

Review

Neuronal ensembles: Building blocks of neural circuits

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SUMMARY

Neuronal ensembles, defined as groups of neurons displaying recurring patterns of coordinated activity, represent an intermediate functional level between individual neurons and brain areas. Novel methods to measure and optically manipulate the activity of neuronal populations have provided evidence of ensembles in the neocortex and hippocampus. Ensembles can be activated intrinsically or in response to sensory stimuli and play a causal role in perception and behavior. Here we review ensemble phenomenology, developmental origin, biophysical and synaptic mechanisms, and potential functional roles across different brain areas and species, including humans. As modular units of neural circuits, ensembles could provide a mechanistic underpinning of fundamental brain processes, including neural coding, motor planning, decision-making, learning, and adaptability.

How neural activity is transformed into perception, cognition, or action is arguably the central question of neuroscience. To answer this question, a circuit-level approach appears necessary to enable bridging the activity of individual neurons with higher-level cognitive processes. But brain circuits are complex, with vast numbers of interconnected neurons of hundreds of different types. The goal of our review is to examine if there are unified functional principles governing the organization of neural circuits, focusing on a structured form of activity within neuronal populations known as “neuronal ensembles.” Neuronal ensembles are defined here as a group of neurons that display recurring patterns of coordinated activity. We argue that ensembles are endogenous building blocks of neural circuits, which act as modular functional units. They could be a versatile mechanism for many brain functions and implement emergent properties, i.e., functions that, by definition, are not present in the individual neurons themselves.

Origin of the ensemble hypothesis

The idea that neurons cooperate to form emergent functional units has a long history. Indeed, Cajal’s drawings already represented cortical circuits as repeated modules, with neurons linked by arrows that illustrated the flow of activity within them¹ (Figure 1A). Providing electrophysiological flesh to these arrows, Sherrington proposed that groups or “ensembles” of neurons form reflex arcs, i.e., synaptic circuits linking sensory stimuli with motor responses.⁵ Combining both anatomical and physiological viewpoints, Lorente de Nó reasoned that “reverberating chains” of neurons, activated via recurrent excitatory feedback connections, would act as modular units of cortical activity and endogenously generate persistent activity, even in the absence of external inputs² (Figure 1B). The idea that cortical activity is

organized and parceled in small groups of neurons was further developed by Hebb, who argued that recurrently connected groups of neurons could form “assemblies” due to synaptic plasticity by strengthening connections from coactive cells³ (Figure 1C). Incorporating Semon’s proposal of the engram as the neural substrate of a memory,⁶ Hebb also hypothesized that assemblies could be triggered by the activation of a subset of key neurons, thus generating “pattern completion,” i.e., the ability of a part of a system to activate the whole (Figure 1D). Pattern completion is a hallmark of memory retrieval and of many brain functions, including speech, motor behavior, emotions, and cognition. Following Hebb’s ideas, Marr built computational models of the cortex and hippocampus and proposed that pattern completion arose in them by the amplification of internal activity due to recurrent network connectivity.⁷ Hopfield provided further mathematical generalization of these models, applying to neural circuits the Ising model of ferromagnetism that explained how the generation of emergent states arises in magnets from the functional coupling among elements. Hopfield predicted that, similarly to magnetic states, strongly coupled neurons in recurrently connected networks naturally form stable activity states, which he called “attractors,” as they “attract” the activity of the population⁸ (Figure 1E). These attractors, endowed with pattern completion, would implement memories and also generate solutions to optimization computations like the traveling salesman problem.^{9,10} Independently, Abeles also reached the conclusion that cortical function must be organized as groups of synchronously active neurons. Realizing that most cortical synapses are weak and stochastic and have short-term depression dynamics, and the fact that the action potential threshold imposes a strong non-linearity in the activation function of the neuron, Abeles proposed that the only way for



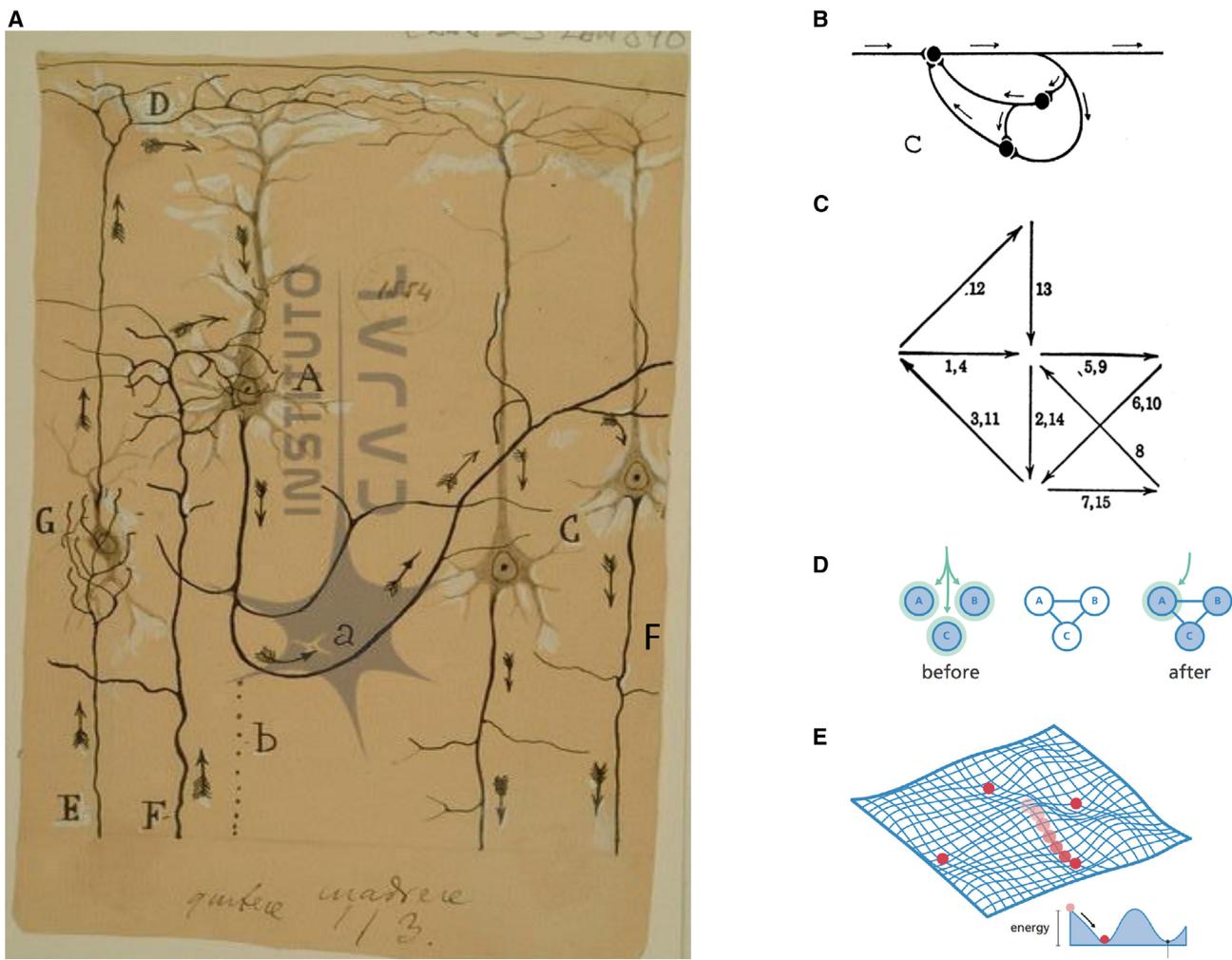


Figure 1. Historical models of ensembles

(A) Cajal's drawing of neuronal activity progressing through a putative cortical module (courtesy of the Cajal Institute, "Cajal Legacy," Spanish National Research Council [CSIC], Madrid, Spain).

(B) Lorente's representation of a reverberating chain, with recurrent feedback excitation. Adapted from De No.²

(C) Hebb's representation of activity flow (arrows) through a neuronal assembly. Adapted from Hebb.³ Note recurrent connectivity.

