Review

Chasing the addicted engram: identifying functional alterations in Fos-expressing neuronal ensembles that mediate drug-related learned behavior

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Given that addiction has been characterized as a disorder of maladaptive learning and memory, one critical question is whether there are unique physical adaptations within neuronal ensembles that support addiction-related learned behavior. The search for the physical mechanisms of encoding these and other memories in the brain, often called the engram as a whole, continues despite decades of research. As we develop new technologies and tools that allow us to study cue- and behavior-activated Fos-expressing neuronal ensembles, the possibility of identifying the engrams of learning and memory is moving into the realm of reality rather than speculation. It has become clear from recent studies that there are specific functional, electrophysiological alterations unique to Fos-expressing ensemble neurons that may participate in encoding memories. The ultimate goal is to identify the addicted engram and reverse the physical changes that support this maladaptive form of learning.

Addiction can be conceptualized as a disorder of learning and memory. Indeed, environmental stimuli such as cues and contexts become strongly associated with drugs of abuse after repeated exposure (Wikler 1973; Goldberg 1976; Stewart et al. 1984; O'Brien et al. 1986; Robinson and Berridge 1993; Siegel 1999; Crombag et al. 2008). Subsequent exposure to these drug-paired stimuli can drive craving and ultimately relapse to drug seeking behavior. Given that this maladaptive form of learning is a powerful driver of relapse, understanding the neurobiological correlates of these associations will be a critical step in reversing this learning and will ultimately aid in identifying novel targets for treatment. The majority of research examining neurobiological correlates of addiction-related behavior has focused on identifying neuroadaptations that occur throughout the general neuronal population in a brain region of interest (Hyman et al. 2006; Koob 2006; Bowers et al. 2010; Scofield et al. 2016). However, associative learning relies on functional alterations within sparsely distributed populations of neurons, commonly referred to as "neuronal ensembles," that are selected by specific cues during learning (Hebb 1949; Pennartz et al. 1994; Nicolelis et al. 1997; Buzsaki 2004; Schwindel and McNaughton 2011; Lansink et al. 2012; Cruz et al. 2015). Recent technologies now permit us to identify and characterize ensembles that are activated enough to activate the immediate early gene *Fos*. However, this review is not about the technologies used; for reviews focused on the Fos-based technologies, see (Garner and Mayford 2012; Cruz et al. 2013; Kawashima et al. 2013, 2014; Ramirez et al. 2013a; Mayford and Reijmers 2015; Tonegawa et al. 2015; Warren et al. 2017). Here we focus on electrophysiological alterations within Fos-expressing neuronal ensembles in appetitive learned behaviors related to addiction research (Cruz et al. 2015). For reviews focused on neuronal ensembles in fear conditioning, readers can refer to (Garner and Mayford 2012; Cruz et al. 2013; Kawashima et al. 2013, 2014; Ramirez et al. 2013a; Warren et al. 2017).

Why study Fos-expressing neuronal ensembles in the context of addiction?

Hebb postulated in 1949 that memories could be encoded in "cell assemblies" or small groups of sparsely distributed neurons (Hebb 1949). Early support for this idea came from in vivo recording data demonstrating ensemble-based coding of location in hippocampus (Mountcastle 1957; O'Keefe 1976; Wilson and McNaughton 1993). Early ensemble-based coding was also demonstrated in the nucleus accumbens, a brain region known to play a critical role in reward-based learning (Carelli et al. 1993; Pennartz et al. 1994). Only a minority of neurons in the accumbens are active during drug seeking (Carelli and Wightman 2004), and separate populations are active in response to drug versus natural reward-related cues (Carelli et al. 2000). Although in vivo recording data support the neuronal ensemble hypothesis, these data are still correlational and not a direct demonstration of neuronal ensembles playing a causal role in mediating behavior.

For many years, expression of the immediate early gene products *Fos* mRNA and protein had been used to label neurons that were strongly activated during behaviors used in addiction research (Morgan and Curran 1991; Kawashima et al. 2014; Cruz et al. 2015). Based on this premise, Koya et al. (2009) used Fos-lacZ transgenic rats to coexpress β-galactosidase in strongly activated Fos-expressing neurons in a context-specific sensitization model. Daun02 injections into nucleus accumbens selectively ablated only the βGal/Fos-expressing neurons that were previously activated during exposure to the cocaine-paired context attenuated expression of the learned behavior, but not when Daun02 ablated a different set of Fos-expressing neurons activated by exposing the rats to a distinct drug-unpaired context. This indicated that the environmental context activates specific Fos-expressing neuronal ensembles that mediate the specific learned behavior.

