Review

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The optogenetic revolution in memory research

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Over the past 5 years, the incorporation of optogenetics into the study of memory has resulted in a tremendous leap in this field, initiating a revolution in our understanding of the networks underlying cognitive processes. This review will present recent breakthroughs in which optogenetics was applied to illuminate, both literally and figuratively, memory research, and describe the technical approach, together with the opportunities it offers. Specifically, a large body of literature has been generated, setting the foundation for deciphering the spatiotemporal organization of hippocampal-based memory processing and its underlying mechanisms, as well as the contribution of cortical and amygdalar regions to cognition.

Contemporary and traditional methods in memory research

One of the biggest mysteries in neuroscience, which attracts a great volume of research, is the way in which memories are acquired, consolidated, and retrieved. In decades of using techniques such as physical, pharmacological, and genetic lesions of specific brain areas, combined with electrical or pharmacological stimulation of these areas, pioneering studies had identified the major brain areas that are involved in various memory tasks. However, the precise functional connections between these different regions (as well as the contribution of other areas, hitherto considered irrelevant) remained only partially understood. Additionally, it was impossible to causally implicate the real-time contribution of specific neuronal populations within these structures in cognitive function. The main obstacle until recently was the inability to specifically control genetically defined neuronal populations in real time in behaving animals performing cognitive tasks. The realization of this seemingly impossible requirement came with the development of optogenetics – allowing real-time control of genetically defined neuronal populations with millisecond precision [1–4] (Figure 1).

Since the first successful use of opsins in neurons in 2005 [5], the technique has been enthusiastically embraced by the neuroscience community and has led to a dramatic renaissance in this field. Specifically, a major scientific effort was invested in studying the neural basis of memory, because optogenetics provided an opportunity to delve into the fine mechanisms underlying this process. Indeed, an impressively elaborate new body of knowledge, including major breakthroughs in this field, has accumulated during less than 5 years. This review will describe the optogenetic toolbox (Box 1, Figure 1), its limitations (Box 2), and the knowledge gained by the implementation of optogenetics in the study of brain regions, functional connectivity, and the identity of neurotransmitters contributing to memory.

Novel findings in hippocampal memory research from optogenetic studies

The vast majority of optogenetic memory studies were performed using the fear conditioning (FC) paradigm (Figure 2). This method offers a defined anatomy of the basic neuronal circuit as the major brain areas involved in fear memory were recognized decades ago. In recent years, the incorporation of optogenetics (Box 1) enabled us to causally pinpoint the real-time roles of specific neuronal populations within these structures and the functional connections between them. Several aspects of hippocampal involvement in memory (Figure 3) will be discussed. In each subsection, examples of the way in which optogenetics has been used to substantiate long-standing hypotheses, modify others, and provide a basis for new theories will be specified.

Diverse roles for hippocampal subregions in memory

The hippocampus comprises several subregions and the basic connections between them are known. However, many past studies had tested the involvement of the hippocampus as a whole in fear memory [6], and only few (e.g., [7–9]) focused on specific regions. Thus, we do not yet fully understand the unique real-time contribution of each region to learning and memory processes. To date, optogenetic experiments have shed light on differences between the roles of the primary region to receive information from the cortex (dentate gyrus, DG) and the final region in hippocampal processing (CA1) in memory.

The exclusive role of CA1 in fear memory acquisition was demonstrated in the past using CA1-specific NMDA knockout [7], and lately its real-time contribution was tested by targeting pyramidal neurons throughout the dorsal CA1 with the inhibitory opsin NpHR [10]. Continuous CA1 optogenetic inhibition during FC training prevented the acquisition of contextual FC, and this effect was reversible, because when these mice were retrained without CA1 optogenetic inhibition they showed no deficit in memory acquisition [10]. Another study disturbed the

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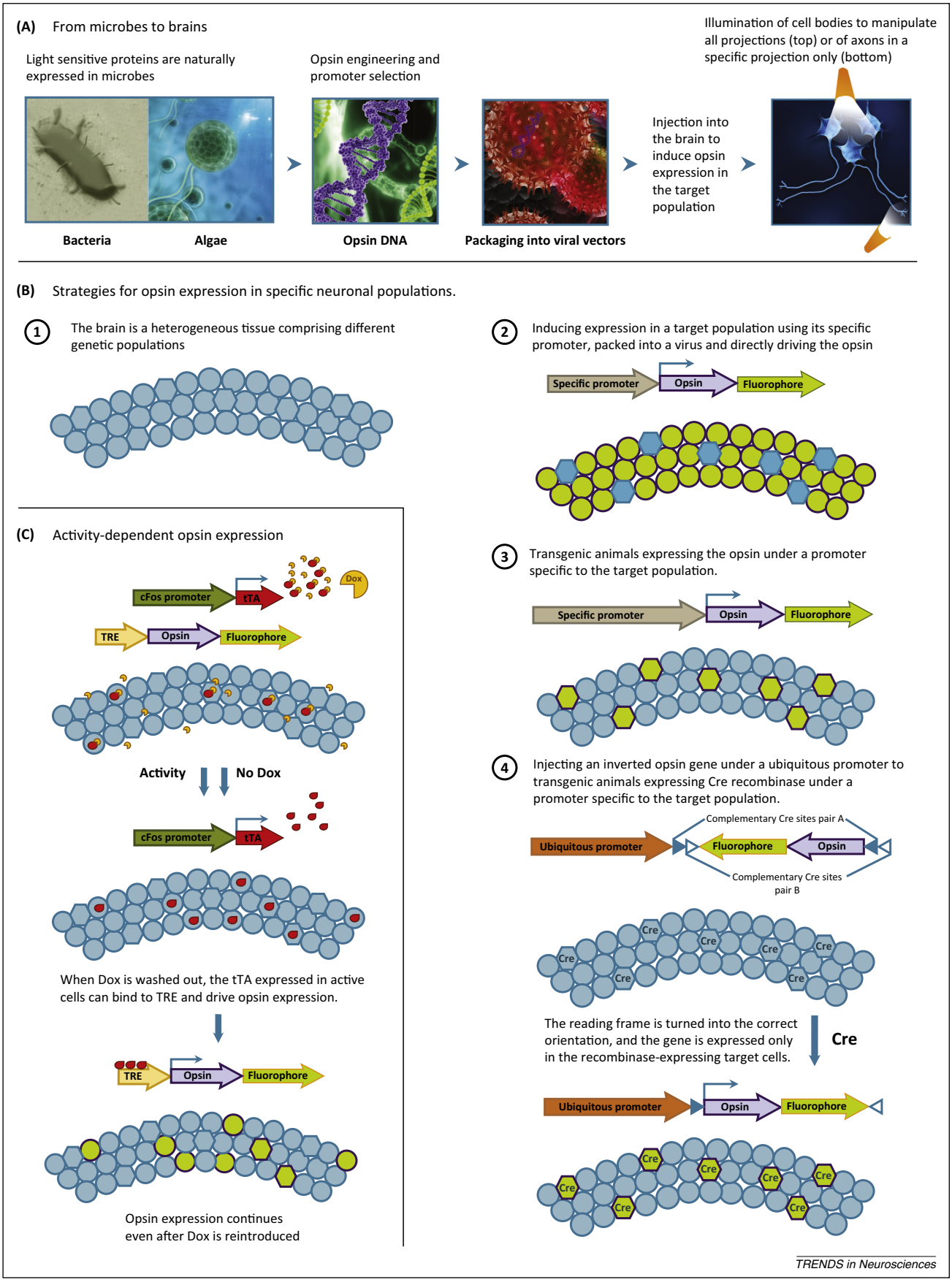

