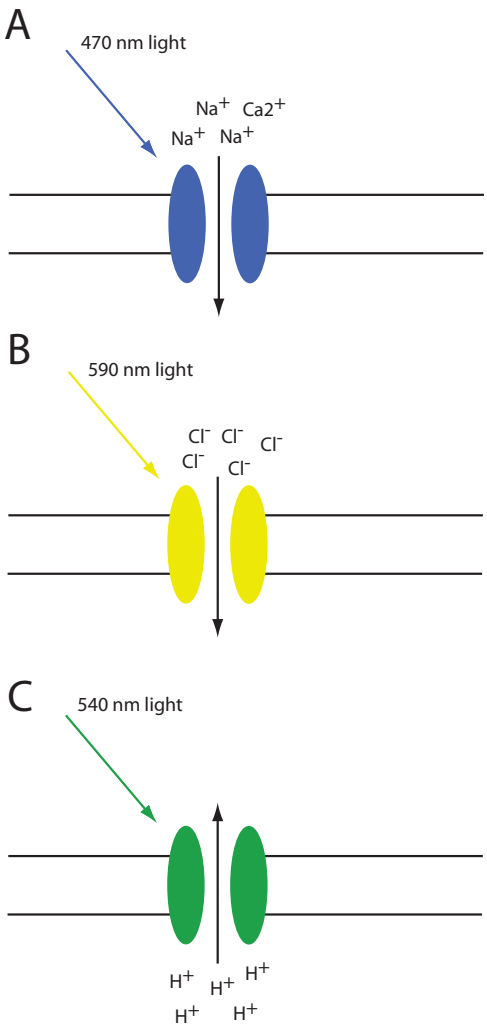


Figure 1. Prototypic opsin proteins for bidirectional manipulation of neuronal activity. **(A)** Channelrhodopsin-2 (ChR2) is a light-gated, nonspecific cation channel (with low Ca2+ permeability) (97) that is activated by blue light (~470 nm). In ChR2-expressing neurons, illumination with blue light causes depolarization and spiking of the cell. Although traditional ChR2 variants produce reliable spiking up to about 20 Hz, modified versions are capable of producing much higher spiking frequencies (see Yizhar *et al.* [98] for review). **(B)** Halorhodopsin is an inward chloride pump that causes hyperpolarization of expressing neurons, inhibiting their activity, upon illumination with yellow light (~590 nm). **(C)** Archaerhodopsin is an outward proton pump that also hyperpolarizes expressing neurons upon illumination with green or yellow light (~540–590 nm).

Luo *et al.* [21] for review of this topic). Lasers or light emitting diodes can then be employed to deliver light to the brain to control the activity of opsin expressing cells. The use of both excitatory and inhibitory opsins in this way can unambiguously demonstrate both necessity and sufficiency of defined circuit elements. This approach has been used to control behavior and has been reviewed previously in (17,27).

The optogenetic approach provides the capability to control neural activity in the fear circuit with millisecond precision and to manipulate specific cell populations and afferent inputs to a given brain region. In this article, we first provide a brief introduction to the functional anatomy of the fear circuit and the computations performed by neurons in this circuit during fear conditioning. We then discuss the potential applications of optogenetics to the study of the neural circuits of fear.

Fear Conditioning Circuits

A rough working model of the fear circuit (Figure 3) has been developed through a variety of approaches including brain lesion and pharmacologic manipulations as well as electrophysiologic measurements. Studies examining the circuit architecture of fear conditioning have focused on pathways that transmit auditory CS information, aversive somatosensory US information, those which integrate CS and US information, and those involved in producing fear CRs.

Auditory CS Pathways for Fear Conditioning

The auditory CS pathways involved in simple forms of fear conditioning (those in which a pure tone or other acoustic stimuli with simple features is used) require the medial geniculate (MGm) and the posterior intralaminar thalamic nuclei (PIN) (28–31) (but see Campeau and Davis [30] and Boatman and Kim [32]), whereas fear conditioning to more complex CSs recruits both thalamic and auditory cortical pathways (31,33). Neurons in both the MGm/PIN and primary auditory cortex and auditory association cortex (temporal association cortex, TeA) are responsive to auditory CSs and some neurons in the MGm/PIN and in TeA also respond to somatosensory stimuli (34–38). Many MGm/PIN neurons do not exhibit precise frequency tuning to auditory stimuli before learning (37,39,40), but auditory cortical neurons are more finely tuned to frequency (38). Neurons in both auditory thalamus and cortex increase their CS-evoked responses following fear conditioning (34–38,41,42) and tuning in both of these regions is sharpened to the specific tone frequencies that are paired with the aversive US (37,38).