(D) Pattern completion: after neurons are activated by a synchronous input (left), connections between neurons are generated, or strengthened, (middle), and stimulation of one neuron triggers the rest. Adapted with permission from Yuste.⁴

(E) Attractor landscape generated by a recurrently connected neural network. Population activity is represented as a ball that rolls over to the lower energy point, where a group of neurons is coactive, defining an attractor. Adapted with permission from Yuste.⁴

neuronal activity to propagate through the cortex was via coactive groups of neurons. These “synfires chains,” another conceptualization of the idea of an ensemble, would sequentially activate each other, forming temporal chains of synchronous activity.¹¹

One can capture the essence of these proposals with the previously mentioned working definition of a neuronal ensemble as a “group of neurons displaying recurring patterns of endogenously coordinated activity.” Thus, these groups of coactive neurons can occur with or without external sensory stimulus, and their reproducible patterns of neural activation can be categorized as circuit attractors. It is important to note that the neurons of an ensemble could also be involved in different types of activity, unrelated to the group. For homogeneity and concor-

dance with the majority of the literature, we use Sherrington's original term of “ensemble” to describe circuit modules with synchronous, or closely correlated, coordinated activity. But, differently from Sherrington reflex arcs, ensembles would be intrinsically generated and be activated either endogenously or by an outside stimulus, as proposed by Lorente, Hebb, Hopfield, Abeles, and many others. Therefore, in the ensemble hypothesis, the emphasis switches from a neural circuit that responds to the outside environment as an input-output system to one that has ingrained activity patterns embedded into it. This distinction is important because it highlights the possibility of activity states generated by neural circuits that could be independent of the outside world and can therefore be used to implement internal representations of objects,¹² memories, or cognitive states.³

Novel methods to study ensembles

Experimental search for ensembles has been hampered by the inability to record the activity of many neurons simultaneously. Neuroscientists traditionally used single-cell recordings and correspondingly focused on describing responses of individual neurons such as receptive fields. But ensembles, which, as an emergent property, reflect the interaction between neurons, cannot be captured by recording one neuron at a time. The recent developments of multielectrode recordings and voltage or calcium imaging from large numbers of individual neurons have transformed our ability to identify and characterize multi-neuronal activity patterns^{13–47} (Table 1). The phenomena described using these methods are diverse and so is the nomenclature used to define or describe them. Besides ensembles, other terms have been used to describe patterns of coordinated neural activity, such as assemblies, attractors, reverberations, synfires, domains, oscillations, trajectories, clusters, groups, packets, domains, flashes, songs, bumps, avalanches, trajectories, and states, among others (Box 1).

How do all these different descriptions relate to neuronal ensembles? These recent results describe how neuronal activity propagates through circuits by engaging specific groupings of neurons, and different studies highlight different aspects of this multineuronal activity. A core feature of ensembles is that they can become endogenously active, in the absence of external stimuli.^{54,58} And indeed, many of these studies have revealed that coactive, or sequentially active, groups of cells work together as endogenous functional modules. As we will discuss, data from both cortex and hippocampus demonstrate organized spontaneous activity in the absence of sensory stimulation or motor behavior. The origin and function of this ongoing spontaneous activity, which is present throughout the nervous system^{59–64} and in species ranging from humans⁶⁵ to fish⁴⁷ and cnidarians,⁶⁶ are still poorly understood and could reflect different physiological or cognitive processes. But it is clear that spontaneous activity is not “noise” in the system but has a particular structure. Indeed, as we will see below, the groups of neurons, activated are in some cases can be the same as the ones that are activated during perceptual tasks or behavior, consistent with the idea of ensembles as internal circuit units that can be engaged by external stimulus.

In most experimental descriptions of ensembles, they are fine-grained and structured units with a particular spatiotemporal scale, involving a small percentage of the neurons, activated for a characteristic duration of time, constrained by the method to record neuronal activity (Table 1) and the analytical approaches used (Box 2). Although there is no set rule, most studies define ensembles as groups of neurons that are repeatedly activated within a period of a few milliseconds to a few seconds. In addition, individual neurons can join different ensembles, which enables combinatorial, compositional, and hierarchical arrangement of ensembles, just like in other emergent systems.^{90,91}

Neuronal ensembles in the hippocampus

The hippocampus is one of the first brain regions where experimental evidence for the ensemble existence was collected *in vivo* in the form of repeated coordinated firing of subsets of pyramidal cells, potentially carrying together more information than single

neurons³⁵ (Figure 2A). Coordinated neuronal activity was sequential, with neurons firing one after another within the period of a theta cycle.³⁵ Recurring sequences of neuronal activation forming hippocampal ensembles can span from a few milliseconds, nested within sharp-wave ripples, to several seconds.^{55,80,81,92–94} When the same neurons are recorded across different behavioral states, larger hippocampal ensembles can be segmented into smaller functional units. For example, seconds-long hippocampal sequences observed during locomotion are in fact formed by chains of successive ensembles activation³⁶, Figure 2B).

Importantly, these hippocampal sequences can be reactivated offline during sharp-wave ripple oscillations recorded during quiet rest or sleep. This is known as “replay,”^{77,78,95} and agrees with the idea that ensembles arise from intrinsic neuronal dynamics, as external stimulation is minimal during rest. Consistent with this, the same ensembles observed during behavior can be artificially activated through optogenetic activation of local excitatory neurons.²³ Hippocampal ensembles are therefore structured independently from external inputs. In addition, they appear remarkably resilient to transient perturbations^{81,96–99} and also stable across days.^{42,97} It is possible that hippocampal ensembles are recruited from a predefined reservoir constrained by developmental programs before any contextual spatiotemporal information is mapped into them.^{100–103}

Hippocampal ensembles appear anatomically scattered and sparse.³⁶ Although the data are still preliminary, hippocampal ensembles may be functionally orthogonal, meaning that the activation of one ensemble may suppress the others.³⁶ Several circuit mechanisms could support ensemble formation and competition, including changes in intrinsic excitability (see below) and local interneurons,¹⁰⁴ which could also provide a temporal backbone for sequential spiking.^{23,105}

Importantly, hippocampal ensembles can be causally linked to behavior. Indeed, activating or inhibiting neuronal ensembles associated with SWRs or theta sequences can impair spatial memory acquisition or recall.^{106–109} This suggests that hippocampal ensembles could also function as engrams. Experimentally, engram cells are often defined as groups of neurons expressing immediate early genes following a given experience, that when reactivated, reproduce the behavior observed during that experience.¹¹⁰ The link between engrams and ensembles has been analyzed in two studies.^{45,50} In novel environments, engram neurons encode context rather than space,⁵⁰ whereas in familiar environments, they represent stable place cells.⁴⁵ Therefore, engrams could be hippocampal ensembles dedicated to context rather than groups of neurons encoding spatiotemporal information into sequences. But, although the coordinated engram cell activation can reactivate a memory, the population dynamics evoked by the synchronous activation of c-Fos expressing neurons remains unknown and could activate endogenous ensembles, independently from the engram cells.¹¹¹ Thus, the relation between engrams and ensembles still remains unclear. Engrams would qualify as ensembles if the activation of the cells forming the engram was repeatedly coordinated. Future studies examining the temporal order of activation of the cells recruited in engrams may certainly help clarifying this point.