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Since 2009, the Daun02 inactivation procedure has been used to inactivate Fos-expressing neuronal ensembles in different prefrontal cortex areas, nucleus accumbens, striatum, and amygdala and found causal roles for these ensembles in the expression of learned behaviors related to heroin (Bossert et al. 2011; Fanous et al. 2012), cocaine (Cruz et al. 2014), methamphetamine (Caprioli et al. 2017), ethanol (Pfarr et al. 2015; de Guglielmo et al. 2016) nicotine (Funk et al. 2016), and food (Suto et al. 2016; Warren et al. 2016; Whitaker et al. 2017). These ensembles may be involved in encoding the learned task itself (e.g., lever pressing) or in the learned motivation to perform the task, such as learning which cues inform the animal when it is appropriate or inappropriate to perform the task. Overall, whether encoding the task itself or the motivation to perform the task, the cellular mechanisms for encoding information within these neuronal ensembles are likely to be fundamentally the same.

A number of other transgenic, viral, and chemogenetic strategies based on activation of the *Fos* promoter have been used to manipulate these small populations of strongly active neurons and assess their role in learned behaviors in the addiction and fear conditioning fields (Garner and Mayford 2012; Cruz et al. 2013; Guenthner et al. 2013; Kawashima et al. 2013, 2014; Ramirez et al. 2013a; Warren et al. 2017). Most of these systems used the *Fos* promoter to drive expression of other proteins of interest (e.g., GFP, LacZ, tTA) to identify, manipulate and characterize the Fos-expressing neuronal ensembles (Reijmers et al. 2007; Liu et al. 2012; Cruz et al. 2013; Guenthner et al. 2013; Ramirez et al. 2013b; Pfarr et al. 2018). However, some strategies used promoters from other neural activity-dependent IEGs, such as *Arc* (Guenthner et al. 2013) and *FosB* (Engeln et al. 2016) or the synthetic promoter SARE (Kawashima et al. 2013) to identify and manipulate activated neuronal ensembles in learned behaviors. Nevertheless, all the studies regarding addiction related ensembles and engrams described below were based on neuronal ensembles identified by activation of the *Fos* promoter.

Neuronal ensembles and engrams

The term neuronal ensemble (or cell assembly) has been used to define small percentages of sparsely distributed neurons that undergo coordinated activation during behavior or stimuli presentation. When using in vivo electrophysiology recordings, neuronal ensembles can be populations of neurons that fire acutely with coordinated population firing rates and phase synchronization during ongoing behavior (Russo and Durstewitz 2017) without the necessity for learning or memory-related alterations. Since the term "engram" or "mneme" is defined as the memory trace, or the long-lasting, physical mechanism that encodes a learned association (Semon 1921; Eichenbaum 2016), then this acute activation during behavior is not the equivalent of the engram. Neuronal ensembles can be temporarily active during information processing with without long-term alterations.

We hypothesize that Fos-expressing ensembles comprise a small subset of the acutely activated ensembles that are detected using in vivo electrophysiology. Please see a more comprehensive discussion in (Cruz et al. 2013, 2015). We and others hypothesize that Fos induction in these neurons requires strong persistent activation of the neurons. The most strongly activated neurons cross a threshold when sufficient levels of calcium influx activate the ERK/MAPK pathway that phosphorylates transcription factors on the *Fos* promoter to enable transcription (Morgan and Curran 1991; Brami-Cherrier et al. 2009; Cahill et al. 2014). This high activity threshold for Fos induction may explain the discrepancy between the low percentages of neurons detected using Fos expression and the higher percentages of ensemble-related neurons detected using in vivo electrophysiology. Only the most strongly and persistently activated neurons become part of the Fos-expressing ensemble. Based on the fact that selective Daun02-mediated ablation of Fos-expressing ensembles alters recall of memories in addiction research (Cruz et al. 2015), it appears that the neural activity thresholds seem to be similar for Fos expression and for recruiting a neuron into a persistent ensemble that mediates learned behaviors in addiction research.