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Box 1. The principles of optogenetics

Optogenetics allows specific subpopulations of cells to be controlled by light with millisecond precision using microbe-derived light sensitive proteins (opsins) [1–4] (Figure 1A). In recent years, opsins have been engineered to offer a diverse toolbox for neuronal excitation or inhibition, as well as control of intracellular signaling [1,91,92]. The microbial opsins used for neuronal excitation are light-sensitive cation channels that depolarize the cell upon illumination, typically with blue light. Proteins from the channelrhodopsin (ChR) family have been engineered to allow a better imitation of the physiological activity of different cell populations by manipulating their kinetics, preferred wavelength, and ionic selectivity [91]. Inhibition is mediated by light-activated ion pumps that hyperpolarize the cells in which they are expressed upon illumination [93–95]. The inhibitory opsins NpHR (*Natronomonas pharaonis* Halorhodopsin) and Arch (*Halorubrum sodomense* Archaerhodopsin-3) are sensitive to yellow light, thus allowing combinatorial manipulation of excitation (by blue light) and inhibition (by yellow light) within a single experimental setup [95].

To induce opsin expression in discrete cell populations, a foreign DNA sequence encoding for the opsin has to be delivered into the brain. In most cases, this DNA is delivered using viral vectors injected into the area of interest. Within days to weeks, the opsins are expressed throughout the cell membrane, including distant projections. Illumination is achieved by implanting an optical fiber right above the region of interest and connecting this fiber into a light source [1]. In experiments requiring projection targeting, cell bodies are infected in one brain region and their axonal projections are illuminated in another (Figure 1A).

Genetically defined cell type specificity can be achieved by three main strategies (Figure 1B): if the known unique promoter of a specific cellular population is strong and small enough, it can be packed into the viral vector together with the opsin gene and directly drive expression in the relevant cells [1]. Alternatively, if the relevant promoter is too weak to drive sufficient expression, or too large to be packed into a virus, transgenic animals can be used. Indeed, several mouse lines expressing opsins in the brain were developed [1]. A more versatile strategy is to use transgenic mice that express Cre recombinase in a specific cellular population, and then introduce a virus containing any opsin DNA in an inverted orientation, flanked by two sets of incompatible Cre recombinase recognition sequences [1,2]. Only in the cells that transgenically express Cre will the DNA sequence be flipped into the correct orientation, allowing transcription of the coding region under a strong ubiquitous promoter [1,2]. Lastly, new genetic tools now allow opsin expression based on cellular activity, providing an opportunity to specifically control a population of neurons that participated in a certain behavioral task using optogenetics (Figure 1C) [77,78].

The use of optogenetics in behavioral studies offers a combination of advantages (cell type specificity, high temporal and spatial resolution) without the tradeoffs between them that are inherent in other methods. The optogenetic toolbox is rapidly expanding, offering a range of potential modulations.

normal function of CA1 not by inhibiting this region but rather by increasing its activity by NpHR inhibition of somatostatin-expressing dendrites in CA1, causing disinhibition of the pyramidal neurons [11]. This manipulation also resulted in fear acquisition impairment [11]. Together, these two experiments show that any deviation from the normal function of CA1 neurons (either inhibition or excitation via disinhibition) during FC acquisition results in impaired performance, demonstrating the importance of this region in conditioning.

When light was delivered to mice expressing NpHR in the CA1 during recall (rather than acquisition), the memory that had been previously present became unavailable under illumination [10]. Thus, CA1 excitatory neurons are involved in both acquisition and recall of contextual fear memory. Conversely, this is not the case for the DG. Kheirbek *et al.* [12] expressed opsins specifically in DG granular neurons using the proopiomelanocortin (POMC)-Cre mouse line [8]. When the activity of dorsal DG neurons was inhibited by NpHR during contextual FC acquisition, memory was impaired, but when the DG was inhibited during memory recall, no effect was observed [12]. Interestingly, different results were obtained when DG cells were activated with ChR2 rather than inhibited. Specifically, illumination of ChR2-expressing DG granule cells during conditioning disrupted both acquisition and recall [12]. A similar result was obtained when DG activation was indirectly induced by using NpHR to inhibit the hilar GABAergic input to granular cells [13]. In that case, DG hyperactivity resulted in impaired recall of the position of an escape platform in a water maze probe test, but had no effect on the gradual acquisition of spatial memory [13]. Furthermore, DG inhibition had no effect either on the gradual acquisition or on the retrieval in an active place avoidance task, where mice were trained to avoid a stationary shock zone based on spatial cues in a circular arena [12]. However, when the position of the shock zone was switched, requiring a rapid encoding of a new contingency and resolution of two conflicting memories, DG inhibition resulted in a marked cognitive impairment. Together, these data suggest that the DG is necessary for rapid memory acquisition and cognitive flexibility, but not for gradual memory acquisition or the recall of established memories. The latter, however, can be disturbed by nonsense hyperactivation of the DG during recall.