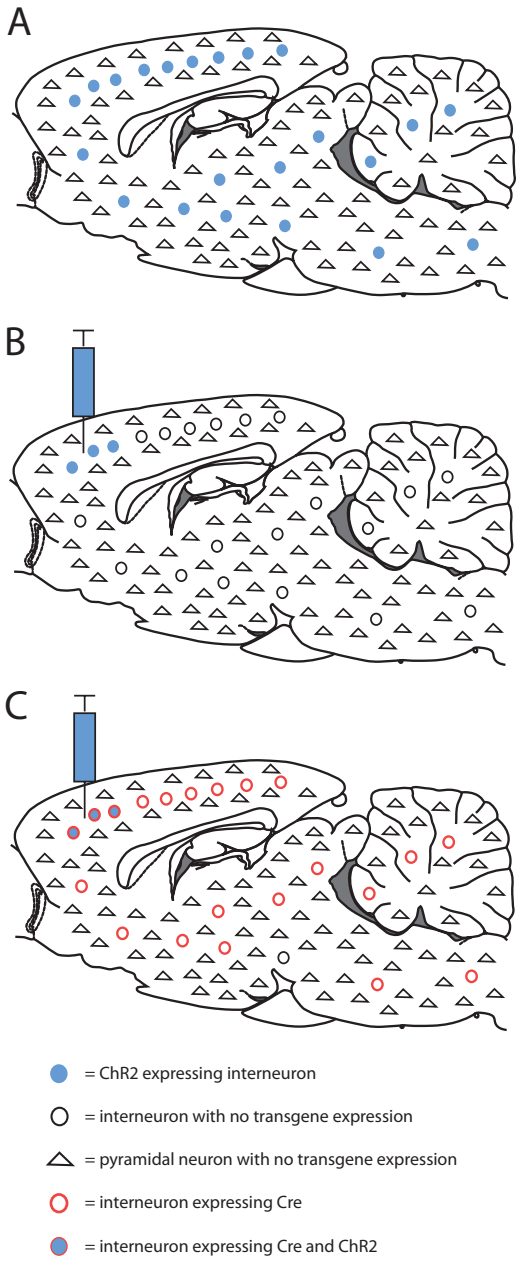
Lateral Amygdala Is a Critical Site of Associative Plasticity for Fear Conditioning

The lateral nucleus of the amygdala (LA) is known to be a critical site of CS-US convergence and associative plasticity and memory storage during auditory fear conditioning, although it is likely not the only site of plasticity in the fear circuit (37,43). Because it receives input from a variety of sensory systems, amygdala neurons may participate in fear conditioning induced by a wide range of sensory stimuli (44–46). Both MGm/PIN and TeA project to and form synapses with neurons in the LA (see LeDoux [1] for review). LA neurons receive convergent input from auditory CSs and somatosensory USs (47,48), and before fear conditioning, LA neurons code auditory frequency crudely, exhibiting large auditory frequency receptive fields (49,50). Importantly, fear conditioning induces an enhancement of CS-evoked responding as measured by electrophysiologic recordings (36,48,51–55). Central to our understanding of the fear circuit is the idea that the LA is a key site of associative plasticity during fear conditioning. According to this model, CS–US convergence in LA pyramidal cells induces associative plasticity such that the CS more effectively drives postsynaptic neurons in the LA after pairing with the US. Supporting this, both fear conditioning at the level of behavior and associative plasticity of auditory CS inputs to postsynaptic LA neurons requires activation of various intracellular signaling molecules or processes that are thought to be important for synaptic plasticity in LA (see Sah *et al.*, [5], Pape and Pare [6], and LeDoux *et al.* [11], for review).

The LA is made up of pyramidal cells and interneurons, and because a larger percentage of LA neurons are pyramidal cells, it is possible that this cell population was preferentially sampled in most of the in vivo recording studies. However, the specific contri-

bution of LA interneurons and pyramidal cells to neural coding and behavior during fear conditioning is largely unknown.

Although the US pathways that trigger LA plasticity are not clearly defined (56–60), it does appear that the periaqueductal gray (PAG) may be a part of the circuit that transmits US information to the LA (12,48). Like CS coding, US information processing in LA neurons is also modulated by learning but in the opposite direction from learning related modulation of CS-evoked responding (12,48,61). Namely, US-evoked responding is reduced as animals learn that the CS predicts the US. This type of expectancy-modulated coding of US information is also seen in the PAG and PAG inactivation attenuates this US signal in LA neurons (47). This suggests that the expectancy modulated US signal in the LA is encoded in (or before) the PAG and then directly or indirectly transmitted to the LA.



Output Circuits for the Production of Fear Behaviors

Following fear conditioning, the CS gains access to the output circuits responsible for producing fear responses. Projections from the LA to the central nucleus of the amygdala (CE), directly and indirectly (possibly through the basal nucleus of the amygdala (62,63) (but see also Herry *et al.* [64]), the prelimbic cortex (for review, see Sotres-Bayon and Quirk [65]) and through the amygdala intercalated cells (see Pare *et al.* [63] and Hitchcock and Davis [66] for review) may provide an output pathway from the LA for the elicitation of fear CRs (67,68). Recent studies (69,70) suggest that a pathway from the lateral division of the CE (CEl) to the medial division of the CE (CEm) transmits CS information through the CE. The CEm is then thought to project to the PAG, hypothalamus, and directly or indirectly to other brainstem effector sites to control specific components of the concerted fear response (1–7).