Table 1. Experimental descriptions of multineuronal activity patterns

Terminology	Brain region	Duration and frequency	Stability	Proposed function	Species	References
Attractors	V1, entorhinal cortex, anterior lateral motor cortex, frontal cortex, hippocampus	10 ms–1 s, <<1 Hz	hours to days	spontaneous, decision-making, motor planning, working memory	mouse, human	Cossart et al. ⁵⁴ Finkelstein et al. ²⁸ Inagaki et al., ³⁰ Kamiński et al., ³³ and Gardner et al. ³⁴
Ensembles	sensory and motor cortex, hippocampus	100 ms-s	days	perceptual states, awareness	mouse, human, monkey	Carrillo-Reid et al., ³⁷ Carrillo-Reid et al., ³⁸ Wenzel et al., ⁴⁰ Wenzel et al., ⁴¹ Pettit et al., ⁴⁵ and Truccolo et al. ⁴⁶
Assemblies	adult hippocampus, developing barrel cortex prefrontal cortex, striatum	50–200 ms, 0.1 Hz	days	substrate for internal cognitive processes, including memory and planning, circuit development	rat, mouse	Harris, ²¹ van de Ven et al., ²⁵ Holtmaat and Caroni, ²⁶ Buzsáki, ²⁷ Harris et al., ³⁵ Malvache et al., ³⁶ Módol et al., ³⁹ Haimerl et al., ⁴² Sheintuch et al., ⁴³ and Oberto et al. ⁴⁸
Avalanches	organotypic cultures	10 s ms, <1 Hz	>10 h	a critical state serving information transmission and network stability	rat	Beggs and Plenz ²⁰
Clusters	developing habenula	seconds, <0.1 Hz	days	learning	zebrafish	Fore et al., ⁴⁴ Cherng et al., ⁴⁹ and Jetti et al. ⁶¹
Domains	neocortex slices	seconds, <0.1 Hz	days	circuit formation	rat	Yuste et al. ^{17,18}
Engrams	hippocampus	seconds, <0.1 Hz	days	memory encoding and retrieval	mouse	Pettit et al., ⁴⁵ Tanaka et al., ⁵⁰ Josselyn and Tonegawa, ⁵¹ Liu et al., ⁵² and Ramirez et al. ⁵³
Flashes	V1 slices	100 ms-s	hours	spontaneous states	mouse	Cossart et al. ⁵⁴
packets	sensory cortex	50–500 ms, 0.1 Hz	hours	cortical communication	rat	Luczak et al. ²² and Luczak et al. ³²
Sequences	motor cortex hippocampus	10 ms-s, <0.1 Hz	hours	choice-decision integration of information	mouse	Harvey et al. ²⁹ Aery Jones and Giocomo ⁵⁵
Songs	neocortical slices	10 ms-s, <0.1 Hz	hours	spontaneous	mouse	Ikegaya et al. ¹⁹
Trajectories	motor cortex	10 ms-s, <0.1 Hz	days	motor reaching	monkey	Churchland et al. ³¹

Box 1. Previous definitions of ensemble-related phenomenology

A cell assembly is defined as a group of neurons that meet four criteria: (1) display structured temporal patterns of spiking even without temporally structured stimuli; (2) spiking is not strictly controlled by sensory input; (3) coordinated spike times; (4) assembly activity correlates with internal cognitive processes.²¹

Cell assemblies are transiently active ensembles of neurons.²⁷

Assemblies are the result of the dynamic congregation and segregation of excitatory principal cells into functional groups.²⁴

Temporally organized packet of population activity lasting 50–200 ms.²²

Cell assemblies are formed by the co-activation of groups of neurons thought to underpin information representation in the brain.²⁵

Neuronal assembly: a group of neurons that can be recruited together due to synaptic connections between them, usually as a consequence of a learning process. Neurons belonging to one assembly can be distributed between several interconnected brain areas. Neuronal assemblies can be viewed as the smallest counterparts of representations in the brain and might represent the physical bases of memories. This term is mostly used in the context of learning and memory.²⁶

Neuronal ensemble: a population of neurons involved in a particular computation. The notion of ensemble implies that coding is produced by populations of neurons whose individual contributions are noisy but that together produce coherent outputs. The term is mostly used within systems and computational neuroscience to describe a neural network with a particular function.

Engram: the hypothetical physical means through which memories are stored in the brain. Engrams are thought to reflect biochemical and biophysical reactions in the brain induced upon learning, which are maintained as latent traces to allow subsequent memory retrieval.

Ensembles are collections of principal neurons that fire together over a time scale of tens of ms.⁵⁶

Neuronal ensembles are coactive groups of cortical neurons, found in spontaneous and evoked activity that can mediate perception and behavior.⁵⁷

In summary, multineuronal recordings reveal that the hippocampal system is organized into discrete ensembles involving sequentially firing groups of neurons, whose activation can be linked to spatial cognition.

Neuronal ensembles in the neocortex

Results from sensory areas in the cerebral cortex parallel many of the findings from hippocampal ensembles. Coactive, or sequentially active, groups of neurons consistent with the ensemble definition have been described in neocortex *in vitro* and *in vivo*, using voltage or calcium imaging^{16,19,37,38,54,58,63,64,70,112–116} and electrical recordings^{19,21,32,59,79,113,117} (Figure 3A). Coordinated and repeated neuronal activation was found under spontaneous and sensory-evoked neuronal activity and lasted from tens of millisecond up to a second, involving a minority (<10%) of neurons in a given cortical territory.^{38,54,58} In calcium imaging data, ensemble neurons are coactive in individual imaging frames (~100 Hz), whereas voltage imaging and electrical recordings reveal millisecond accurate temporal substructures with repeated sequences of neuronal firing.^{112,113,118} Like in hippocampus, individual neurons participated in multiple neocortical ensembles,^{54,58} providing a substrate for combinatorial coding. Consistent with the hippocampal replay, cortical ensembles evoked by sensory stimuli can also become spontaneously coactive in the absence of stimuli^{58,71,112,113} (Figure 3A). Cortical ensemble replay can also occur during sleep.¹¹⁹ Also, like in hippocampus,^{36,81} ongoing ensembles block the activation of other ensembles,¹⁰⁴ and persist during sensory stimulation.¹²⁰ These results imply that cortical ensembles are endogenous modular blocks of circuit activity, which can be recruited by sensory inputs.^{63,112,113,121}

In primary sensory cortex, the function of these endogenous cortical building blocks has been causally linked to perceptual and behavioral tasks. Visual ensembles can be observed under anesthesia but are particularly common when the animals are

awake and viewing natural scenes,⁵⁸ supporting the possibility that ensembles have a physiological role. Indeed, precise firing patterns, as potential cellular signatures of structured ensemble activity, are seen during stimulus processing⁷⁵ and delayed localization tasks.¹²² Ensembles also encode cognitive variables in operant tasks.^{123,124} Ensembles can also be created *de novo* by inducing correlated activity in groups of neurons in a behavioral context.¹²⁵ In agreement with this, activating neurons synchronously with holographic optogenetics can imprint an ensemble into visual cortex, and the imprinted ensemble can be triggered by the stimulation of a single cell, demonstrating pattern completion³⁷ (Figure 3B). Imprinted ensembles and their pattern completion persist in the cortex for at least several days, as if the microcircuit had been reprogrammed by the optogenetic stimulation.³⁷ Similarly, ensembles created artificially can be assigned different stimulus valences.¹²⁶ Holographic optogenetics can also be used to manipulate behaviorally relevant ensembles in Go/No-Go visual discrimination tasks. In these experiments, inactivating visually evoked ensembles blocked visually guided behavior, whereas their activation substituted for the presence of the visual stimulus, and triggered the behavioral task^{38,70,127} (Figure 4A). Thus, these experiments demonstrated that ensembles are both necessary and sufficient for perceptual tasks, consistent with the hypothesis that they are building blocks of perception.⁷⁰ Cortical ensembles, both visually evoked and spontaneously active ones, are also stable for weeks⁵⁷ (Figure 4D), providing a potential substrate for perceptual stability, overcoming the representational drift generated by individual neurons, whose activity varies over time.^{128–131}

In summary, cortical ensembles echo many of the properties from hippocampal ensembles, including their sequential structure and endogenous nature, as they can occur during ongoing activity but be recruited by behavioral tasks or evoked by sensory information. Moreover, the use of holographic optogenetics

Box 2. Analysis challenges to detect ensembles

One standard method to analyze neuronal ensembles is to calculate correlations between multi-neuronal firing or calcium signals and identify statistically significant clusters of synchronous neurons.^{47,64,67–69} Estimating the probability of occurrence of such synchronous firing of neurons is possible by using simple multi-variate statistical tools and variations of singular value decomposition and factor analysis.^{36,67,69–74} Yet, especially for spiking data, one needs to consider jitter in neuronal firing in order to build proper statistical models.^{75,76} Although such jitter in neural activity is less of a problem for calcium imaging with low temporal resolution, calcium signals with relatively high levels of noise impose an additional challenge. Hence, in both electrical and optical methods, different kinds of noise could result in an underestimation of the components of neural ensembles and ideally require several independent statistical approaches for identifying ensembles.