To conclude, it seems that both DG and CA1 are necessary for the acquisition of contextual memory traces. However, during recall, CA1 is necessary whereas DG silencing

Figure 1. The principles of optogenetics. **(A)** DNA encoding light-sensitive proteins is extracted from microbes (algae or bacteria) and tailored to the desired use. Engineering can modify the kinetics, wavelength sensitivity, expression pattern, and ionic selectivity, for example. The genetic material is then packed into a virus and injected into the brain. The opsin is expressed in the targeted cell bodies and throughout their processes (dendrites and axons), and illumination can then be delivered either to the cell bodies or to a specific projection, depending on the scientific question. **(B)** Opsin targeting to genetically defined populations: (1) the brain is a non-homogeneous tissue comprising different genetic populations of neurons and glia. For simplicity, only two cell types (round and hexagonal) are presented. (2) A known strong, short, and unique promoter of a specific cellular population can be packed into the viral vector together with the opsin DNA to directly drive expression in the relevant cells. (3) An opsin can be transgenically expressed in a specific population under a strong promoter of unlimited size. (4) Transgenic mice can express Cre recombinase in a specific cellular population under a large or relatively weak promoter. These mice can be injected with a virus containing an opsin gene in an inverted orientation, flanked by two sets of incompatible Cre recombinase recognition sequences driven by a strong ubiquitous promoter (top). Only in the cells that transgenically express Cre the DNA sequence is flipped into the correct orientation, allowing transcription of the coding region (bottom). **(C)** Opsin targeting to activity defined populations can be achieved by using the cFos-tTA mouse line, expressing a tetracycline transactivator (tTA) under the promoter for the neuronal activity-dependent gene cFos. tTA is expressed in cells that were recently active following a specific task. The cFos-tTA mice can be injected with a virus encoding for an opsin, under the tetracycline responsive element (TRE), which depends on tTA binding to drive the opsin. Thus, neuronal activity will drive the expression of tTA, which in turn will enable the expression of the opsin in the active cells only. To restrict this expression to a specific time window, mice are kept on a diet containing doxycycline (Dox), which prevents tTA from binding to

Box 2. Challenges and limitations in optogenetics

Although optogenetics offers a variety of advantages, described throughout this review, the technique has several limitations. Some of the challenges and possible solutions where such may exist are detailed here.

Invasiveness. Optogenetics requires fiber insertion into the brain, usually combined with intracranial virus injection. These technical issues are the simplest to tackle. As new red-shifted opsins are developed, the fiber position can move farther away from the area of interest (even above the dura, e.g., [64]), as longer wavelengths better penetrate the tissue. Second, the effect that a strong expression of a foreign protein may have on cellular function should always be controlled for by expressing a fluorophore alone under the same promoter in the control group.

Cellular specificity versus inherent complexity. A major strength of optogenetics is the ability to perturb the function of a specific neuronal population in real time. However, it is likely that normal activity in brain circuits is based on the combined simultaneous contribution of several cell types and that combinatorial manipulation of two (or even more) distinct populations will provide a deeper understanding of the natural circuit. As the optogenetic toolbox is expanding to include opsins with a wide range of spectral sensitivity such experiments gradually become possible (e.g., [64]), because several opsins with different spectral preference can now be used together in different populations. Such experiments bring us closer to a more comprehensive circuit perturbation.

Forced activation patterns. The major (and most challenging) obstacle in optogenetic excitation is that the illuminated neurons are simultaneously 'locked' into a certain frequency. Optogenetic activation *in vivo* is currently achieved by using constant frequencies (changing between, but not within, experiments) of square light pulses, illuminating relatively large populations. Such illumination does not provide a good resemblance to real-life stimulation patterns in a specific region (and certainly not in a specific cell). Because the differences between simple tonic and phasic stimulation patterns yielded interesting insights [96], further increasing the complexity of the light stimulus by varying the frequency and intensity within the stimulation pattern, as done electrically in dynamic clamp [97], may yield interesting results. Similarly, future *in vivo* application of patterned illumination of specific neurons will also bring us closer to a true perturbation of complex circuits [4,94].

Brief illumination – permanent effect? Surprisingly, little is known about the long-term effects of optogenetic stimulation. However, it is likely that a modulation that efficiently recruits a population of cells will result in plastic changes, especially as ChRs allow a direct influx of Ca^{2+} through the channel, which may directly influence long-term processes in the cell. A few studies have already shown that optogenetic activation can independently alter neuronal networks in culture [98] and support long-term potentiation in slices [99]. Interestingly, effects on synaptic plasticity were also observed following neuronal inhibition with NpHR, as a result of changes in the $GABA_A$ receptor reversal potential [100]. Thus, optogenetic modulation may remodel the perturbed circuit during the experiment. This issue cannot be avoided, but can be monitored by comparing baseline and post-illumination neuronal activity and/or behavior whenever possible.

has no effect (Figure 3). What could be the reason for that difference? Currently, not enough data are available to fully answer this question. It could be hypothesized that because CA1 is the main output region of the hippocampus, any disruption of its activity during a hippocampal-dependent task will have a significant effect, even if the actual computation required for recall is performed elsewhere, and the CA1 only serves as a conduit. The DG, however, could be bypassed via direct connections between the entorhinal cortex and CA3 or CA1 [14,15]. New techniques to optogenetically target other hippocampal regions such as CA2 [16,17] and CA3 may resolve this question. There are, however, several structural and functional differences between DG and CA1 that may contribute to the different roles these regions play in contextual memory. First, there are significant differences in the ways in which neuronal ensembles in these structures encode specific information: the DG is characterized by sparse coding [18,19], that is, only a small fraction of the neurons in this area are activated by any specific behavioral experience. Consequently, the detrimental effect of DG hyperactivation [12] is probably due to the fact that the information conveyed by the DG to the following hippocampal stations is now meaningless, because not only the neuronal ensemble encoding the specific context is activated but also many other neurons.

Another unique characteristic of the DG is neurogenesis: the continuous differentiation and integration of newly formed neurons into this structure. Ablation of these new neurons results in memory impairments and their function evolves as they integrate into the existing network [20,21]. To examine the time-dependent contribution of newly formed DG neurons to memory, Gu *et al.* [22] targeted the inhibitory opsin Arch to these neurons by using retroviruses (which only affect dividing cells). Four weeks later, mice were trained in an FC paradigm and the 4-week-old neurons (which already form synapses with CA3 pyramidal neurons and demonstrate enhanced plasticity) were optically inhibited during FC recall, resulting in impaired contextual memory [22]. Interestingly, when 2-week-old or 8-week-old neurons were silenced no behavioral effect was observed, thus narrowing the time window of functionally relevant enhanced plasticity in newly formed cells. This is another elegant demonstration of the power of optogenetics – allowing the first opportunity for real-time control of a specific cellular population, in this case based on the age of the cells.