In the search for an "addiction engram," it is important to be even more precise about what we are seeking. Daun02 inactivation and other related technologies suggest that Fos-expressing neurons in a given region of interest contain some alterations that allow them to be part of the engram. However, the Fos-expressing neurons per se do not necessarily constitute the engram. More precisely, the engram is the set of long-lasting molecular and cellular alterations (e.g., changes in synaptic efficacy) that are induced within these neurons during learning that alters the neurons' responses to specific afferent inputs to encode a memory.

In light of these definitions, most of the work with Fos-expressing ensembles in behavior has only identified Fos-expressing neurons that are "involved" in mediating a memory (Han et al. 2007; Reijmers et al. 2007; Bossert et al. 2011; Fanous et al. 2012; Liu et al. 2012; Cruz et al. 2014; Hsiang et al. 2014; Redondo et al. 2014; Pfarr et al. 2015; Ramirez et al. 2015; de Guglielmo et al. 2016; Funk et al. 2016; Suto et al. 2016; Warren et al. 2016; Caprioli et al. 2017). Most of these studies have not identified the actual engrams or alterations induced within these neurons that physically store the memories. Thus, the critical question now is what specific functional and physical changes were induced within the neuronal ensembles that drive learned behavior? New viral and transgenic methods have permitted the investigation of such questions (Barth et al. 2004; Reijmers et al. 2007; Koya et al. 2009; Cifani et al. 2012). Again, most of these technologies have relied on the Fos promoter as an indicator of strong neuronal activation to identify activated ensemble neurons (Morgan and Curran 1986; Cruz et al. 2015). Here we focus on electrophysiological alterations in Fos-expressing ensembles that may contribute functionally to the engram in animal models used in drug addiction research.

Electrophysiological alterations

The advent of new technologies permitting targeted recording from activity-dependent ensemble neurons has afforded the opportunity to determine specific functional, electrophysiological alterations within cells strongly activated by a particular event or experience. Both intrinsic and synaptic properties of ensemble neurons are beginning to be characterized (Fig. 1). We focused on neuronal ensembles within the nucleus accumbens and prefrontal cortex since these regions are known to be important for addiction-related learned behaviors (Peters et al. 2009; Feltenstein and See 2013; Marchant et al. 2015).

Synaptic alterations

The first demonstration of glutamatergic synaptic plasticity specific to Fos-expressing neurons identified an increase in silent synapses (synapses that contain functional NMDA but no functional AMPA receptors) (Koya et al. 2012). This study used FosGFP mice (Barth et al. 2004) to identify ensemble neurons within the nucleus accumbens following cocaine-induced locomotor sensitization. A follow-up study determined that silent synapses in NAc ensembles arose in a context-specific manner such that only animals trained and tested in the same context showed an increase in silent synapses (Whitaker et al. 2016). Previous work examining global neuroadaptations within the NAc also identified an up-regulation of silent synapses in the general population of neurons regardless of