Aside from the statistical significance of the patterns, another matter to consider, when identifying neural ensembles, is the choice of temporal bin or period selected for analyzing relationships between neurons. Different neural and behavioral processes occur in different time windows. For example, synfire chain of neurons can last for few hundreds of milliseconds,^{19,77–79} but some other types of ensemble activity can last several seconds.^{19,47,59,66,80–82} Such long-lasting sequential activation of neurons poses a major analytical challenge for identifying neural sequences that might appear randomly and cannot be studied by zero-lag correlation-based analysis. Applying graph-based methods might offer some solutions, yet the longer the time windows of neural sequences, the exponentially larger number of iterations one needs to run to identify such sequential activations of neural ensembles. One potential approach could be to identify ensembles triggered by distinct tasks or stimuli (sensory, optogenetic, microstimulation), and then to use these reliably activated sequences of ensembles⁷⁹ as a “Rosetta Stone” for deciphering other ensemble sequences that might precede or follow these reliable ensembles. Hence, multiple analytical and experimental strategies are needed for investigating the temporal dynamics of ensemble sequences occurring in different time scales.

Similarly, slower and state dependent alterations of neural excitability through neuromodulators, metabotropic receptors as well as astroglia could have a major impact on the type of ensembles one might observe. For instance, ensembles could grow and shrink in size depending on the reach of available neural excitation, and on the changing excitability thresholds of neurons to be recruited in the ensembles. This might in fact be one of the major strengths of neuronal ensembles that can arise by recruiting permutations of neurons within the same population and by utilizing those permutations for different tasks and purposes. Such multiplexing of available neural populations doesn't require building new synapses through Hebbian processes or other slow alterations of circuit architecture and instead can be achieved fast and at very low cost by altering neural excitability. Probing such processes pre-associated with distinct behavioral programs and neural processes might not require very specific circuit manipulations. For example, one strategy to test these processes might be to “heat” or “cool down” neural networks by gradual alterations of ambient neurotransmitters levels or cations, as well as manipulations of synaptic receptors and neural excitability through pharmacology or optogenetics. In such a scenario, one would not expect a linear expansion for the number of neurons participating in ensemble activity but instead critical jump points that expand and change the composition of neurons within ensemble. Perhaps, facilitation or disruption of specific behaviors or spontaneous activity via broad opto-/chemogenetic activation or inhibition of neurons and even astroglia in specific brain regions^{83–85} might similarly result in unspecific alteration of local excitability.

Such critical jump points and nonlinear expansion of ensemble size could also relate to a hierarchical order, where number of smaller ensembles are nested within larger ensemble and are merged only when neural excitability reaches to a critical state. The sequential recruitment of motor patterns, or behavioral fixed action patterns, is a hallmark of most complex behaviors, all the way to cnidarian locomotion.^{86,87} Moreover, similar hierarchies of ensembles have also been observed throughout development, where larger ensembles eventually give rise to smaller and more specific ensembles, through maturation of neurons and synapses.^{44,88,89} Finally, it is important to add that such nested ensembles do not have to exist within only a single brain region but can involve interconnected ensembles across distant brain regions.⁷²

has confirmed that cortical ensembles can be causally related to behavioral tasks.

Mechanisms of ensemble generation and recruitment

In spite of the importance of ensembles for hippocampal and cortical function, little is known about their biological properties. Many questions remain open: How are ensembles generated? Do ensembles arise from strengthening of existing synaptic connections? How do neurons within an ensemble coordinate their activity? How are ensembles recruited, once formed?

Since cortical ensembles can be generated by simultaneous stimulation of groups of cells,^{37,125} it is widely assumed that Hebbian plasticity is involved in establishing cortical ensembles, by synaptic strengthening generated by joint firing of pre-and post-synaptic neurons, following, for example, spike-timing dependent

plasticity (STDP).^{132,133} But, as an alternative, and not necessarily contradictory, hypothesis, intrinsic changes in excitability could also help generate coordinated activity in an ensemble. For example, if neurons become more excitable, the same synapse could have a stronger effect, and thus result in an enhanced synaptic input, an analogous effect to synaptic strengthening. In fact, several experiments have revealed cell-intrinsic changes in excitability in neurons after periods of neuronal activity.^{134–137} Moreover, increases in excitability are found following long-term potential (LTP) induction protocols in visual cortical neurons¹³⁸ and hippocampal CA1 pyramidal cells.¹³⁹ The excitability of cortical neurons is also modified after behavior training,¹⁴⁰ exposure to novel sensorial experience¹⁴¹ or environmental enrichment.¹⁴² Interestingly, changes in intrinsic excitability appear to underlie the selection of place cell in the hippocampus.^{143–145}

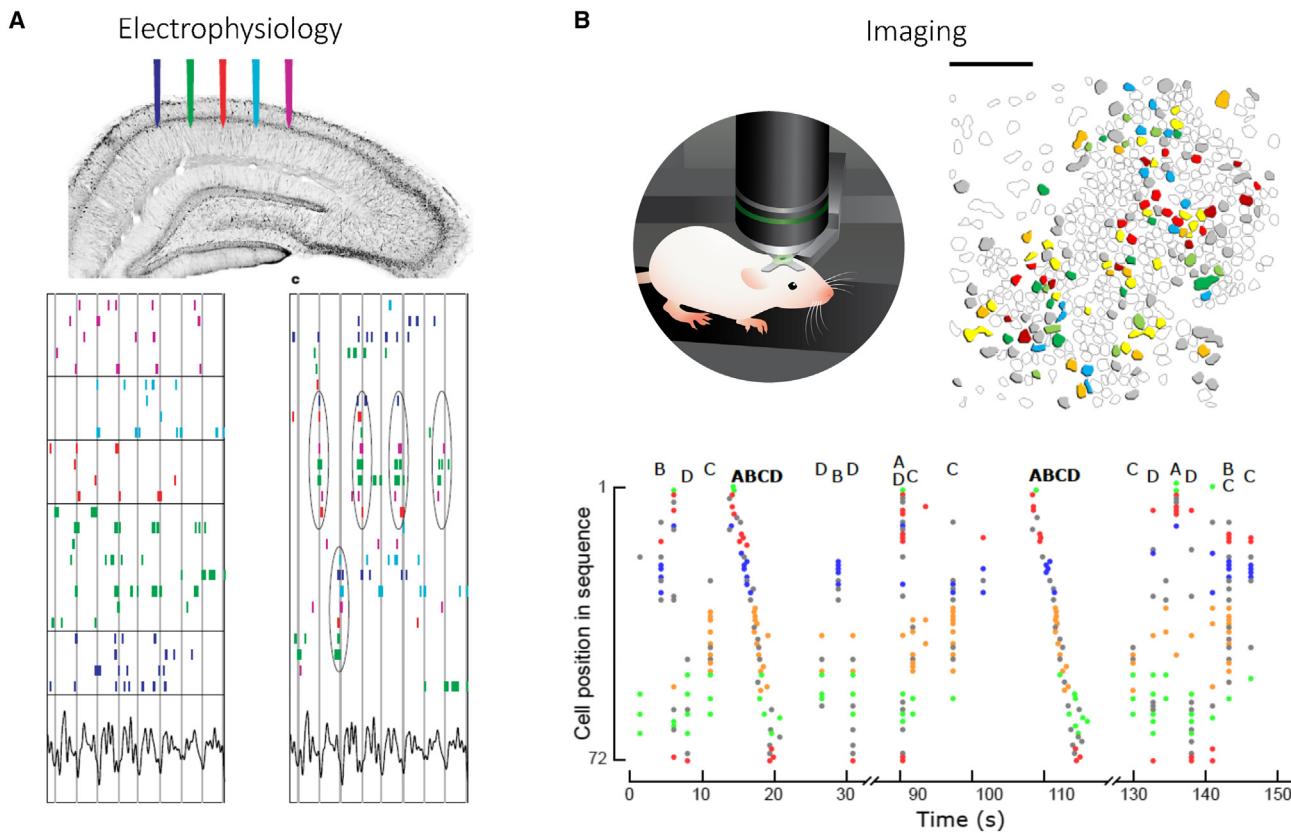


Figure 2. Hippocampal ensembles

(A) Top: extracellular electrophysiological recordings *in vivo*. Bottom left: raster plots of spiking during spatial exploration arranged in order of physical position along CA1 pyramidal layer (vertical lines indicate troughs of theta wave). Bottom right: spike raster rearranged to highlight synchrony demonstrate ensembles, with repeatedly synchronous firing of neuronal subpopulations (circled). Adapted with permission from Harris et al.³⁵