A number of studies have reported that fear conditioning produces changes in CS processing by CE neurons (69–73). Several recent articles demonstrate that subpopulations of CEl neurons (which are mainly inhibitory) are altered differentially by fear conditioning (69,70,73). CE "on" cells exhibit fear-conditioned enhancement of CS-evoked excitatory responding, whereas "off" neurons show conditioned enhancement of CS-evoked inhibitory responding. In addition to different electrophysiologic subtypes of CEl neurons, there are also many molecularly defined subclasses of CEl neurons (70,74,75). One study (70) identified a molecular marker for CEl-off cells, opening the possibility for genetic and optogenetic manipulation of these neurons (see below). In contrast to CEl neurons, CEm neurons (which are known to receive input from CEl) were primarily excited by a fear conditioned CS. To date, there have been no in vivo physiologic recordings of CS processing during fear conditioning in any CE projection targets involved in producing the individual fear responses.

Figure 2. Strategies for opsin expression. **(A)** Opsins can be expressed using a transgenic approach in specific subpopulations of neurons with tissue-specific promoters such as the interneuron cell specific promoter parvalbumin (PV; circular cells are interneurons and triangles are pyramidal cells). Illustrated here is hypothetical channelrhodopsin-2 (ChR2) expression (filled blue cells) in all PV interneurons in the brain driven by the PV promoter. **(B)** Specific cell populations can also be targeted using a virus-only approach in which a virus can be injected into particular brain regions and produce expression of opsins in specific cells types using cell type restricted promoters such as the PV promoter to target PV interneurons neurons (as illustrated here). This approach has not been demonstrated for PV interneurons, however, and can be nonoptimal for targeting specific cell populations. This is because most viruses have limited packaging capacity, making it necessary to use truncated versions of tissue specific promoters, which can reduce cell-type specificity. Furthermore, only some promoters can be appropriately truncated, which limits the number of cell populations that can be targeted using this approach. **(C)** Specific cell populations can also be targeted using a combined transgenic and virus based approach. In this method, transgenic animals can be constructed that express Cre-recombinase (Cre; see Luo *et al.* [21] for review, and Carlen *et al.* [99] and Sohal *et al.* [100] for recent application) under the control of tissue-specific promoters such as the PV promoter (pictured here as red outlined circular cells). Viruses with expression that is dependent on Cre can then be injected into the specific brain region in which opsin expression is desired. Because the opsins will only be expressed in Cre-expressing neurons (blue filled and red outlined cells), this approach adds cell type-specificity to the spatial selectivity. Overall, this approach offers increased cell type specificity because of the use of endogenous, full-length promoters driving Cre expression and allows better spatial and temporal control of opsin expression than transgenic opsin-expressing animals. Fiber-optic cables attached to a light source can then be inserted into the brain region in which opsins are expressed and optogenetic control is desired.