Dorsoventral axial variations in hippocampal activity

Based on the great variance in afferent and efferent connectivity along the dorsoventral axis of the hippocampus, it was hypothesized that the dorsal and ventral hippocampus also have different functions [23,24]. Specifically, the dorsal hippocampus projects extensively to associative cortical regions, suggesting a role for this area in spatial and contextual memory, whereas the ventral hippocampus projects to the prefrontal cortex (PFC), amygdala, and hypothalamus, suggesting a role in emotional processing [24–26]. Indeed, most studies had shown disrupted spatial memory after dorsal hippocampus lesions, whereas lesions of the ventral pole spared spatial learning but had an anxiolytic effect [23,27,28]. However, other studies reported opposite results [29,30]. Further, the real-time involvement of the dorsal and ventral hippocampus in memory and anxiety remains unclear. A recent study addressed these open questions by using optogenetic manipulation of either the dorsal or ventral DG and testing the effect of illumination on cognitive and emotional behaviors. As discussed above, Kheirbek *et al.* [12] expressed NpHR or ChR2 specifically in DG granular neurons and found the dorsal DG to be involved in contextual FC acquisition and in the resolution of conflicting memories. Ventral DG manipulation, by contrast, had no effect on contextual memory acquisition.

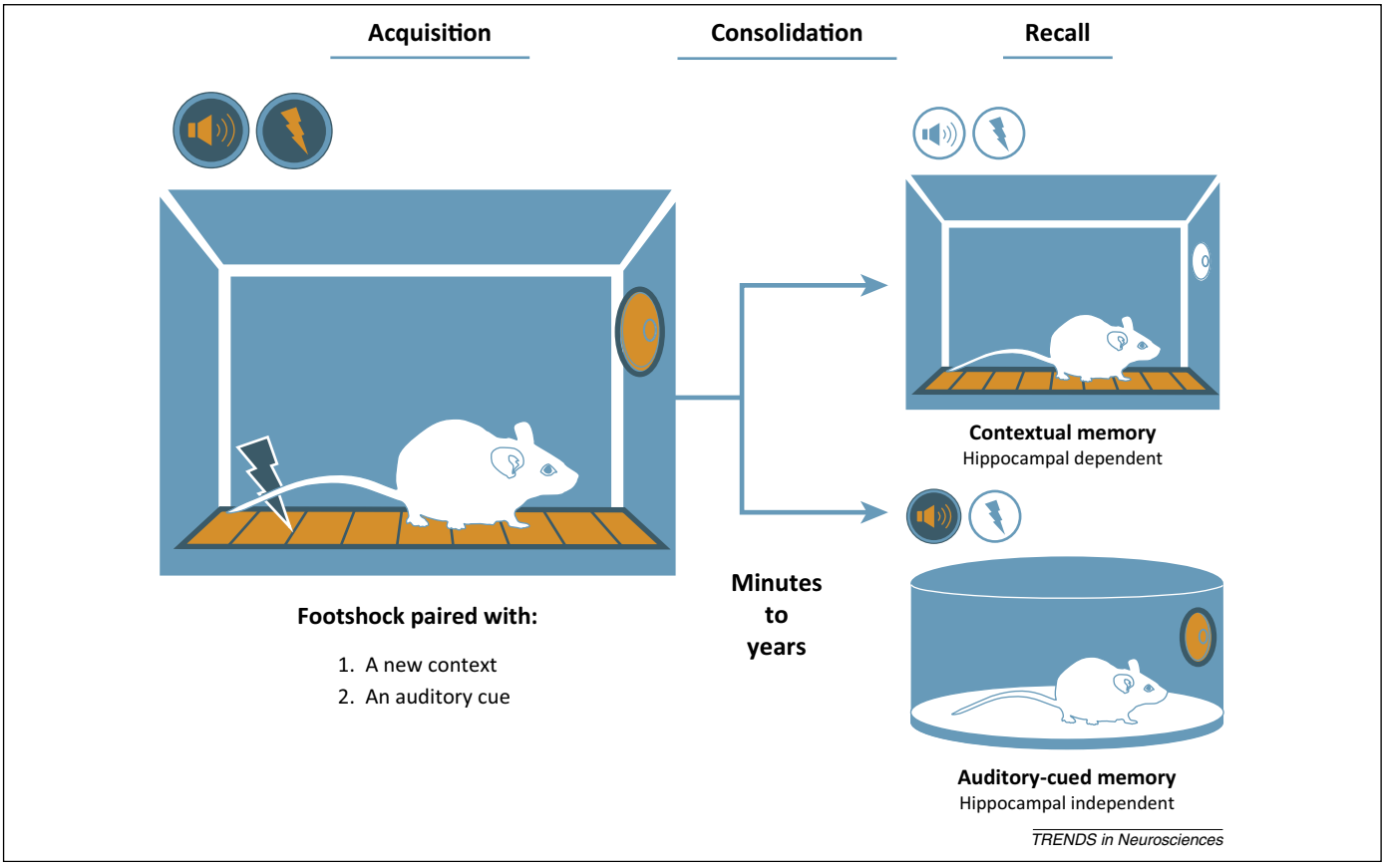

Figure 2. Fear memory induction and testing. Classical fear conditioning (FC) is one of the most powerful experimental models for studying the neural basis of associative learning and memory formation in mammals. The simultaneous presentation of a neutral conditioned stimulus (CS) and an aversive unconditioned stimulus (US) endows the formerly neutral stimulus with a frightful quality, so that when it later appears by itself without the aversive stimulus, it will elicit a fearful conditioned response. FC can be rapidly formed in humans and animals, even following a single conditioning trial, and is usually maintained for long periods. In the classical protocol for FC in rodents, two neutral CSs (a context and an auditory cue) are paired with a mild footshock as a US. Following consolidation (spanning in duration from minutes to months), the presentation of either CS will elicit a fear response, as measured by freezing (complete immobility), even when no US is present. The conditioning process itself (i.e., the association between the neutral and aversive stimuli) is mediated primarily by the amygdala, and the use of different types of CS enables differentiation between hippocampal-dependent memory (e.g., of a complex context) and hippocampal-independent memory (e.g., of an auditory cue). To test contextual memory, animals are placed again in the original conditioning cage and freezing is measured. Recall depends on the intact functioning of the hippocampus (to create a mental representation of the context) and the amygdala. To test auditory-cued memory, the tone is presented in a differently shaped context. This simpler task requires the amygdala alone and is not affected by hippocampal lesions.