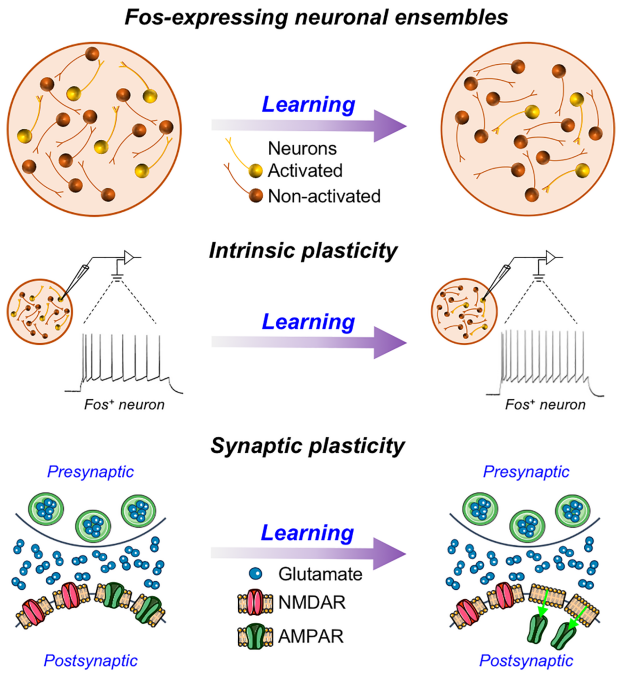

Figure 1. (*Top*) Schematic representation of Fos-expressing neuronal ensembles before and after learning. The number of Fos-expressing neurons in the ensemble decreases with further training (Whitaker et al. 2017). (*Middle*) Example of spiking activity in Fos-expressing neurons before and after incorporation into a behaviorally relevant ensemble. Neuronal excitability increases after learning (Whitaker et al. 2017; Ziminski et al. 2017, 2018). (*Bottom*) Example of synaptic changes in a Fos neuron before and shortly after drug-based learning experience. There is an increase of silent synapses that contain NMDA receptors but no functional AMPA receptors (Koya et al. 2012; Whitaker et al. 2016).

activation state following cocaine-induced locomotor sensitization (Huang et al. 2009; Brown et al. 2011; Lee and Dong 2011) as well as incubation of cocaine craving (Lee et al. 2013; Ma et al. 2014). However, the mechanism that leads to the appearance of silent synapses in the general population appears to be different from silent synapses in Fos-expressing neurons. In the general population of NAc neurons, there is evidence that silent synapses appear following cocaine sensitization due to the emergence of new NR2B-containing dendritic spines (Huang et al. 2009). Neurons that express Fos-driven GFP following exposure to the cocaine-paired context show no evidence of increased NR2B or new spine generation. Thus, a likely mechanism by which silent synapses are increased in ensemble neurons is via internalization of AMPA receptors. One limitation of these studies is that the time point required for Fos-driven expression of GFP is 90–120 min following context exposure. Thus, changes that occur before or after this time point were not assessed. An interesting question for future research is whether silent synapses appear as a homeostatic response to a preponderance of glutamatergic input or following context re-exposure or via some other separate mechanism. Additionally, the question remains as to whether silent synapses in activity-driven accumbens neuronal ensembles play a causal role in the expression of sensitization.

Intrinsic properties

Although an overwhelming focus of the literature on electrophysiological alterations in drug addiction has been on synaptic properties, more recently studies have begun to focus on changes in intrinsic membrane properties (Kourrich et al. 2015). One recent study using FosGFP mice showed that following exposure to a context previously paired with cocaine, there was a significant difference in firing properties of FosGFP+ and FosGFP– neurons (Ziminski et al. 2018). The effect was due to suppression of firing activity in the FosGFP– neurons. This difference was not present when mice were exposed to the saline-paired context suggesting that this change in firing may be important for the encoding of the learned association between cocaine and the locomotor chamber. A similar effect was found in an earlier study examining changes in prefrontal cortex firing activity in FosGFP rats following operant food self-administration training. In this study, FosGFP+ neurons showed increased firing whereas FosGFP– neurons suppressed their firing activity when injected somatically with depolarizing current (Whitaker et al. 2017). Taken together, these data support the idea that ensemble and nonensemble neurons together modulate the gain of the system such that the signal from ensemble neurons is given preference over signal from nonensemble neurons. Such a mechanism allows for increased signal to noise ratio in relevant brain regions that may contribute to encoding of learned associations. An important question that remains is how to reconcile findings reported in Fos-expressing ensembles as compared to the general population of neurons within a given brain region. Fos-expressing ensembles comprise a small percentage of cells within a particular brain region, encompassing between 2%–10% of neurons (Koya et al. 2009; Fanous et al. 2013; Cruz et al. 2014; Warren et al. 2016; Whitaker et al. 2016). Therefore, it is most likely that a cell selected at random within a particular brain region will be one of the nonactivated or weakly activated neurons and not a part of the neuronal ensemble. So global adaptation within randomly recorded cells are most likely to reflect the general trend within the Fos-negative population of neurons. Thus far there have been two studies in which Fos-negative neurons display opposite effects of Fos-positive neurons after behavioral training, but it remains to be seen whether this effect will generalize across different behaviors, brain regions, and cell types.