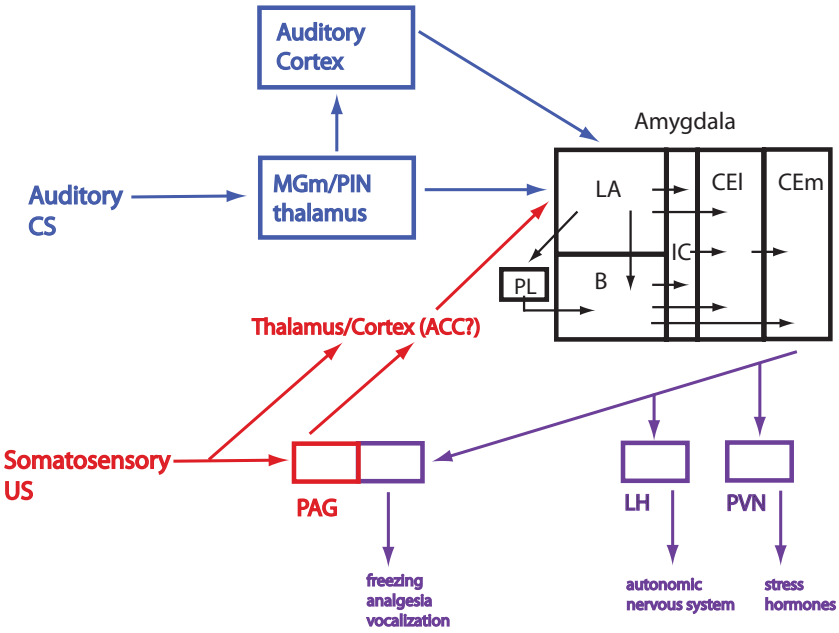


Figure 3. Working model of the fear conditioning circuit. Auditory conditioned stimulus (CS) information is conveyed through medial geniculate (MGm) and posterior intralaminar nucleus (PIN) of the thalamus and auditory cortical regions to the lateral nucleus of the amygdala (LA). Unconditioned stimulus (US) information is conveyed through a pathway that includes the periaqueductal gray (PAG) and other relays, possibly in the thalamus and/or anterior cingulate cortex (ACC), to the LA. Single LA neurons receive convergent CS and US information and undergo associative synaptic plasticity during fear conditioning. Plasticity may also occur in the central nucleus of the amygdala and in the MGm/PIN. LA connects with the central lateral nucleus of the amygdala (CEl) directly and indirectly by way of amygdala connections in the prelimbic (PL), basal (B), and intercalated (IC) amygdala subregions. The central medial nucleus of the amygdala (CEm) receives input from the B and CEl and is an output nucleus which projects to other regions including the PAG, lateral hypothalamus (LH), and paraventricular nucleus of the hypothalamus (PVN) that control the expression of conditioned fear responses, including freezing, autonomic, and hormonal responses. Neural processing of CS and US information has been examined in several of these regions and is described in the text.

Optogenetics and Fear Conditioning

Although a rough outline of the fear circuit has been delineated using traditional techniques, there is still much to be discovered. For example, neurons within particular areas of the fear circuit are known to be activated during specific time periods of fear conditioning (example, CS or US periods), but in most cases, their temporally limited, functional role in behavior and neural processing is unknown. In addition, within specific areas of the fear circuit, there are neuronal subpopulations (some of which were discussed earlier). These subpopulations may be distinguishable on the basis of their unique molecular identity or anatomic projection patterns, but before the advent of optogenetics, it was difficult to target these specific neural elements. Optogenetics offers a means to surmount these issues by providing the ability to 1) manipulate neural firing rate with high temporal precision during specific time epochs of fear conditioning; 2) target manipulations to particular subclasses of neurons, specific afferent input terminals to a given brain region, or specific cell types based on their projection patterns or molecular markers (Figure 4); 3) identify specific cell types during extracellular recordings (Figure 5); and 4) map the detailed connectivity of defined inputs to cells in a given brain region (Figure 6).

Cell-Type-Specific Manipulations with Precise Temporal Control

As mentioned earlier, lesion, pharmacologic inhibition, and electrical/pharmacologic techniques have a number of limitations. Lesion and pharmacologic manipulations affect cell processing permanently or throughout the entire behavioral training or testing session and commonly modulate activity across all cell populations in a given region. Electrical stimulation, although more temporally precise, stimulates all cell types and fibers of passage. Optogenetics provides the ability to manipulate defined cell types (using tissue specific promoters or conditional viruses in combination with Cre lines to drive expression of opsins; see Figures 2 and 4) during specific temporal epochs of fear conditioning.

Although the use of this technique is in its early stages, it has already been exploited successfully in a few fear conditioning papers. Using a minimal version of the Ca^{2+}/Calmodulin-dependent protein kinase II promoter, one set of studies preferentially targeted LA pyramidal cells (as opposed to LA inhibitory interneurons) using a viral approach in rats during fear conditioning to examine the mechanisms through which the aversive US triggers learning (76,77). It has generally been assumed that LA plasticity and fear

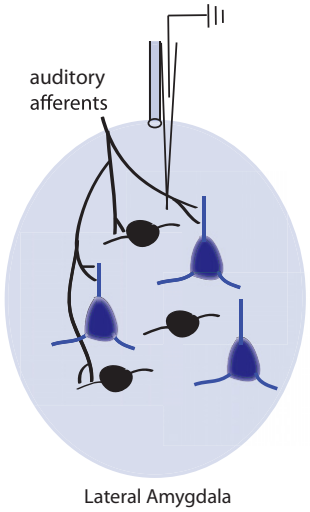


Figure 4. Virus mediated targeting of lateral amygdala (LA) pyramidal neurons and not interneurons. LA pyramidal neurons can be targeted (75) using a minimal Ca2+/calmodulin-dependent protein kinase II (CAMKII) promoter that can be used to preferentially drive expression in CaMKII+ (illustrated here as blue cells), as opposed to gamma-aminobutyric acid +, neurons. Laser light (473-nm wavelength) can then be shone into the LA (blue sphere in figure) through a fiber-optic cable to manipulate fear learning and behavior. This technique could also be used in conjunction with in vivo physiology to record single neurons or field potential responses. It would then be possible to manipulate activity specifically in LA pyramidal cells (blue cells) and examine the effects of these manipulations on neural processing and associative plasticity in the LA. Other populations of LA neurons, such as interneurons (black cells), could also be targeted. However, this would likely require a transgenic approach or the combination of transgenic mouse or rat lines expressing Cre-recombinase in defined neuronal populations with Cre-dependent viruses for opsin expression (as has been done previously) (99,100).