It did, however, have a striking effect on emotional behavior. Ventral DG excitation with ChR2 exerted an anxiolytic effect in two different tests of anxiety, the elevated plus maze and the open field [12]. Conversely, ventral DG inhibition with NpHR had no effect on anxiety [12], suggesting this region is not necessary for the expression of baseline anxiety levels. Dorsal DG inhibition also had no effect on baseline anxiety and the stimulation of this region with ChR2 resulted in a general increase in exploratory behavior, accompanied by a mild anxiolytic effect.

To conclude, this study used both inhibition and excitation of either the dorsal or the ventral DG to demonstrate the changing roles performed differentially by the hippocampus along its dorsoventral axis (Figure 3). It completes and clarifies the exclusive role of the dorsal DG in acute FC acquisition suggested by previous studies. It also shows that the ventral DG is neither necessary nor sufficient for this process, but it does contribute to emotional responses. Given the different roles that the ventral DG and CA1 play in memory retrieval, it may be that in the case of ventral DG modulation, the lack of effect on cognition stems from the ability of CA3 or CA1 to bypass the DG and support this process, and not because the ventral hippocampus is not involved in FC. The finding that the ventral DG is not necessary for emotional behavior (as its inhibition had no effect) is even harder to interpret in light of previous literature using lesions [28,31], thus further research will be required to fully clarify this issue.

Different retrieval strategies for recent and remote memories

Many findings from both human studies and animal research suggest that long-term contextual fear memory consolidation requires early involvement of the hippocampus, later replaced by the neocortex. Hippocampal lesions impair memory 1 day after training, but the same lesions have no effect on memory several weeks after training [7,32–41]. Similar graded retrograde amnesia is observed in human patients with bilateral medial temporal lobe injuries [42]. Conversely, nongraded retrograde amnesia was reported in some human patients [43] and in animal studies involving extensive hippocampal damage [44]. Furthermore, human imaging studies show hippocampal activation during the recall of both recent and remote memories [43]. Together, these seemingly conflicting findings have led to the 'multiple trace theory', suggesting

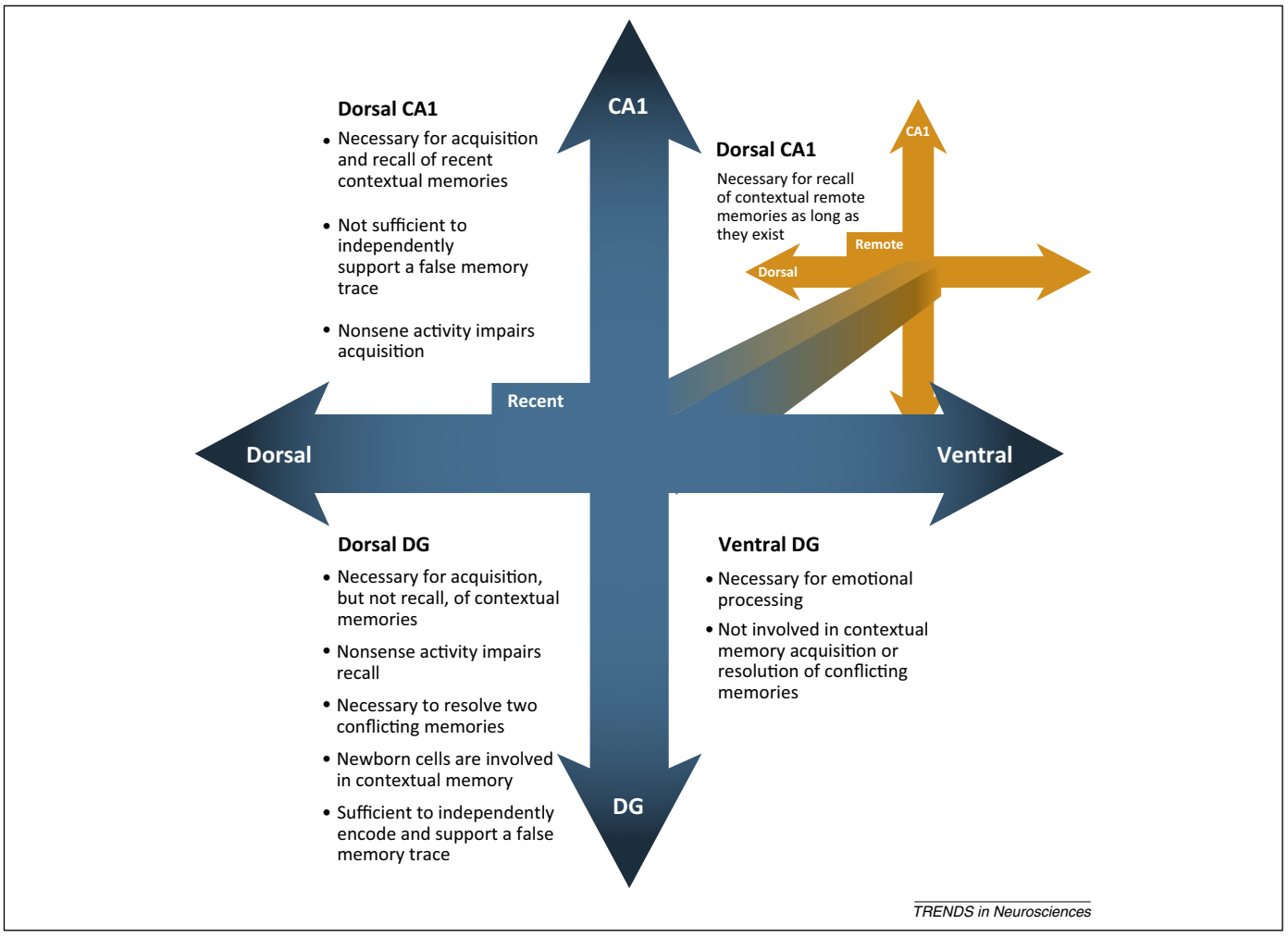

Figure 3. Novel findings in hippocampal memory research, facilitated by optogenetic studies. The incorporation of optogenetic methods into hippocampal memory research had enhanced our understanding of this region on three theoretical axes. The first two are anatomical – pointing to functional differences between hippocampal subregions (DG vs CA1) and to the distinct functions of dorsal and ventral hippocampus. The third is on the time axis showing the dynamic change in hippocampal contributions to recent and remote memories. Clearly, this picture contains more questions than answers, and in years to come this matrix may be completed and new dimensions may be added (see section on 'Concluding remarks and future directions'). Abbreviation: DG, dentate gyrus.

that in the process of system-wide consolidation the memory is not merely transferred from the hippocampus to the cortex, but rather transformed and possibly saved in different variations in several cortical regions and remain available with continuous interplay [43,45].