The role of the SK channel in Fos-expressing ensembles and nonensembles

An interesting potential player that has emerged from the literature on activity-dependent ensembles that mediate learned associations is the small conductance calcium-activated potassium channel. Disterhoft and colleagues found that neuronal excitability increases in CA1 pyramidal neurons following trace eyelid conditioning via a reduction in SK currents (Disterhoft et al. 1988; Moyer et al. 1996). Furthermore, the SK channel is known to interact with the NMDA receptor (Faber et al. 2005; Ngo-Anh et al. 2005), which is critical for many different types of learning and memory (Morris et al. 1986), and is necessary for the induction of many forms of long term potentiation (Bliss and Collingridge 1993). More recently, the SK channel has been implicated in drug-related associative learning. Hopf et al. described a reduction in SK channel function following withdrawal from alcohol seeking that led to increased excitability in randomly selected nucleus accumbens neurons (Hopf et al. 2010). More recently, Ziminski et al. (2018) discovered a reduction in the magnitude of the medium AHP in Fos-expressing neurons of nucleus accumbens core after cocaine conditioned locomotion, which may suggest a change in SK channel expression or function. Interestingly, SK channels were down-regulated in Fos-negative neurons of the medial PFC following operant food self-administration (Whitaker et al. 2017). While it is clear that the SK channel is an important modulator of excitability and a frequent target in studies of associative learning, its role in activity-dependent ensembles and addiction is yet to be explored.

Technical considerations

Fos-expressing neuronal ensembles in addiction research are comprised of a heterogeneous population of cell types that reflect the general make-up of the brain region examined. Colabeling with RNAscope in situ hybridization or immunohistochemistry have found that Fos-expressing cortical ensembles were approximately 90% glutamatergic and 10% GABAergic, in line with the overall composition of the prefrontal cortex (Leao et al. 2015; Warren et al. 2016). Fos-expressing striatal ensembles were primarily DARPP32-containing medium spiny neurons of both D1 and D2 receptor types, along with small percentages of the different GABAergic interneurons (Leao et al. 2015; Rubio et al. 2015; Caprioli et al. 2017), again in line with the overall composition of the striatum. Future studies will be necessary to examine whether different electrophysiological alterations exist within different cell types of Fos-expressing neurons.

An additional consideration when comparing Fos-positive and Fos-negative neurons is to confirm that any electrophysiological differences result from the learning process and are not simply due to baseline differences in spontaneous activity of the neurons. To rule out this alternative explanation, a separate control group of animals should be tested with different set of cues or contexts than those used during learning and thus activate a set of Fos-expressing neurons different from those activated during learning.

Functional engram formation during learning

Although the idea that learning may be encoded in small populations of sparsely distributed neurons is not new, the tools to study these neuronal ensembles have only been recently developed. Given that this field is in its relative infancy, many questions remain to be answered. First, how are neurons selected to participate in a given ensemble? Most likely there is a population of neurons that happens to receive the highest numbers of activated cue-specific afferents that converge on what was an initially random pattern of neurons (Pennartz et al. 1994). Synaptic alterations are likely induced during subsequent training sessions so that the relevant cue-specific inputs can more reliably and efficiently activate the most strongly activated ensemble neurons (Koya et al. 2012; Yiu et al. 2014; Whitaker et al. 2016). This coupled with shifts in the intrinsic membrane properties of cue-selected ensemble neurons across training may lead to the selection of particular neurons as ensemble neurons (Whitaker et al. 2017; Ziminski et al. 2017, 2018). However, a different line of studies found that constitutive overexpression of the transcription factor CREB "before" learning or cue experience led to preferential recruitment of lateral amygdala neurons into an ensemble encoding cue-cocaine associations, or a "cocaine engram" (Hsiang et al. 2014). Subsequent work from the same laboratory found that constitutive CREB overexpression led to corresponding increases of excitability in lateral amygdala neurons, and that manipulating the level of excitability in LA changed the probability of neurons being incorporated into the ensemble (Yiu et al. 2014). They postulated that CREB-mediated increases of excitability may drive particular neurons to be incorporated into an ensemble. However, while transient CREB phosphorylation is probably an important step in forming engrams, it seems unlikely that neurons are allocated (e.g., via increased CREB function) for a particular memory prior to learning; the learning specific alterations cannot exist prior to cue-exposure and learning. To this point, Whitaker et al. (2017) measured excitability in Fos+ and Fos− neurons of the prefrontal cortex before and after operant training and found no difference in excitability in Fos+ and Fos− neurons on the first day of training, suggesting that neurons that go on to become part of the ensemble do not necessarily start at a higher baseline firing frequency or resting membrane potential. Nevertheless, these constitutive CREB overexpression studies support the idea that CREB may play an important role in the formation of naturally formed neuronal ensembles. Future studies are also necessary to determine whether naturally formed ensembles are selected differently based on brain region and cell type.