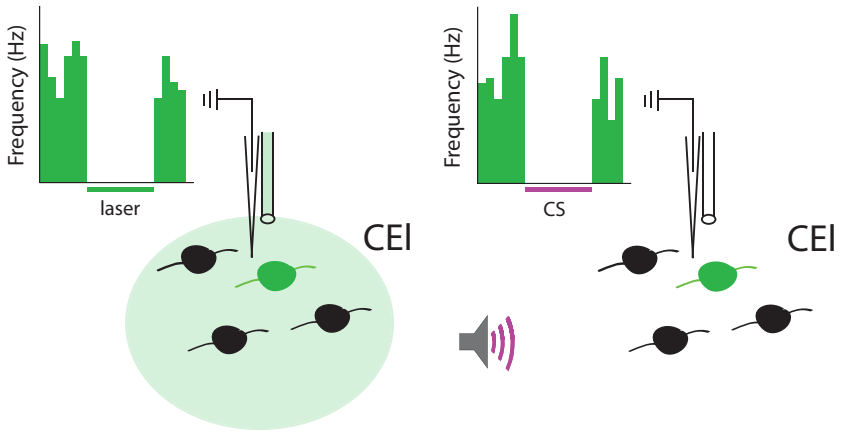


Figure 5. Optogenetic identification and characterization of molecularly defined cell types in the central lateral nucleus of the amygdala (CEl). Molecularly or anatomically defined cell populations can be identified through optogenetic manipulations, and their neural coding can be assayed. In this example, protein kinase Cδ positive (PKCδ+) cells in CEl are targeted (green cells) with an inhibitory opsin using a transgenic mouse expressing Cre-recombinase driven by a PKCδ promoter combined with injection of a Cre-dependent virus encoding an inhibitory opsin into the CEl. After a single cell has been isolated, light can be shone onto it (left panel). Inhibition of neural activity by green or yellow light (green sphere in figure) identifies the cell as PKCδ+. This is shown in the perievent time histogram of hypothetical data in which firing rate (y axis) is reduced during the laser on period (green bar under x axis). The neural response to various experimental manipulations can then be assessed (right panel). In this illustrative case, conditioned stimulus (CS) presentation (purple line under x axis) inhibits neural activity in the cell. A nonoptogenetic technique has been used previously to identify PKCδ cells in CEl as being CEloff cells (70), and it is shown here to illustrate the potential for optogenetic identification of specific cell populations. Thus this technique allows online identification of individual cell populations while recording in the awake, behaving animal, which will then facilitate the study of how these specific cell populations encode sensory (in this case) or other types of information.

learning involve associative Hebbian mechanisms in LA pyramidal cells. Thus it is thought that US-evoked depolarization in LA pyramidal neurons triggers plasticity of coactive CS inputs onto the same cells resulting in fear memory formation. If this is true, then pairing an auditory CS with direct depolarization of LA pyramidal neurons, in place of an actual footshock US, should produce learning. By targeting expression of ChR2 to LA pyramidal neurons and delivering laser light to this population of cells, this study (76) showed that large numbers of CS-laser US pairings produced some fear learning and memory. However, this learning was weak, and more recent preliminary work (77) has found that under normal training conditions (i.e., lower numbers of CS–US pairings) US-evoked depolarization of LA pyramidal cells is not sufficient to produce normal levels of fear learning and that a multiprocess mechanism involving US-evoked depolarization and activation of the noradrenergic system is important in triggering fear memory formation. The use of optogenetics in these studies to manipulate cell activity in a specific subpopulation of neurons (LA pyramidal cells) during a temporally defined period made it possible to test a question that was not addressable previously (Figure 4).

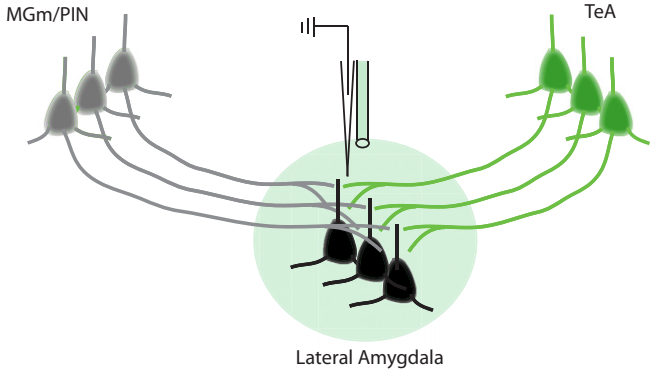


Figure 6. Optogenetic control of specific afferent inputs to the lateral nucleus of the amygdala (LA). Infection of temporal association cortex (TeA) cells with an opsin-expressing virus (an inhibitory opsin in this case) will produce opsin expression in TeA terminals in the LA. Green or yellow light (green sphere in figure) delivered through an in-dwelling fiber-optic cable can then be used to inhibit the release of neurotransmitter specifically from TeA terminals while not affecting other inputs such as those from medial geniculate/posterior intralaminar thalamic nuclei (MGm/PIN, gray cells). When combined with in vivo neural recording in awake, behaving animals, it would be possible to determine the contribution of TeA inputs to neural coding (e.g., of conditioned stimulus information) in LA neurons (black cells) and to fear behavior.