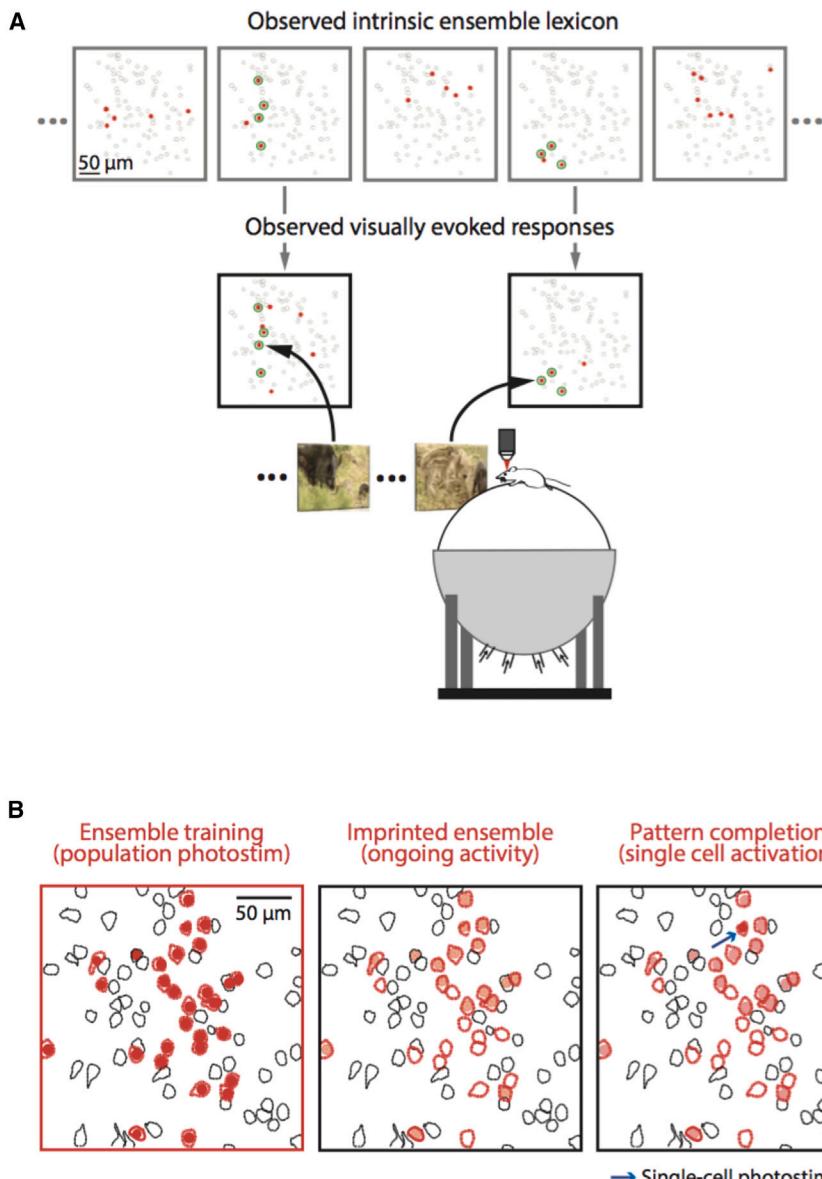
(B) Top left: two-photon calcium imaging of CA1 neurons in head-fixed mice *in vivo*. Top right: Contour map of imaged neurons shows spatially intermingled ensembles (color-coded according to ensemble membership). Scale = 100 μ m. Bottom: rasterplot displaying neuronal activation, color-coded according to ensemble affiliation (4 ensembles labeled from A to D). Ensembles are reactivated in the same order during sequences.

Also in hippocampus, a short-term increase of excitability of engram cells (as defined by c-Fos expression) enhances the subsequent retrieval of specific memories.¹³⁷ This is consistent with experiments where the activation of hippocampal engram cells recalls stored memories under protein synthesis inhibitors, which should prevent synaptic plasticity.^{135,146}

Demonstrating an important role for excitability in ensemble formation, cortical neurons display increases in intrinsic excitability after optogenetic or electrical stimulation of groups of neurons that generates ensembles.¹⁴⁷ In those ensembles, paired recordings showed fast increases in excitability, input resistance, and rheobase, but without evidence of new synapses. A small synaptic enhancement was observed only after longer periods of stimulation, suggesting that intrinsic excitability changes create ensembles, whereas synaptic plasticity could become important at later stages, perhaps during learning or consolidation. Altogether, these results indicate that, in addition to Hebbian synaptic plasticity, the formation of an ensemble could rely on cell-autonomous intrinsic mechanisms.

A related question is the ensemble composition. How are neurons assigned to an ensemble? Is ensemble membership random or are there specific neurons, or subtypes of neurons, that are key

to turn an ensemble on, or perhaps to turn it off? Although ensembles can be built in neocortex *in vivo* by activating a group of pyramidal neurons (Figure 3D),³⁷ it remains unclear whether these cells belong to a specific subtype of neurons or have stronger interconnections to the rest of the neuron in the ensemble, a property that could reflect an early developmental program.¹⁴⁸ Inhibitory neurons could also be important for generating ensembles. Cortical and hippocampal interneurons belong to many different subtypes,¹⁴⁹ so it is quite likely that interneurons subtypes will play different roles in generating and modulating ensemble activity. Although it may seem paradoxical, GABAergic interneurons can coordinate groups of excitatory cells in hippocampus.^{150,151} Indeed, during development, GABAergic “hub neurons” that are highly connected to the rest of the ensemble play a key role in ensemble generation.^{151,152} GABAergic interneurons also parse the temporal order of firing within hippocampal ensembles^{23,105} and suppress competing hippocampal ensembles.^{97,98,104,153} Perturbing interneurons also disrupt grid cell ensembles in entorhinal cortex.^{83,154} Consistent with an important role of interneurons in ensembles, bidirectional plasticity of parvalbumin (PV) and cholecystokinin (CCK)-expressing interneurons was observed in c-Fos expressing engrams neurons.¹⁵⁵



Development of ensembles

As Cajal pointed out, how a system develops can often give deep insights into its function.¹⁵⁶ Thus, research into the development of ensembles could help understanding their structure, mechanisms, and computational purpose. We propose that early developmental programs scaffold the spatial extent and composition of neuronal ensembles. This is supported by two converging lines of evidence: the first one relates to the preconfigured nature of circuit activity, and the second links the diversity in intrinsic excitability and connectivity with developmental origin.