The groundbreaking studies on the circuitry of remote memory involved lesion studies (physical, pharmacological, and genetic), which lack fine temporal resolution [8,40,46,47]. An optogenetic approach, by contrast, allows real-time modulation of specific cell populations with high temporal and spatial resolution. For example, silencing CA1 neurons by illuminating NpHR during recall of recent contextual fear memories resulted in a significant impairment[10]. Surprisingly, such precise real-time inhibition of the CA1 region during recall also blocks remote fear memory when administered 4 to 12 weeks after acquisition [10], suggesting a permanent role of the hippocampus in memory recall, as long as a memory trace exists (Figure 3). Furthermore, remote fear memory expression could be interrupted even after the context was already recalled by optogenetic CA1 inhibition in the midst of a recall session, which resulted in an immediate termination of the fear response [10]. Thus, CA1 is necessary not only for the recall of remote memories but also for their maintenance throughout the recall session.

The reason for the contradictory results obtained using optogenetic tools suggesting permanent involvement of CA1 in remote recall compared with the pharmacological and genetic studies suggesting the hippocampus is not necessary for remote recall may be the temporal precision of the former technique: pharmacological or genetic manipulations are orders of magnitudes slower than the typical neuronal activity and thus allow compensatory mechanisms to commence before the behavioral test takes place. Indeed, when illumination was delivered in a pharmacological timescale (for 30 min before testing as well as during the test) to allow time for compensatory mechanisms to be engaged, no effect on recall was observed, whereas precise illumination disrupted recall [10]. This compensation occurred only for remote memory, probably after extrahippocampal consolidation, but not for recent memory, which relies solely on the hippocampus and thus was not rescued during prolonged illumination [10].

Which brain areas may support such a rapid compensation process for hippocampal inactivation? One possible

candidate is the anterior cingulate cortex (ACC), which was shown to be involved in remote, but not recent, recall [38]. Indeed, the increase in ACC activity (inferred by cFos expression) observed during remote recall was blunted by precise optogenetic CA1 inhibition [10]. However, when given enough time to compensate for the absence of hippocampal activity during prolonged illumination, ACC activity not only returned to control levels but exceeded them [10], suggesting an active compensation role for this structure. Furthermore, either precise or prolonged optogenetic ACC inhibition at a remote time point impaired contextual fear memory [10], suggesting that a part of the contribution of the ACC to the recall of the context is unique and cannot be compensated for by other areas, which supports the multiple trace theory.

To conclude, in the findings described above, the use of optogenetics resulted in modification, rather than validation, of a long-standing hypothesis, by showing that contrary to the prevalent belief of a gradual transition from hippocampal-mediated recall of recent memories to cortical-mediated recall of remote memories, the hippocampus is in fact the default activator of contextual memory traces at all time points. However, the hippocampus dynamically shifts its recall strategy between recent and remote memories – relying on different brain areas following cortical reorganization. An elegant demonstration of this dynamic process was recently provided by Wheeler *et al.* [48] using brain-wide mapping of cFos expression to quantify the evolution of functional connectivity between recent and remote fear memory recall. They reported increased intracortical correlations during remote recall as well as an increase in correlated activity between the PFC and other cortical and hippocampal regions, consistent with systems consolidation [48]. Importantly, the correlation of hippocampal activity with other brain regions was not decreased at the remote time point, but rather strengthened, as was especially apparent for hippocampal–neocortical connections [48]. The combination of behavioral, optogenetic, and imaging studies presented above suggests that the hippocampus mediates both recent and remote recall, but that the additional brain structures it cooperates with are continuously changing. Some of these structures and new findings regarding their involvement in memory will be discussed in the next section.

The role of non-hippocampal regions in memory circuitry

Even very simple memories, such as auditory fear conditioning (Figure 2), rely on a complex brain circuitry, involving amygdalar, cortical, striatal, and thalamic contributions. More complex tasks may require additional hippocampal involvement. This section will briefly describe new findings regarding amygdalar and cortical contributions to learning and memory. Other impressive studies, describing striatal and thalamic circuits, will not be discussed here [49–51].

Cortical involvement in learning and memory

The PFC is involved in a variety of cognitive processes (e.g., executive functions, attention, decision making) [52]; however, this short section will focus exclusively on its role in fear memory. The importance of frontal cortical areas such as the ACC and PFC to systems consolidation and FC extinction was well established in past decades [38,53,54]. Recent optogenetic studies support these findings and provide insight to the modulatory processes affecting them. For example, parvalbumin (PV)-expressing interneurons in the PFC were shown to inhibit fear expression, because their optogenetic inhibition using Arch (subsequently disinhibiting pyramidal PFC neurons) resulted in spontaneous fear expression and could even induce place aversion [55], whereas activation of PV neurons in the PFC with ChR2 (consequently inhibiting pyramidal neurons in this region) reduced conditioned fear expression [55]. Interestingly, one recent optogenetic study described a surprising role for the primary auditory cortex in auditory-cued FC. Letzkus *et al.* [56] defined a circuit in which cholinergic inputs increase the activity of GABAergic cells in cortical layer 1 in the auditory cortex, which in turn inhibit the activity of PV interneurons in layer 2/3. This PV inhibition results in disinhibition of layer 2/3 pyramidal neurons, which consequently show increased firing. When PV interneurons were optogenetically activated by ChR2 following footshock (at the time in which their activity is naturally suppressed), auditory-cued FC is dramatically impaired [56]. Fear acquisition (auditory-cued and contextual) was not affected by optogenetic activation of PV neurons in the PFC [57], but was severely impaired by prolonged hyperactivation of pyramidal neurons using a slow ChR2 variant (stable step function opsin, SSFO) during FC [57], probably due to masking of the informative PFC trace with a noisier opsin-induced signaling. The PFC was also implicated in trace FC by optogenetically inhibiting this area using ArchT during the 'trace interval', a 20-s delay between the conditioned stimulus (CS; auditory cue) and the unconditioned stimulus (US; electric shock). PFC inhibition at that specific time significantly impaired acquisition [58], providing yet another powerful demonstration of the power of temporal precision when using optogenetics. Finally, inhibition of pyramidal ACC neurons with NpHR has no effect on recent FC recall, but impaired remote recall [10].