Another recent experiment that elucidated the functional microcircuitry of CE (as discussed in Ciocchi *et al.* [69]) used a viral based optogenetic approach in mice to determine whether stimulation of CEm neurons is sufficient to produce freezing behavior (the most well-studied response to fear inducing stimuli). As discussed, CEm is thought to be an important output nucleus of the amygdala for producing conditioned fear responses, but it was not clear, before this study, whether excitation or inhibition of CEm cells produced fear responses such as freezing. Previous work did find evidence that stimulation of CEm drives freezing behavior, but these studies used electrical stimulation, which excites fibers of passage in addition to cell bodies. Furthermore, one previous experiment found that putative projection neurons in the CE were inhibited by CSs, suggesting that inhibition of CE neurons may produce freezing behaviors. The recent study demonstrated that CEm projection neurons were robustly excited by CSs following fear conditioning and that direct, optical stimulation of CEm neurons was sufficient to produce freezing responses.

These are just the first few studies using optogenetics to study fear, but the potential for its use in understanding the function of specific cell populations during temporally defined periods of fear conditioning is impressive. For example, one potential application would be to elucidate the functional and temporal contribution of the many subpopulations of neurons in the CEl (70,75,78,79) to the learning and performance of fear conditioning by using Cre lines specific for these subpopulations. The recent genetic engineering of PKCδ and CRF promoter driven Cre mice (70,80) (and other Cre

lines are available through commercial suppliers) could be used to optogenetically target specific cell populations in the CE. It will also be possible to combine recordings from single neurons in the awake, behaving animal with optogenetics (81–84) and examine the contribution of these populations of CEl neurons to coding in CEm neurons and cells in areas that receive CE projections such as the PAG. Furthermore, optogenetics allows for identifying cell types during extracellular single unit recordings (85). Using this approach, molecularly or anatomically defined subtypes of neurons can be recorded from awake animals during fear conditioning, and their information coding capabilities can be assayed (Figure 5).

Subtype and Afferent-Specific Control of Neural Circuits Based on Anatomic Connectivity

Another advantage that the optogenetic approach affords is the ability to control the activation of specific afferent inputs in a given brain nucleus. Expressing excitatory or inhibitory opsins in neurons in one brain region, results in expression of the corresponding opsins throughout the cell, including in the axons and synaptic terminals in brain structures distant from the region that was originally infected/transduced. Synaptic release can then be controlled by shining light onto the terminals of these neurons (86–94). Optogenetic control of afferent terminals expressing opsins has been used to map circuit structure (see below) and to manipulate behavior. For example, using a viral approach in rats, one study found that optical excitation of basal amygdala projections to CEm reduced anxiety-like behavior and inhibition of these same terminals enhanced such behavior (90). Interestingly, this effect was not seen when the cell bodies of these neurons were manipulated, demonstrating afferent-specific modulation of behavior.

Other viral-based approaches have recently been used that allow control of specific cell populations in a given brain region based on their projection patterns to other brain regions. For example, several recent studies have taken advantage of certain viruses that are taken up preferentially by synaptic terminals and transported retrogradely to the cell bodies of these terminals in other brain regions (25,85). This makes it possible to express opsins and control neural activity in cells that project to the brain region in which a virus is introduced. Another similar approach uses transsynaptic rabies viruses to express opsins in retrogradely transduced cell populations that project to a specific subpopulation of target neurons (95). To date no one has used either of these approaches to manipulate behavior. However, they could allow light control of particular subpopulations of neurons in a given brain region based on their anatomic connectivity with other brain regions or with specific postsynaptic neurons.

Combined with the temporal control that optogenetics allows, these strategies have obvious advantages for studying the circuits and computations mediating fear conditioning. For example, the MGm/PIN and TeA both project to the LA and likely provide different types of information to LA neurons (36–38) during specific temporal epochs. However, both of these regions contain heterogeneous subpopulations of cells that project to brain regions other than the LA, making it difficult to interpret the results of manipulations that target all subpopulations of neurons in these regions. To examine the specific role of the thalamic and cortical projections to the LA, a virus encoding excitatory or inhibitory opsins could be injected into the MGm/PIN or TeA. This would allow control of the terminals of these neurons in the LA and make it possible to determine the functional/temporal contribution of these inputs to fear behaviors and to neural coding in LA (Figure 6). Alternatively, a virus that is taken up by synaptic terminals could be injected into the LA where it would travel retrogradely to the MGm/PIN and TeA neurons that project there. This technique has not been used to control behavior (25,85), but it could allow light control of the specific MGm/PIN or TeA neural subpopulations that project to the LA. Although in early stages of development, these two complementary approaches could be widely used in the fear circuit to determine the functional involvement of anatomically defined cell populations and their synaptic inputs in specific brain regions to fear conditioning and to neural processing.