Let's recall the definition of an ensemble as a group of neurons that display recurring patterns of coordinated activity. Thus, an essential feature of the ensembles is that they can occur spontaneously, without external stimulation. Consistent with ensembles engaging preexisting existing circuits, despite being prone to experience-dependent plasticity, the cell composition

Figure 3. Neocortical ensembles and pattern completion

(A) Cortical ensembles activated spontaneously (top) or by naturalistic visual stimuli (*in vivo*) in mouse primary visual cortex *in vivo*. Red cells are members of an ensemble, and green are those active in both conditions. Note similarity (arrows) between two visually evoked and spontaneous ensembles. Adapted with permission from Miller et al.⁵⁸

(B) Ensemble imprinting and pattern completion. Left: two-photon optogenetic activation of a group of neurons (red). Middle: most stimulated neurons become spontaneously active, forming an ensemble (light red). Right: ensemble is reactivated by optogenetic photostimulation of one neuron (arrow), demonstrating pattern completion. Adapted with permission from Carrillo-Reid et al.³⁷

and functional wiring (i.e., activation order) of neural ensembles are remarkably stable across environments and time.^{19,22,23,32,42,47,54,57,59,71,97,113,157,158} In addition, ensemble membership is likely non-random^{23,97,100} and activated within local circuits with minimal reliance on external inputs.^{23,63,97,99,113} Thus, there could be a finite repertoire of preexisting ensembles within a given circuit in the adult brain. The fact that neuronal ensembles can be orthogonal or non-overlapping^{36,104} further constrains the number of possible ensembles a given circuit can produce.

With these constraints, it is interesting to discuss when and how a predetermined pool of ensembles emerges during development and to disentangle the respective contributions of the developmental genetic program and experience in this process. There is a growing body of indirect evidence suggesting that the repertoire of neuronal ensembles may be partly genetically predetermined as early as embryogenesis. As discussed, ensemble membership and latency to fire within a given ensemble

appear mostly determined by intrinsic excitability and local connectivity^{23,97,104,153,159} with both parameters partly rooted in the time of neurogenesis of individual neurons.^{44,160–164} For example, the intrinsic excitability of CA1 pyramidal neurons reflects their embryonic birthdate,¹⁶¹ which may explain their differential participation to neuronal ensembles.^{97,165} The idea that ensembles are partly constrained by genetic programs is further supported by lineage analysis indicating that sister pyramidal neurons in the neocortex are most likely to be synaptically coupled within cortical columns, the same way that neurons in ensembles share similar receptive fields.^{168–170}

In our view, “proto-ensembles” would first emerge from sparse groups of coactive neurons coupled through electrical synapses (Figure 5-1). These are observed around birth in the cortex of rodents, which would correspond to the start of the last trimester of gestation in humans. Transient electrically coupled ensembles

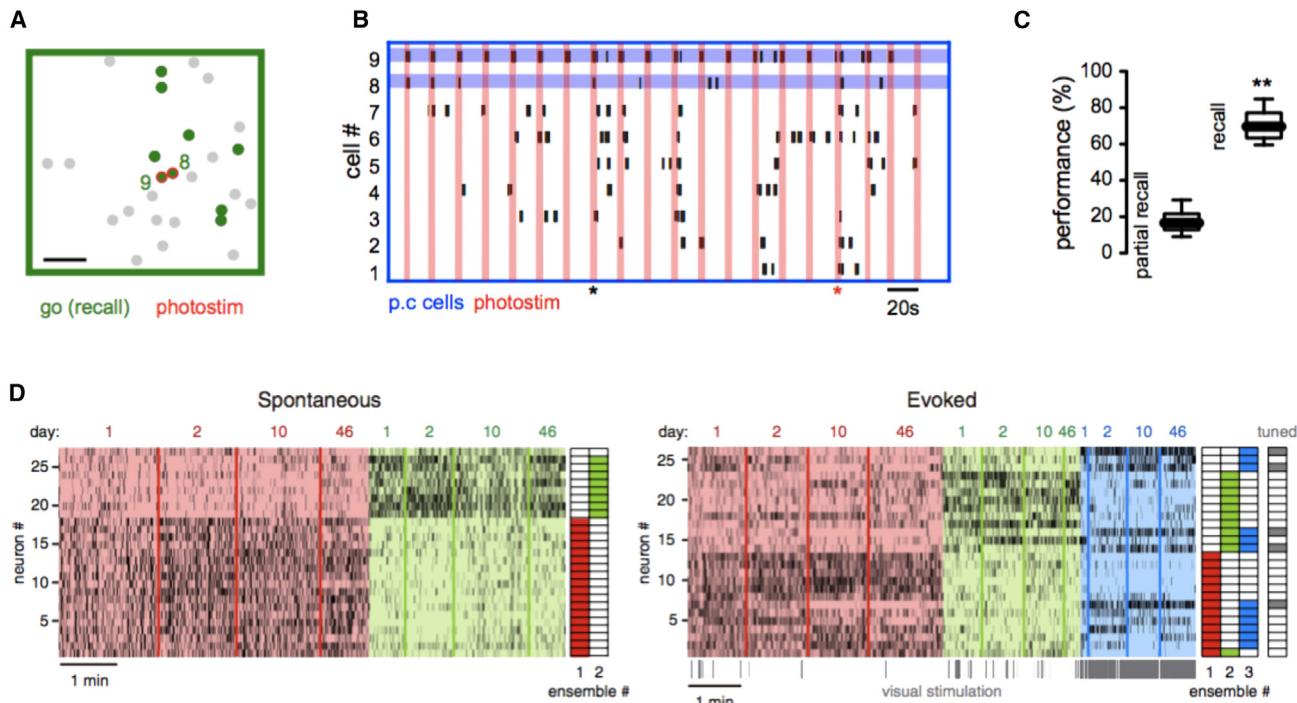


Figure 4. Neocortical ensembles can trigger behavior and are long-lasting

- (A) Ensemble activated by Go stimulus in a visual discrimination licking task (green neurons). This ensemble can be reactivated by two-photon optogenetic stimulation of two cells (red).
- (B) Behavior induced by recalling a Go ensemble in absence of visual stimuli. Raster plot of activity of ensemble neurons during holographic stimulation of those two pattern completion cells (blue lines). Vertical red lines indicate optogenetic photostimulation. Red star shows successful recall of Go ensemble and licking behavior. Black star shows partial recall with no behavior associated.
- (C) Behavioral performance evoked by recalling Go ensemble by optogenetic stimulation in the absence of visual stimuli (right) is significantly higher than performance in partially recalled trials (left). Adapted with permission from Carrillo-Reid et al.³⁸
- (D) Long-term stability of ensembles. Rasterplots of spontaneous (left) and visually evoked (right) neuronal activity over 46 days in mouse visual cortex *in vivo*. Neurons sorted based on ensemble identity (color-coded; right columns illustrate ensemble assignment). Adapted with permission from Pérez-Ortega et al.⁵⁷

have been reported in different species and throughout the nervous system including the spinal cord, neocortex, hippocampus, and retina.^{17,18,171–174} Gap-junctional coupling may therefore represent a universal phenomenon to generate a developmental blueprint for ensemble construction. Which neurons join an ensemble remains to be established but there is a bias for spatially clustered cells to belong to the same ensemble (Figure 5-1),^{17,44} as well as for cells sharing a similar lineage¹⁷⁵ or birthdate.^{44,176} However, early ensembles, including cortical synchronous plateau assemblies (SPAs) have pyramidal cells and interneurons^{171,176} a composition that is necessary for adult ensembles given the dual role of interneurons in organizing and suppressing ensembles.^{23,27,104,177} These early coactive groups of neurons could thus represent proto-ensembles,^{178,179} as suggested by early experiments in the developing neocortex where coactive neurons by means of gap-junctions span several layers in a column-like fashion.¹⁷ Activity within these proto-ensembles mainly takes the form of calcium transients, associated^{171,172,176,180} or not¹⁸ with action potential bursts (Figure 5-1). In the former case, calcium transients are critically influenced by intrinsic excitability as determined by active conductances including the H-current.¹⁷¹ The minimal reliance on external inputs for the formation of these proto-ensembles is supported by the fact that they do not

match the barrels in the somatosensory cortex.¹⁷ However, pioneer transient neurons, such as subplate cells or subcerebral projecting neurons, may be critically involved in organizing these proto-ensembles.^{172,181} The function of these early proto-ensembles remains to be established but is likely to be linked to the integration of cells into local circuits as well as their long-range axonal path-finding. An example of the latter can be found in the developing spinal cord where motoneurons coupled by gap-junctions innervate the same muscle, and gap junction elimination gates the transition between multiple to single innervation.¹⁸² Regarding the former, the integrated activity within proto-ensembles may locally regulate the balance between excitation and inhibition before crystallizing as functional units, a process that may involve developmental apoptosis, which was also regulated at ensemble level.¹⁸³