To summarize, these studies support the classical involvement of frontal cortices in complex processes such as systems consolidation and trace conditioning, but also demonstrate their involvement in 'simpler' processes such as fear acquisition and expression, in which they were thought not to be involved [53]. Further research is necessary to tie these findings together and provide a full characterization of the involvement of the PFC in memory processes.

The amygdala in fear memory

Because the amygdala is a core player in fear acquisition and expression [59], a major effort was invested in an excellent examination of its role in FC using optogenetics. This structure as a whole and the various nuclei it comprises deserve a separate review [60]; therefore, only the main findings will be presented here to provide a full picture of fear circuitry and the contribution of optogenetics to its deconstruction. Is the amygdala sufficient to independently support fear? Several studies [61–63] used optogenetics to directly stimulate or indirectly disinhibit

amygdalar nuclei [61–63] to show their ability to directly induce fear [61,62] (but see [64]). Amygdala stimulation can even serve as an independent US when paired with a tone, that is, it induced conditioned fear of the tone at a later time point, when illumination was absent [61]. Optogenetic studies also supported the role of the amygdala in fear memory acquisition and retention [10,63,65] special interest are studies that pointed to specific neuronal populations that are involved in amygdalar fear learning [63,66,67] example, differential roles were shown for two different interneuron populations (PV and somatostatin) in the amygdala. These populations respond differently to the US and the CS, and gate the activity of amygdalar excitatory neurons in response to these stimuli, thus supporting associative learning [66]

A huge body of data obtained from lesion, genetic, electrophysiological, molecular, and pharmacological techniques provided an excellent framework describing prefrontal and amygdalar involvement in fear memory [52,53,68]. The incorporation of optogenetics allowed the demonstration of the real-time contribution of these areas and specific populations within them to associative learning. Without the combination of high temporal resolution, genetic specificity, and the opportunity for opponent modulation (excitation/inhibition) offered by optogenetics, it would have been impossible, for example, to demonstrate the differential roles of different interneuron populations in fear learning [66]. The first optogenetics works in this field served more as a 'proof of principle', but as the experimental complexity increases, novel and surprising findings arise. Because no single brain area can independently support even the simplest memory, in years to come elaborate works examining the functional connectivity between the cortex, the amygdala, and the hippocampus are likely to shed more light on the circuitry of memory. Indeed, such attempts to target the connections between the amygdala and different cortical regions were recently performed [69,70].

The neuronal code of memory

A major hypothesis in neuroscience is that sparse discrete neuronal populations underlie any specific memory trace and are reactivated upon its retrieval. Indeed, the existence of such populations in the amygdala and hippocampus has been demonstrated [71–74], and their necessity for the integrity of memory traces was established by ablating these neurons and consequently erasing a specific memory trace [75,76]. However, prior to the application of optogenetics to this question no demonstration that these neuronal ensembles are not only necessary but also sufficient to encode a specific memory trace was available. This section will present approaches for the generation of false memory traces and ask whether cracking the neuronal code is truly necessary in order to create such memories.

Generation of memory traces using optogenetics

Liu *et al.* [77] provided the first proof that the stimulation of a specific memory engram in the hippocampus can in fact support the memory of a specific context. They expressed ChR2 specifically in the DG neurons that were activated in response to a specific context, using the cFos-tTA mouse line, and controlled the timing of this expression by removing doxycycline (Dox) from the diet (Figure 1), such that only when Dox is not present ChR2 is expressed [72,77]. A major caveat of this system is the low temporal resolution of tTA expression due to the long half-life of Dox. Consequently, the shortest 'off Dox' window used was 24 h, in which mice were probably exposed to many experiences in addition to the one relevant to the experiment. Another limitation is that cFos is an indicator of increased activity only in excitatory neurons, and consequently fast spiking neurons that may be involved in a specific engram will be neglected. Nevertheless, this system was successfully employed to express ChR2 in the DG neurons activated by a specific context A during FC. The mice were then returned to a Dox-supplemented diet and introduced into a different context B while the ChR2-expressing neurons were illuminated. Despite the fact that no aversive stimulus was ever present in context B, ChR2-expressing mice demonstrated fear. The interpretation of these results is that the light activation of the memory engram of context A resulted in a fear response even though the mice were actually in the neutral context B [77]. Ramirez *et al.* [78] then took this idea one step further, and instead of activating an existing memory trace (in which a specific context was actually paired with a shock) created a false memory trace by first tagging a specific neuronal ensemble in context A, and then activating this population with light while administering a footshock in context B. Thus, an offline association between the engram of A and an aversive stimulus was created, which resulted in fear of the previously neutral context A upon reintroduction to this context (with no light stimulation of the ChR2-expressing cells) [78].

The generation of a false fear memory was also demonstrated in the conditioned place avoidance paradigm, in which mice encounter an aversive experience in a specific compartment in a three-chamber arena, and later avoid entering this compartment and exploring it [79]. To create a false fear memory of one of the chambers (chamber X), mice were allowed to explore it off Dox, thus allowing the expression of ChR2 in the DG neurons that encode this context. Mice were then placed back on Dox and 2 days later received a footshock in a different cage with light stimulation, again creating an offline association between the footshock and the neuronal ensemble encoding the specific chamber X. Consequently, these mice showed a clear avoidance of chamber X that was associated offline with an aversive stimulus and strongly preferred the other two chambers [78]. Thus, it is possible to elicit the behavioral output of a specific memory by directly activating the population of neurons in the DG that were active during its acquisition or even create such a memory offline, suggesting that activation of a specific sparse neuronal ensemble is sufficient for the establishment and recall of a contextual memory.

In a parallel study by Garner *et al.* [80], the cFos-tTA mice were employed to express the hM_3D_q designer receptor in activated neurons in a specific context A in the absence of Dox [72,80,81]. This receptor was then activated by a systemic injection of its ligand, CNO, during

conditioning in a different context B [81]. This pharmacogenetic false trace interfered with the 'real' memory of the conditioning context B, but could not by itself support a behavioral expression of fear in context A [80]. Both the pharmacogenetic and the optogenetic techniques employed an activity-dependent expression of a protein that can later be activated at will. Two reasons may explain the robustness of the optogenetic false trace, which elicited a behavioral response, compared with the pharmacogenetic false trace that could not independently support behavior. The first is the temporal precision of light activation compared with drug administration that is significantly slower. The latter activates the false trace for a long duration, which may allow online extinction. Another possible factor is the higher spatial resolution of the optogenetic false memory trace (generated in areas with sparse coding of the context) compared with the forebrain-wide pharmacogenetic trace, possibly diluting the pure representation of the context. It could be speculated that a DG-specific local administration of CNO may have bypassed the temporal and spatial obstacles, and independently support a pharmacogenetic false memory trace.