Mapping Circuit Connectivity

Optogenetic control of specific synaptic afferents to a given brain region has also been used to map circuit connectivity. For example, ChR2 has been expressed in various thalamic and cortical regions as well as in basal ganglia circuits, and the afferent axons of these cells were stimulated in projection regions to determine the distinct connectivity of these inputs in target neurons (86–89,91,92). Using this approach combined with imaging of cortical neurons, one study mapped out both the laminar specificity of different inputs to the barrel cortex as well as the subcellular specificity of these inputs onto different regions of the dendritic arbor (86).

This technique has also been applied to the amygdala. One study (89) used a viral approach in mice and infected TeA or anterior cingulate cortex (ACC, which may convey US information to the LA) neurons with ChR2 and strongly stimulated TeA or ACC inputs in the LA to produce synaptic plasticity. The authors found that high-frequency stimulation-induced long-term potentiation (a cellular model of synaptic plasticity) only occurs in the TeA-LA pathway if feedforward inhibition is blocked but that ACC-LA long-term potentiation does not recruit feedforward inhibitory circuits in the LA. This suggests that synaptic plasticity in TeA-LA CS input pathway may be modulated by feedforward inhibitory circuits. This approach has also been used to reveal the connectivity between a particular subclass of CEl neurons and CEm output neurons and to elucidate a specific intra-amygdalar pathway that includes basal nucleus-CEl-CEm connections (70,90). These types of approaches along with traditional techniques (96) can be used in future studies to, for example, map out the detailed connectivity of different afferent inputs to the LA (and to other parts of the fear circuit) and reveal how postsynaptic LA neurons integrate information from these input pathways. This approach could also be used to study how the local LA circuits and integrative properties of the postsynaptic cells together contribute to synaptic plasticity at particular input pathways.

Future Directions

We have limited our discussion here to a few optogenetic applications that we believe will be most advantageous for studying the circuits and computations underlying behavioral fear conditioning. Although there are some caveats to consider (Supplement 1) advances in molecular biology will help to refine and expand this technology and will likely offer new unexplored avenues of study to researchers from a broad range of disciplines. Using optogenetic manipulations in combination with behavior and physiology, it will be possible to reveal, in much greater detail, the temporal contribution of specific inputs and cell types to fear behavior and to neural coding. Eventually, this will provide an avenue toward the ultimate goal of understanding how brain circuits and computations within these circuits mediate fear behavior and may suggest general mechanisms of circuit and computational coding that are shared by many neural systems.

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Controlling the Elements: An Optogenetic Approach to Understanding the Neural Circuits of Fear

Supplemental Information

Description of All Amygdala Related Optogenetic Studies

Tang W, Ehrlich I, Wolff SB, Michalski AM, Wolfl S, Hasan MT, *et al.* (2009): Faithful expression of multiple proteins via 2A-peptide self-processing: a versatile and reliable method for manipulating brain circuits. *J Neurosci* 29:8621-8629.

-Demonstrated functional ChR2 and Halorhodopsin expression in lateral amygdala neurons from a construct which used 2A peptide bridges to express both of these proteins off of the same promoter.

Johansen JP, Hamanaka H, Monfils MH, Behnia R, Deisseroth K, Blair HT, LeDoux JE (2010): Optical activation of lateral amygdala pyramidal cells instructs associative fear learning. *Proc Natl Acad Sci U S A* 107:12692-12697.

-Expressed ChR2 in pyramidal cells in the lateral amygdala and demonstrated in-vivo control of neural activity using light in amygdala neurons. In behavioral experiments, the authors were able to produce behavioral fear conditioning by pairing an auditory stimulus with optogenetic activation of lateral amygdala (LA) pyramidal neurons in place of an actual shock unconditioned stimulus.

Ciocchi S, Herry C, Grenier F, Wolff SB, Letzkus JJ, Vlachos I, *et al.* (2011): Encoding of conditioned fear in central amygdala inhibitory circuits. *Nature* 468:277-282.

-Elucidated the functional subcircuitry of central nucleus of the amygdala (CE) during fear conditioning. This study showed that fear conditioning induces differential changes in auditory conditioned stimulus (CS) processing in CE neurons and electrophysiologically defined two subclasses of neurons in the lateral division of the CE (CEl). In addition, this work showed that neural activity in the medial division of the CE (CEm) is necessary for expression of previously learned fear responses and activation of CEm neurons is sufficient to produce freezing behavior. Furthermore, this work demonstrated that CEl neural activity is necessary for the acquisition of fear conditioning.

Haubensak W, Kunwar PS, Cai H, Ciocchi S, Wall NR, Ponnusamy R, *et al.* (2010): Genetic dissection of an amygdala microcircuit that gates conditioned fear. *Nature* 468:270-276.