After proto-ensembles, the next step in the development of neuronal ensembles starts with the emergence of chemical synaptic transmission and more specifically of local recurrent connections.^{171,172,176,184} This happens toward the end of the first postnatal week in rodents, which roughly corresponds to the end of gestation in humans. This is likely a two-stage process, with first an expansion of neuronal ensembles from local spatially isolated clusters to larger territories^{39,185} (Figure 5-2) and later a

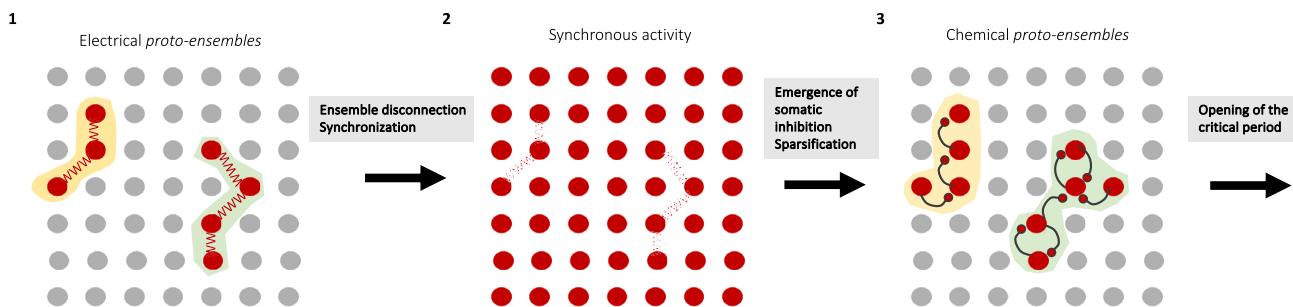


Figure 5. Stepwise model of ensemble development

Cartoon schematizing three steps of cortical ensemble development taking place before the start of the critical period: proto-ensembles connected through electrical synapses (1) are followed by highly synchronous population activity (2) and sparse, synaptically connected ensembles, constrained by somatic inhibition (3), resulting in two ensembles.

sparsification through the maturation of GABAergic assemblies just before the start of active exploration (Figure 5–3). Of note, chemical synapses actively suppress electrical ones,^{171,186} whereas in turn, electrical synapses contribute to the maturation of chemical synapses within ensembles.¹⁸⁷ Hence, blockade of gap-junction-mediated communication between sister neurons impairs the subsequent formation of specific chemical synapses between them within ontogenetic cortical columns.¹⁷⁵ Whether some of these electrical synapses are maintained, for example, in the axon initial segment (AIS) or axon, their location, and the mechanisms by which they are progressively replaced by chemical ones remain open questions.¹⁸⁸ After this, chemical proto-ensembles receive spontaneous, synapse-driven activity conveying bottom-up information from the sensory periphery, which itself is still insensitive to environmental sensory stimuli (visual and auditory) or from spontaneous movement feedback in the somatosensory system. This enables the pre-calibration of the connectivity and excitability of proto-ensembles to the statistics of the body and sensory organs prior to exploration of the external world.

As a final step, the start of active behavioral exploration, which happens toward the end of the second postnatal week in rodents, marks the opening of experience-dependent plasticity of cortical ensembles.^{189,190} It remains to be established when ensembles comparable to the ones reported in the adult emerge as well as their evolution from the proto-ensemble blueprint during critical periods. One possibility could be that the realm of plasticity available for a given adult ensemble will be included in the initial extent of proto-ensembles and regulated by intrinsic excitability and local inhibition.

Challenges and open questions

Ensembles are not, by any means, the only circuit basis for brain function, yet they provide an interesting framework to describe the functional organization of neural circuits and a possible bridge from single neuronal activity to brain function, discretizing population activity into modular units. Thus, ensembles could help solve a fundamental tension in neuroscience between the roles of individual neurons vs. neuronal populations in cognition.¹⁹¹ Conceptual solutions, anchored on the behavior of the animal, are critical to finding a middle ground between reductionism and emergent phenomena.¹⁹²

The correlation between the activation of ensembles and sensory responses or behavior^{58,75,122–124} suggests that ensembles

have a functional role. This is supported by the optogenetic manipulation of ensembles leading to the control of perceptual states,^{38,70,127} which demonstrated that ensemble activity was both necessary and sufficient for a visual discrimination behavior.^{38,70} This confirms that ensembles are not epiphenomena of population activity but veritable functional circuit units. Nevertheless, our understanding of the biology of ensembles is still limited, and many of the questions raised at the beginning of this article remain open. Adding to the results from mammalian hippocampus and neocortex reviewed above, it is necessary to explore the potential existence and function of ensembles in other brain areas. Initial results suggest that ensembles are present in many brain areas and species (Box 3), and the study of simpler preparations could help solve experimental challenges. For example, neurons in ensembles are not contiguous, and ensembles are often not constrained to particular circuit territories or even to particular regions of the nervous system. Therefore, to identify ensembles rigorously, one would need to sample the activity of the entire nervous system with cellular resolution, including both neurons and glia. Whole-brain cellular imaging is already possible in small, transparent animals, like *Hydra*,⁶⁶ *C. elegans*,²¹⁰ or zebrafish larva²¹¹ and the development of large-scale imaging methods fueled by the US and International Brain Research Through Advancing Innovative Neurotechnologies (BRAIN) initiatives^{212–214} could help extend whole-brain cellular resolution imaging to other preparations (Figure 6). Similar challenges apply to experimental methods to perturb ensembles, necessary to explore their functional roles. These methods need to have adequate temporal resolution, single-cell resolution, and be deployable in a parallel fashion throughout the extent of an ensemble, potentially encompassing the entire brain. Classical physiology used electrical macrostimulation, or pharmacology (or neuromodulation) to probe alterations in ensemble connectivity and function. More recently, holographic two-photon optogenetics^{152,215–217} or optochemistry,²¹⁸ have been used and combined with imaging in an all-optical fashion, with single-cell precision.²¹⁹ These functional approaches should be complemented with anatomical methods to reconstruct the connectivity of ensembles. Dense electron microscopy (EM) reconstructions of connectivity^{220,221} and molecular methods such as green fluorescent protein reconstitution across synaptic partners (GRASP)²²² appear promising

Box 3. Ensembles beyond cortex and hippocampus

Recurrent connectivity, and neural ensembles are observed across the brain, beyond the mammalian neocortex and hippocampus (Figure 6). Hence, motor pattern generating circuits, are good examples of how ensemble activity can lead to distinct programs not only in the motor cortex¹⁹³ but also in the rest of the nervous system. In humans, multi-unit recordings of subthalamic nuclei in Parkinson's disease patients revealed pairwise synchronous activation of tremor and movement direction-related neuronal ensembles that are partially overlapping.¹⁹⁴ Striatal ensembles were shown to generate sequentially patterned activity during motor learning¹⁹⁴ and to be organized spatiotemporally for encoding action space.¹⁹⁵ Similarly, ensembles of cerebellar Purkinje cells are associated with distinct motor actions, and they are regulated by animals' behavioral states.¹⁹⁶ Highly coordinated ensemble activity that is associated with locomotor rhythms is also observed across vertebrate spinal cord.¹⁹⁷ Finally, although birds do not have a motor cortex, high vocal center (HVC) and robust nucleus of the archistriatum (RA) in the forebrain of songbirds are good examples of how neural ensembles can learn and generate sequences^{82,166} (Figure 6D).