As discussed above, CA1 and DG inhibition elicit different effects on memory recall. Ramirez *et al.* [78] provided an elegant demonstration of the importance of sparse coding in the DG compared with CA1. Mice were exposed to a novel context when off Dox, and 24 h later while back on Dox, and were exposed to either the same context or a different context, and their brains were stained for cFos activity. In the DG, an overlap of only approximately 1% of the cell populations representing the new and old context was found, demonstrating the sparse coding in this area, whereas in CA1 a 30% overlap was found between the cells active in the different contexts [78]. This high selectivity of neuronal representation in the DG compared with CA1 has a functional consequence: a false memory trace could be generated by DG illumination but not by selective CA1 light activation [78]. The same was true for conditioned place aversion – mice showed avoidance of the falsely aversive chamber upon tagging and activation of DG, but not CA1, neurons [78].

Cracking the neural code: always a necessity?

The generation of true or false memory traces in a sophisticatedly targeted group of recently active neurons is a truly remarkable achievement. Interestingly, researchers from the Axel group managed to create a fear memory, and even more remarkably drive an appetitive behavior in response to nonspecific neuronal targeting [82]. ChR2 was expressed in neurons in the piriform cortex in three ways: (i) mixed neuronal expression – both excitatory and inhibitory; (ii) excitatory neuron expression; and (iii) sparse mixed expression [82]. Choi *et al.* [82] used the active avoidance paradigm, in which a mouse is exposed to a shock (US) paired to a previously neutral CS (e.g., smell) in one side of an arena and can terminate the shock by fleeing to the other side of the arena. After several pairings, the CS itself will generate a flight response [83]. After using light stimulation of piriform cortex neurons expressing ChR2 as the CS, mice were placed in the context, and the flight response to piriform stimulation alone (with no shock) was recorded. Remarkably, using all three random expression methods, the conditioning resulted in a strong escape behavior in response to the light stimulation alone [82]. Importantly, randomly targeted piriform cortex neurons could also drive appetitive behavior when paired to a socially rewarding stimulus (a female mouse). When a random piriform ensemble in males is activated in a cage compartment in which a female is present, they will later prefer to be in a compartment that offers light stimulation even when no female is present [82]. Alternatively, mice can be trained to associate light delivery with a water reward and show increased licking in response to light delivery [82]. Interestingly, these associations are very flexible because the same random population can be entrained to support both appetitive and aversive behaviors [82].

The studies presented in this section all used optogenetics to create memory traces that can drive behavior [77,78,82]. The Tonegawa group [77,78] targeted the specific neurons encoding the context, whereas the Axel group [82] stimulated random neuronal populations to drive behavior as efficiently. Together, the results suggest that in order to powerfully modulate behavior in a context-specific manner one does not necessarily need to decipher the original neural code, but can simply force an alternative code onto the system, and drive behavior efficiently. Clearly, this level of permissibility is region-dependent: it seems to be lenient in areas that provide the input on which a memory is later constructed, such as the piriform cortex, but much less permissive in areas that are tightly related to long-term memory formation such as the hippocampus.

Concluding remarks and future directions

In an extremely short period of time, an impressive variety of new findings regarding memory was obtained using optogenetics, proving some long-standing hypotheses, modulating others, and providing insight into new hitherto unexplored circuits. Every exciting new finding in memory research is also a source of new mysteries, which in turn stimulate further innovating studies. The combination of new research questions and hypotheses, hand in hand with rapidly evolving technological developments, is an extremely efficient driving force in memory research. A few examples for questions arising from current research would be: What is the computational function of the CA3 region, the 'information bottleneck' of the hippocampus? What underlies the compensation mechanism for hippocampal inactivation during remote recall? How do brain areas upstream and downstream from the dorsal/ventral hippocampus contribute to their functional variation?

Discovering the answers to these and many other challenging questions may require continuous adoption of new tools and increased complexity in the use of current tools. Several such modulations are suggested as follows.

Increasing the methodological complexity

The vast majority of memory research to date employed simple cognitive tasks and basic light stimulation strategies. Increasing the complexity of both may further push

the boundaries of our understanding of cognitive processes. (i) Targeting novel populations: an impressive body of knowledge about the function of certain populations such as glutamatergic, dopaminergic, cholinergic, and GABAergic (specifically PV) cells has recently accumulated. The development of new Cre driver lines, as well as improved and smaller promoters for viral vectors, will hopefully expand our ability to examine new populations and their contribution to the known circuits. (ii) Connectivity based targeting: optogenetic memory research to date employed mostly viruses that infect the soma, followed by illumination of cell bodies or projections. Integration of circuit-based targeting is also possible (e.g., [69]), using viruses that can infect terminals (such as the herpes simplex virus), and travel trans-synaptically (such as the rabies virus) [84]. (iii) Challenging cognitive tasks: the beauty of FC is in its simplicity, but more elaborate tasks (examining spatial navigation and the assimilation of memory schemas, for example) may provide interesting insights. This process seems more realistic owing to the development of Cre driver rat lines [85], because these animals are capable of performing more sophisticated tasks and carry bigger devices on their skulls. Indeed, pioneering studies are already in progress [49].

As the complexity of experiments continuously increases, researchers should be careful not to neglect to estimate as precisely as possible the size of the modulated area. The spread of opsin expression can be easily quantified, and the parameters governing light spread through the brain tissue are generally known, allowing a good estimation of the upper boundaries of the modulated area size. However, these data are often missing from papers, making it difficult to precisely interpret and compare them.

Combination of optogenetics and imaging techniques

One of the advantages of optogenetics is that it allows simultaneous optical stimulation and electrical recording in behaving animals (e.g., [86]). In the near future, all-optical stimulation and imaging during the performance of cognitive tasks may become achievable. Concomitantly with the leap in tool development for neuronal manipulation, recent years had seen a tremendous progress in the development and application of neuronal activity imaging tools [4,87–90]. Future combination of calcium imaging in behaving mice [87] with new genetically encoded indicators [95] that can be optogenetics compatible [88] can provide a better understanding of the exact spatiotemporal effect of optogenetic manipulation and later help to refine and optimize it.

Optogenetics has been employed to demonstrate the necessity of specific neuronal populations in defined brain regions to fear memory, and in some cases the sufficiency of a certain population to independently support a memory trace. Further advances in this field are sure to arise in the very near future.

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