Functional alterations in neuronal ensembles in other brain areas

Although research is being done to characterize ensemble neurons relevant for drug addiction, much of this research is relegated to the nucleus accumbens or prefrontal cortex (Koya et al. 2012; Whitaker et al. 2016; Ziminski et al. 2017, 2018). While these two areas are known to be of critical importance in a number of addiction-related learned behaviors, other critical brain regions need to be examined. As mentioned, one study found that an ensemble in lateral amygdala may play a role in retrieval of a cocaine-related memory (Yiu et al. 2014), although this ensemble was induced by viral transduction prior to learning and cue exposure. It will be interesting to see if a naturally formed ensemble that is selected by cues during learning will also play a role in this memory. Recent studies indicate a role for the central amygdala (Venniro et al. 2017) and in particular neuronal ensembles in the central amygdala in mediating nicotine seeking (Funk et al. 2016) and alcohol dependence (de Guglielmo et al. 2016) in rats. Neuronal ensembles in the dorsal hippocampal CA1 region play a role in conditioned place preference to cocaine (Trouche et al. 2016). Neuronal ensembles in dorsomedial striatum have also been shown to mediate methamphetamine seeking (Caprioli et al. 2017). Thus, it will be important to assess functional electrophysiological alterations in these latter brain areas.

Conclusion

Identifying neurons that comprise activity-dependent ensembles has been a significant advancement in the past 10 years. The next step is to answer the question of "what is the engram." Given that it has been repeatedly shown that Fos-expressing neuronal ensembles play a causal role in the expression of learned behavior (Koya et al. 2009; Bossert et al. 2011; Fanous et al. 2012; Cruz et al. 2014; Pfarr et al. 2015; de Guglielmo et al. 2016; Funk et al. 2016; Warren et al. 2016; Caprioli et al. 2017; Whitaker et al. 2017), it is likely that the answer to this question involves the electrophysiological alterations that have been identified here (and in future studies) in Fos-expressing ensemble neurons. In addition to the electrophysiological alterations within the Fos-expressing neurons described above, a number of molecular alterations have also been identified only within Fos-expressing neuronal ensembles and not in the majority of neurons that are Fos-negative (Guez-Barber et al. 2011; Fanous et al. 2013; Liu et al. 2014; Li et al. 2015; Rubio et al. 2015, 2016). We now need to move past demonstrating causal roles for ensemble neurons per se and on to manipulations of identified alterations within these ensemble neurons to identify which specific electrophysiological and molecular alterations play causal roles in encoding the learned associations. With a focus on these long-lasting molecular and cellular alterations within the Fos-expressing neurons that are mediating behavior, we may finally elucidate the real engram and how it forms.

Although speculative, these findings could permit us to more accurately treat the maladaptive learned associations that drive addiction. There may be alterations that are relatively unique to drug addiction-related engrams that can then be targeted selectively within the relevant drug-associated ensembles. Alternatively, drug craving-related memory ensembles can be reactivated by

drug-related cues, which puts these ensemble neurons in a unique highly activated state that distinguishes them from the surrounding less activated neurons. This high neural activity state creates new pharmacological targets such as activated NMDA receptor, which bind preferentially to ketamine and memantine when in the activated open state (Wood 2005; Johnson and Kotermanski 2006; Sinner and Graf 2008), or some pharmacological agents could bind selectively to activated MAP kinases within these activated ensemble neurons. This would add a new dimension of context-specific and ensemble-specific pharmacology to the more commonly used context-independent cell, receptor or neurotransmitter-targeted pharmacology.

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