Brain regions associated with switching animals' internal states were also reported to utilize neural ensembles for various functions. Calcium imaging in mammalian amygdala²⁰¹ and zebrafish raphe²⁰² revealed distinct ensemble activation, when animals switch between exploratory and nonexploratory states. Similarly, coactivated ensembles of neurons associated with animals' adaptive behaviors were observed in the habenula^{44,61,203,204} as well as its inputs at the lateral hypothalamus.^{205,206} It is yet to be discovered whether these ensembles are generated due to recurrent connectivity of these brain regions or driven by the inputs from higher brain centers⁴⁷ (Figure 6C). In fact, highly distributed neural ensemble activity associated with internal states of animals was demonstrated successfully in invertebrate brains such as *Caenorhabditis elegans*,²⁰⁷ *Drosophila melanogaster*,^{208,209} and evolutionary distant animals without even ganglia, such as the cnidarians *Hydra vulgaris*⁶⁶ and *Clytia hemisphaerica*¹⁶⁷ (Figures 6A and 6B).

but are also hampered by the potential need to extend these reconstructions to the entire brains. With these large-scale methods, many practical questions could be tackled. For example, what minimum number of neurons constitutes an ensemble? What exactly is the space and time extent of ensembles? Does one need to record from every neuron? Can the size of ensembles change due to plasticity or state alterations? Solving these questions can provide rigorous arguments to evaluate the role of ensembles in neural circuits. Given the importance of development in shaping the functional organization of adult circuits, relating ensemble generation or activation to their developmental origin could help guide some of these questions.

There are also challenges with the analytical and computational methods employed to detect ensembles. There are many different approaches to detect ensembles, ranging from simple thresholds

or template matching, to dimensionality reduction algorithms, to Markovian chains, to graph theory models^{71,223,224} (Box 2). Although many methods are based on detection of zero-lag correlations, covariance, or functional connectivity, the statistical detection of sequential activity has often led to major disagreements.^{118,225–228} A similar analytical problem applies to sequential activity within an ensemble or within hierarchies of ensembles. As of today, there is not an ideal analytical method, as each of them has advantages and disadvantages and is particularly suited to a specific experimental method used. Further statistical or computational research is needed to specifically develop robust methods to capture and characterize ensembles and, more generally, emergent states of circuit activity. Perhaps inspiration and methods could be drawn from other fields where emergent properties have been studied and modeled, such as condensed matter

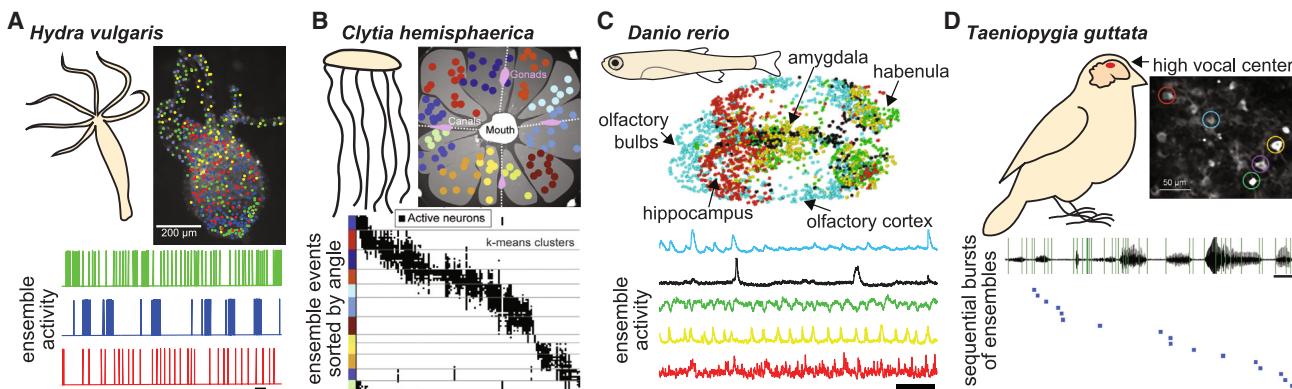


Figure 6. Neuronal ensembles in non-mammalian species

- (A) Calcium imaging of the activity of the entire nervous system of *Hydra vulgaris* reveals ensembles of coactive neurons (color-coded).
- (B) Neural ensembles in distinct divisions of the body of the jellyfish *Clytia hemisphaerica* display synchronized calcium signals (color-coded).
- (C) Ensembles of neurons in anatomically distinct zones of juvenile zebrafish forebrain exhibit highly correlated ongoing calcium activity (color-coded).
- (D) Electrophysiological recordings of sequential activation of neurons in high vocal center of songbird during song generation reveal a stereotyped activity pattern. Figures adapted from Bartoszek et al.,⁴⁷ Dupre and Yuste,⁶⁶ Picardo et al.,¹⁶⁶ and Weissbourd et al.¹⁶⁷

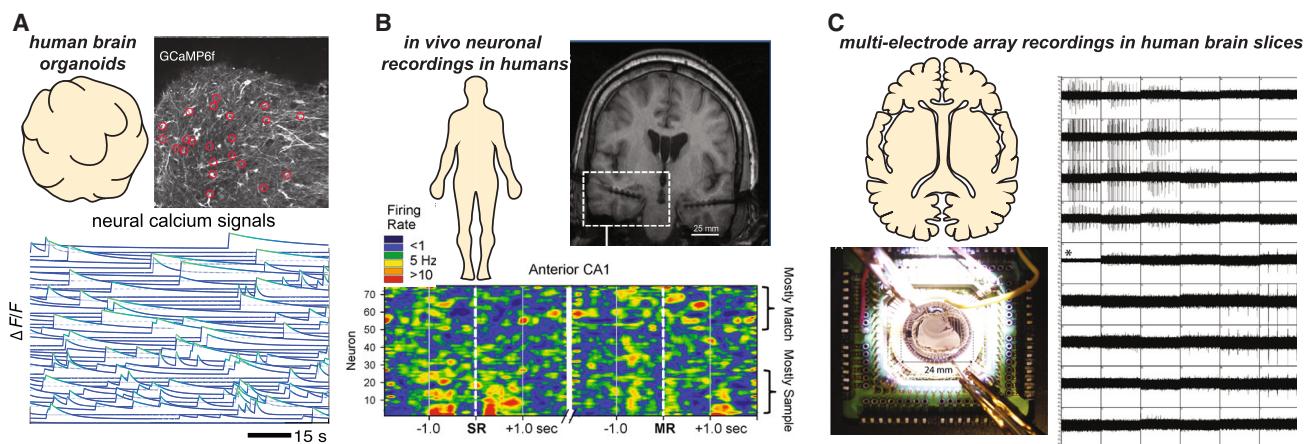


Figure 7. Potential ensembles in human neocortex

(A) Top: example of human brain organoids expressing transgenic calcium indicators. Bottom: spontaneous activity demonstrates coordinated population events.

(B) Top: spatially targeted multi-neural recording electrodes during neurosurgery to record the activity of large neural populations of human neurons, *in vivo*. Bottom: synchronized activity in neuronal populations in hippocampus.

(C) Multicellular extracellular recordings from human brain slices of tissue resected during brain surgery demonstrate synchronized activity of neuronal populations. Figures are adapted from Samarasinghe et al.,¹⁹⁸ Wicks et al.,¹⁹⁹ and Hsiao et al.²⁰⁰

physics, complex systems, network theory, or even linguistics and speech recognition.

Finally, essentially all knowledge gathered about ensembles stems from experimental animal preparations, with an almost complete ignorance of how these mechanisms play out in the human brain. Tantalizing data from human organoids, or recordings from human brain slices or patients appear consistent with the ensemble hypothesis (Figure 7). Although the basic structure and function of ensembles could be conserved across species,²²⁹ the human neocortex has undergone a massive evolutionary expansion with potential major changes in its microcircuits, arising from protracted developmental proliferation phases and the appearance of novel cellular subtypes.²³⁰ Indeed, initial exploration of the structural and functional properties of human cortical circuits reveal important differences with respect to mice.^{231–236} The recent development of methods to functionally probe human circuits^{198–200} besides providing fundamental information to understand the pathophysiology of mental and neurological diseases, and generate potential new therapeutics, could also provide an exciting new field of investigation to potentially build a bridge between the activity of individual neurons and human cognition, as a central goal of neuroscience.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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