-Determined the local anatomical connectivity and functional contribution of a molecularly defined subset of CEl neurons. This work showed that protein kinase Cδ (PKCδ) expressing neurons corresponded to a an electrophysiologically identified subclass of CEl neurons and that

PKCδ+ cells inhibited fear output neurons in the CEm. Finally, this study demonstrated that inhibiting neural activity in PKCδ+ cells enhanced learned fear responses.

Tye KM, Prakash R, Kim SY, Fenno LE, Grosenick L, Zarabi H, *et al.* (2011): Amygdala circuitry mediating reversible and bidirectional control of anxiety. *Nature* 471:358-362.

-Showed that optogenetic excitation of B neuron projection terminals to CEl reduced anxiety-related behaviors and that optogenetic inhibition of these terminals was anxiogenic. Importantly, no effect on anxiety behaviors resulted from manipulations of the cell bodies of these projections in the B. This work was the first to demonstrate an effect of optogenetic manipulation of a specific subset of synaptic inputs to a brain region, and not the cell bodies from which these inputs originated, on behavior.

Morozov A, Sukato D, Ito W (2011): Selective suppression of plasticity in amygdala inputs from temporal association cortex by the external capsule. *J Neurosci* 31:339-345.

-Used optogenetics to stimulate and induce long-term potentiation (LTP) at either anterior cingulate cortex (ACC) or temporal association cortex (TeA) inputs to the LA and found that TeA-LA, but not ACC-LA, LTP was under the control of feedforward inhibitory networks. This work demonstrated pathway specific recruitment of inhibitory networks during the induction of synaptic plasticity in the LA.

Stuber GD, Sparta DR, Stamatakis AM, van Leeuwen WA, Hardjoprajitno JE, Cho S, *et al.* (2011): Excitatory transmission from the amygdala to nucleus accumbens facilitates reward seeking. *Nature* 475(7356):377-80.

-Showed that optogenetic stimulation of B inputs to the nucleus accumbens (NAcc) reinforced lever pressing behavior and that inhibition of B-NAcc inputs reduced cued reward seeking behavior. This effect did not occur when cortical inputs were stimulated demonstrating pathway specific modulation of reward seeking behavior.

Considerations and Caveats

While optogenetics has the potential to revolutionize the study of the nervous system, there are some things to consider when using this technique. For example, lack of tissue specificity and low expression levels can be issues when using minimal tissue specific promoters in combination with viral transduction (1-3). To address these potential problems, it is essential to test any new promoter/virus combination in vivo to determine both its tissue specificity and its infection efficacy. This problem can at least be partially avoided by the use of transgenic animals, which can give high opsin expression in defined neuronal populations. However, such an approach lacks the brain area specificity offered by virus-based methods. Anatomical selectivity

and high expression levels can be obtained, in principle, when using conditional viral vectors in Cre mouse lines. It should be considered, however, that neuronal subpopulations targeted in this manner are often still heterogeneous. In the long run, intersectional strategies, based on more than one molecular marker, may help to refine the targeting of opsins to specific cell types.

Light delivery to large brain structures can also present a problem for the use of optogenetics, especially when using fiber optic approaches to target deep brain structures. While ongoing work is attempting to address this problem (4,5), it is important to keep this in mind when designing optogenetic studies.

In case bidirectional control of neuronal activity is required in the same neurons, it is necessary to use viruses for co-expression of different opsins (see (6)). However, each construct has to be validated for appropriate co-expression. This is especially true for internal ribosome entry site (IRES)-based co-expression constructs, while 2A-based solutions may be more reliable. Furthermore, if the opsins are expressed in different neuronal populations, they have to be carefully chosen to ensure minimal overlap of their activation spectra.

Although the functionality of the optogenetic approach has been shown several times, even in vivo, it should always be tested whether the targeted cells indeed respond to the light stimulus. Electrophysiological recordings in vivo are the most direct way to test this. Furthermore, it is ideal not only to manipulate neuronal activity and to analyze the behavioral effects, but to monitor the light-induced physiological effects in both the transfected and the non-transfected cells. This facilitates much stronger conclusions about the function of defined circuit elements in behavior.

When using optogenetics to identify extracellularly recorded neurons in vivo, it has to be verified that the observed light responses are caused by direct stimulation of the recorded cell rather than indirect network effects. Especially, ChR2-mediated excitation of an entire cell population can cause indirect activation of non-expressing cells which may look similar to direct light-induced activation. A first criterion to address this issue is the latency of the light-response. However, this may also be misleading, since response latency can depend on levels of ChR2 expression and strength and stability of illumination. Therefore, additional criteria (e.g., spike waveform, cross-correlations, spontaneous firing, etc.) can be used to complement the optogenetic identification. Alternatively, one should consider using inhibitory opsins for cell identification because indirect effects are less likely to occur in this case.

Supplemental References

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